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Intrinsic Fluorescence of Chloramphenicol Acetyltransferase: Responses to Ligand Binding and Assignment of the Contributions of Tryptophan Residues by Site-Directed Mutagenesis†

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ABSTRACT: Replacement by tyrosine or phenylalanine was used to assign the additive contributions of each of the three tryptophan residues of chloramphenicol acetyltransferase (CAT) to its intrinsic fluorescence on excitation at 295 nm. During the assessment of the fluorescence responses of the wild-type enzyme to the binding of ligands, it was found that the overlapping absorption spectra of chloramphenicol and tryptophan, with an attendant inner filter effect, required the use of a displacement technique involving an alternative substrate (the *p*-cyano analogue of chloramphenicol) without significant absorption at 295 nm. By the use of two-Trp, one-Trp, and Trp-less variants, in combination with this displacement technique, it was possible to demonstrate that Trp-86 and Trp-152 are involved in the fluorescence quenching associated with the binding of chloramphenicol, most likely via nonradiative energy transfer from these residues to the bound substrate. Trp-152 is mainly responsible for the fluorescence enhancement accompanying the binding of acetyl-CoA (and CoA) through proximity effects and solvent exclusion on substrate association.

Chloramphenicol acetyltransferase (CAT;[1] EC 2.3.1.28) catalyzes acetyl transfer from acetyl-CoA to the 3-hydroxyl of chloramphenicol, an inhibitor of the peptidyltransferase activity of prokaryotic ribosomes (Shaw, 1967). The product of the reaction, 3-acetylchloramphenicol, fails to bind to bacterial ribosomes (Shaw & Unowsky, 1968) and is thus devoid of antibiotic activity. Genes specifying CAT are the most common determinants of microbial resistance to chlor-

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

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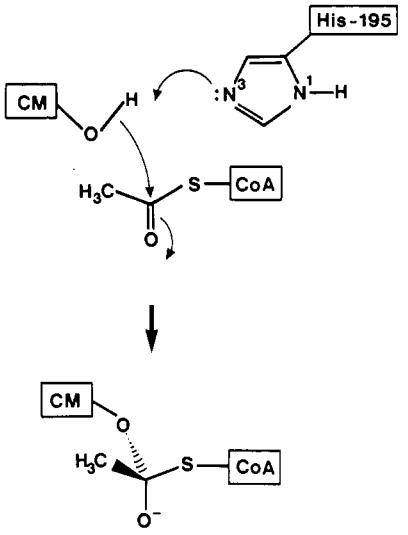
Scheme I



amphenicol and may be borne not only by plasmids and bacteriophages but also by transposable elements [reviewed by Shaw (1983)]. An understanding of the CAT mechanism and an appreciation of the structural determinants of ligand binding and catalysis have come in the main from studies of the type III enzyme, the only variant for which a high-resolution structure is available. That of the complex of CAT_{III} with chloramphenicol has been determined at 1.75-Å resolution (Leslie, 1990) whereas the structure of the CAT_{III}·CoA binary complex is known at 2.4-Å resolution (Leslie et al., 1988). CAT is a homotrimer (3 × 25 kDa) with active sites lying at each of the three interfaces between adjacent subunits. Kinetic and chemical modification studies of CAT_{III} have shown that the reaction proceeds by a rapid equilibrium, random order, ternary complex mechanism wherein the $N^{\epsilon 2}$ of the imidazole of His-195[2] serves as a general base to abstract the proton of the 3-hydroxyl of chloramphenicol (Kleanthous & Shaw, 1984; Kleanthous et al., 1985). The proposed route to a tetrahedral intermediate (Scheme I), yields an oxyanion which is probably stabilized in part by a hydrogen bond from the hydroxyl of Ser-148 in the transition state (Lewendon et al., 1990).

The availability of a high-level expression system for CAT_{III} (Murray et al., 1988) has facilitated studies by site-directed mutagenesis which have addressed enzyme stability (Lewendon et al., 1988; Gibbs & Leslie, 1990), the role of electrostatic and hydrophobic interactions in CoA binding (P. J. Day, A. G. W. Leslie, and W. V. Shaw, submitted for publication), the binding of chloramphenicol analogues (Murray et al., 1991; Cullis et al., 1991), and the chemical properties of a cysteinyl residue in the chloramphenicol binding site (Lewendon & Shaw, 1990). These and other studies bearing on the relation of enzyme structure to function in the CAT "family" of homologous proteins have been reviewed recently (Shaw & Leslie, 1991).

To quantitate the binding of substrates and other ligands to CAT_{III} and as a means to aid the transient kinetic analysis of the enzyme, it became necessary to explore the intrinsic fluorescence of CAT_{III}. In addition, the relative ease by which mutations in the *cat* gene may be introduced made possible the assignment of the contribution to the intrinsic fluorescence of each of the protein's three tryptophan residues. Only Trp-152 is absolutely conserved in the 11 sequenced variants of CAT (Shaw & Leslie, 1991), lying as it does in a polar environment on the surface of a tunnel at the subunit interfaces accommodating the pantetheine arm of CoA (Leslie et al., 1988). Although not absolutely conserved, Trp-16 and Trp-86 are each present in all but one of the naturally occurring variants of CAT for which amino acid sequence data are available. In both cases, replacement is with a tyrosine. The side chains of both Trp-16 and Trp-86 are relatively inaccessible to solvent and are surrounded by mainly hydrophobic residues.

Here we describe the general fluorescence properties of CAT_{III} and assign the contribution of each tryptophan to the observed emission by their sequential replacement with phenylalanine or tyrosine. In addition, we demonstrate the changes in tryptophan-derived fluorescence which accompany the binding of substrates and assign the contribution of individual tryptophan residues to each response. The use of a novel displacement technique, involving an analogue of chloramphenicol which does not absorb at 295 nm, circumvented the inner filter effect observed on excitation of enzyme in the presence of the antibiotic.

[2] Alignment of the amino acid sequences of CAT variants (Leslie & Shaw, 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).

Table I: Yields and Specific Activities of CAT Variants

protein	Trp present at 16	86	152	yield[a] (mg)	sp act. (units/mg)
wild type	+	+	+	149	796
W16Y	−	+	+	137	919
W86F	+	−	+	162	920
W152F	+	+	−	155	266
W86F:W152F	+	−	−	177	231
W16Y:W152F	−	+	−	165	145
W16Y:W86F	−	−	+	132	332
W16Y:W86F:W152F	−	−	−	143	121

[a] Yield of electrophoretically homogeneous CAT obtained from a 500-mL culture, using the methods described under Experimental Procedures.

Experimental Procedures

Site-Directed Mutagenesis and Expression of CAT_{III}. Oligonucleotide-directed mismatch mutagenesis was performed according to the deoxyuridine selection protocol with the *dut ung Escherichia coli* strain RZ1032 (Künkel et al., 1987). The presence of the desired nucleotide substitutions and the absence of second-site mutations were confirmed by determination of the nucleotide sequence of the DNA spanning the *cat* coding sequence and the adjoining 5′ noncoding region. Mutated genes encoding multiple tryptophan substitutions were constructed by repeated cycles of mutagenesis or by recombination using the unique *Sal*I site (GTCGAC) located at codons 79 and 80 (Val-Asp) of *cat*. Mutant and wild-type *cat* determinants were overexpressed in *E. coli* following transfer to plasmid pUC18 (Murray et al., 1988). Table I shows the series of mutations made and the amino acid substitutions at each position.

Purification of CAT_{III}. Purification of wild-type and mutant CAT_{III} proteins from *E. 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; each CAT mutant yielded single bands of mobility identical to that of wild-type CAT. The chloramphenicol used as eluent in the purification procedure was removed by gel permeation chromatography on 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 standard, while the concentration of the latter was obtained either by the method of Lowry (1951) or by absorbance at 280 nm ($\epsilon^{1\%}_{280} = 13.1$). In each case the reference material was a solution of CAT_{III} of known concentration on the basis of amino acid analysis.

Assay of CAT Activity. CAT activity was assayed spectrophotometrically at 25 °C. A modification of the method of Shaw (1975) was used to measure rates of chloramphenicol acetylation. The standard assay mixture contained TSE buffer, pH 7.5, 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 (λ_{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.

In the steady-state kinetic analysis of the forward transacetylation reaction concentrations of chloramphenicol and acetyl-CoA were varied in the standard assay mixture. Linear initial rates were measured over times during which less than 15% depletion of substrates occurred. Kinetic parameters were determined as described previously (Kleanthous & Shaw, 1984).

Assessment of Thermal Stability. The thermal stability of wild-type CAT_{III} and mutant proteins was assessed by incubation in capped 0.5-mL Eppendorf tubes containing 10 μg of enzyme in 100 μL of TSE buffer, pH 7.5, in a covered water bath at 70 °C. At timed invervals (up to 60 min), aliquots were withdrawn and rapidly cooled to 4 °C before being assayed for residual activity by the standard assay at 25 °C.

Fluorescence Spectra. All fluorescence measurements were made at 25 °C with 4-mL clear-sided cuvettes using a Perkin-Elmer LS-5B luminescence spectrometer with a thermostated cuvette block. Excitation and emission slit widths were set at 2.5 nm. A typical sample contained 2 μM protein in TSE buffer, pH 7.5, to which ligands were added at the concentrations indicated. Controls contained TSE buffer (±ligand). Tryptophan fluorescence was selected by excitation at 295 nm. Emission spectra were obtained by scanning between 305 and 410 nm at a rate of 60 nm/min. The data were displayed directly on a Perkin-Elmer GP100 graphics plotter. Spectral data were also digitized by manually tracing spectra on a Cherry graphics tablet for ease of subsequent data manipulation and replotting. Each spectrum was corrected for background fluorescence of the relevant control and then plotted on an Epson HI-80 graphics plotter. No correction was made for possible changes in fluorescence polarization upon ligand binding since no such changes were detected with wild-type CAT_{III} in separate polarization measurements. Possible minor changes in the absorbance of CAT on binding chloramphenicol were judged to be negligible in view of the preponderance of the inner filter effect of the substrate.

Results

Kinetic Properties and Thermostability of Mutant Variants of CAT_{III}. The availability of substantial amounts of the two-Trp, one-Trp, and Trp-less variants of CAT_{III} (see Experimental Procedures) allowed a direct comparison of kinetic, stability, and fluorescence properties of each with those of the wild-type enzyme. The steady-state kinetic parameters are set out in Table II. The W16Y and W86F enzymes, each containing two tryptophans, exhibit virtually wild-type characteristics, whereas W152F and those enzymes possessing two or more substitutions are impaired to varying degrees in catalytic activity. Nonetheless, the affinities of the variants for each of the substrates, as inferred from K_m values, are not significantly compromised in any of the proteins.

Table II: Kinetic Parameters[a] for Acetylation of Chloramphenicol by Wild-Type and Mutant Chloramphenicol Acetyltransferases

protein	k_{cat} (s^{-1})	K_m (μM) chloramphenicol	K_m (μM) acetyl-CoA
wild type	599	12.0	93
W16Y	631	16.6	99
W86F	576	14.9	99
W152F	167	9.8	128
W86F:W152F	137	9.4	130
W16Y:W152F	110	9.8	130
W16Y:W86F	244	20.9	109
W16Y:W86F:W152F	70	15.4	154

protein	k_{cat} (s^{-1})	K_m (μM) *p*-cyano-CM	K_m (μM) acetyl-CoA
wild type	515	12.4	104

[a] Kinetic parameters are the mean of at least three determinations performed as described under Experimental Procedures.

Table III: Stability[a] of Wild-Type and Mutant Chloramphenicol Acetyltransferases at 70 °C

protein	% activity remaining after incubation at 70 °C for 60 min	10 min
wild type	96	–
W16Y	54	–
W86F	97	–
W152F	65	–
W86F:W152F	30	–
W16Y:W152F	–	5
W16Y:W86F	–	8
W16Y:W86F:W152F	–	0.1

[a] All the above CAT_{III} variants retained 100% activity after incubation for 60 min at the standard assay temperature (25 °C).

The thermal stability of CAT (Table III) is substantially diminished by the replacement of Trp-16 and Trp-152 by tyrosine and phenylalanine, respectively. Since Trp-16 is at the center of a predominantly hydrophobic microdomain near the N-terminal arm of CAT_{III}, which is stabilized by a β-sheet interaction involving only three residues, the W16Y substitution may allow unfavorable side-chain movements and/or the introduction of solvent. Trp-152, on the other hand, is an absolutely conserved residue which is positioned at the solvent-accessible subunit interface and which hydrogen bonds to CoA via water molecules (Leslie et al., 1988). Hence, the W152F substitution could promote destabilization via disruption of interactions across the subunit interface.

The effects of the above substitutions on thermostability are compounded in the one-Trp and Trp-less CAT_{III} variants. Although the results in Table III are presented in such a way as to emphasize heat lability, it should be noted that wild-type CAT_{III} is an exceptionally stable enzyme. Hence, under the normal experimental conditions (25 °C) employed for CAT assays, even the Trp-less variant is stable for more than 1 h.

Fluorescence Emission Spectra of CAT_{III} and Variants. The use of 295 nm as the excitation wavelength ensures that the observed fluorescence is attributable almost exclusively to tryptophan residues. The emission spectra in Figure 1 demonstrate the stepwise and cumulative loss of intensity due to the replacement of each tryptophan of CAT_{III} by phenylalanine or tyrosine; the total absence of fluorescence exhibited by the Trp-less variant verifies the efficacy with which tryp-

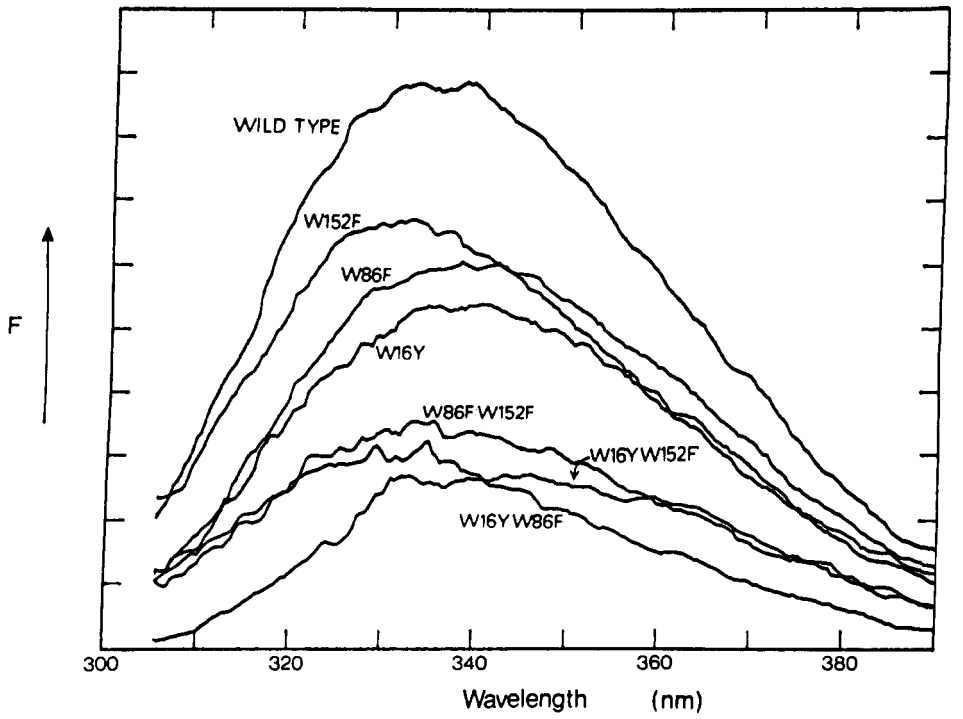


FIGURE 1: Fluorescence emission spectra of CAT_{III} and the tryptophan-deficient variants. The excitation wavelength was 295 nm. The emission spectrum of W16Y:W86F:W152F overlies the abscissa and is not illustrated.

tophan fluorescence is specifically selected. It can be seen that the three tryptophan residues within the CAT_{III} monomer each contribute approximately one-third to the observed fluorescence of the wild-type protein. The simple additive nature of these fluorescence contributions indicates that homogeneous radiationless energy transfer between them does not have a significant effect on their fluorescence properties when excited at 295 nm, near the red edge of the absorption spectrum for tryptophan.

The position of the maximum emission wavelength varies slightly between the CAT variants, reflecting subtle differences in the nature of the local environment of each tryptophan (Figure 1). The spectra of those mutants retaining only Trp-16 or Trp-86 show similar blue shifts (λ_{max} = 331 nm), compared to the wild-type spectrum (λ_{max} = 335 nm), as expected given the relative hydrophobicity of their local environments and solvent accessibility (17 and 7%, respectively, on the basis of the crystal structure) compared to that of Trp-152 (Leslie, 1990). The red shift in the emission peak (λ_{max} = 340 nm) of the protein retaining only Trp-152 indicates the larger percentage of solvent-accessible surface (38%) and correlates with the lower quantum yield from this residue.

Fluorescence Responses to the Binding of Chloramphenicol. The absorption spectrum of chloramphenicol, centered at 278 nm ($\epsilon_M = 9.8 \times 10^3\ M^{-1}\ cm^{-1}$), gives rise to an inner filter effect which makes a significant contribution to any protein fluorescence response occurring upon the addition of this substrate. An analogue of chloramphenicol in which the electron-withdrawing *p*-nitro moiety is replaced by a cyano group (*p*-cyano-CM) was used to circumvent this problem since its absorption is maximal at 234 nm and does not extend beyond ~260 nm. The steady-state kinetic parameters of wild-type CAT_{III} when *p*-cyano-CM replaces chloramphenicol as the acyl acceptor are included in Table II and indicate that the enzyme does not discriminate between the two compounds. The binding of the analogue provokes no fluorescence response from wild-type CAT_{III}. The observation that the fluorescence spectra of CAT_{III} are indistinguishable in the presence and absence of *p*-cyano-CM provided the opportunity to monitor chloramphenicol binding by fluorescence spectroscopy using competition and displacement.

To distinguish between the inner filter effect of chloramphenicol and a genuine fluorescence response to its binding, it was necessary to use *p*-cyano-CM to displace prebound chloramphenicol from the enzyme, thus inverting the chloramphenicol binding response while maintaining the inner filter effect at a constant level. Three emission spectra were recorded and compared for each protein: (1) CAT alone, (2) CAT plus chloramphenicol, and (3) CAT plus chloramphenicol and *p*-cyano-CM. The difference between spectra 1 and 2 is a measure of the inner filter effect *plus* the binding response, whereas the difference between spectrum 2 and spectrum 3, wherein bound chloramphenicol is displaced by an excess of *p*-cyano-CM, provides a measure of the fluorescence response on binding chloramphenicol (Figure 2).

Use of the above experimental protocol revealed that in the presence of 20 μM chloramphenicol, the observed fluorescence intensity of wild-type CAT_{III} decreased by ~56%, of which ~27% is due to the inner filter effect of chloramphenicol and ~29% represents the "true" binding response. The concentration of chloramphenicol used (20 μM) ensured at least 80% saturation of enzyme since the K_d for the binary complex of CAT_{III} and chloramphenicol is 4 μM (Ellis et al., 1991). Higher concentrations of the antibiotic were not used because of the dominant inner filter effect. The concentration of *p*-cyano-CM used (100 μM) allowed for the displacement of approximately 90% of the bound chloramphenicol.

Extension of the chloramphenicol displacement technique to the tryptophan-substituted variants of CAT_{III} showed that, as expected, the inner filter effect remained constant for a given chloramphenicol concentration at ~27% of the initial intrinsic protein fluorescence intensity, whereas the magnitude of the binding response varied according to the combination of tryptophan residues retained in the protein (Table IV).

The fluorescence results obtained with the two-Trp proteins suggest that the removal of Trp-16 has little or no effect on

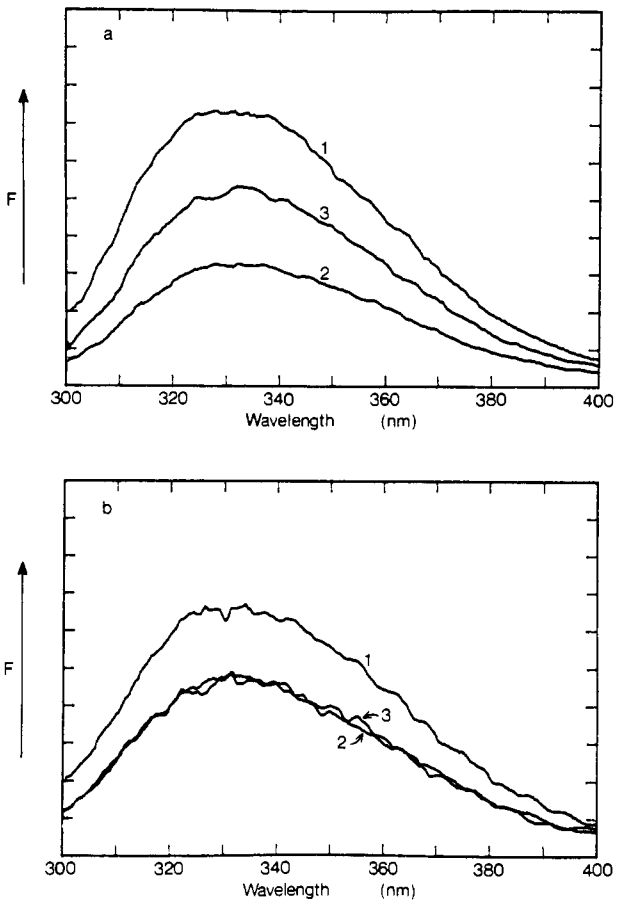


FIGURE 2: Intrintsic protein fluorescence response to the binding of chloramphenicol. Fluorescence emission spectra of (a) wild-type CAT_{III} and (b) W86F:W152F. Additions were (1) none, (2) chloramphenicol (20 μM), and (3) chloramphenicol (20 μM) and *p*-cyano-CM (100 μM). The difference between (1) and (3) is due to the inner filter effect of chloramphenicol, while the difference between (2) and (3) is the quench in protein fluorescence due to the binding of chloramphenicol. The excitation wavelength was 295 nm.

Table IV: Fluorescence Responses[a] of Wild-Type and Mutant Chloramphenicol Acetyltransferases to Ligand Binding

protein	Trp present at 16	86	152	ligand: chloramphenicol (%)	ligand: acetyl-CoA (%)	ligand: CoA (%)
wild type	+	+	+	−28.7	+24.6	+6.8
W16Y	−	+	+	−32.5	+36.1	+8.4
W86F	+	−	+	−17.4	+28.9	+3.9
W152F	+	+	−	−16.7	−3.2	−6.8
W86F:W152F	+	−	−	0	−6.9	−3.0
W16Y:W152F	−	+	−	−16.6	−8.0	−4.0
W16Y:W86F	−	−	+	−16.8	+26.7	+28.0
W16Y:W86F:W152F	−	−	−	0	0	0

[a] The values for enhancement (+) or quenching (−) of fluorescence are given as percent of the intensity change at 335 nm on ligand binding relative to the initial fluorescence intensity of protein alone. In the case of chloramphenicol, the quench was calculated using a displacement technique involving the alternative substrate *p*-cyano-CM (see text).

the response of CAT_{III} to the binding of chloramphenicol, whereas the replacement of either Trp-86 or Trp-152 reduces by half the true quench observed upon the association of chloramphenicol. Use of the single-Trp CAT_{III} variants not only confirmed the above results but also gave a measure of the precise contribution of each tryptophan residue to the quench seen on the binding of chloramphenicol to the wild-type protein. Thus, the fluorescence of both Trp-86 and Trp-152 is quenched by approximately 17%, as measured by the displacement assay of chloramphenicol binding. Together they are responsible for the total response of wild-type CAT_{III} to the binding of chloramphenicol under the conditions defined above. These results are summarized in Table IV. Figure 2b illustrates the absence of response to the association of chloramphenicol exhibited by the double-replacement mutant (W86F:W152F) retaining only Trp-16.

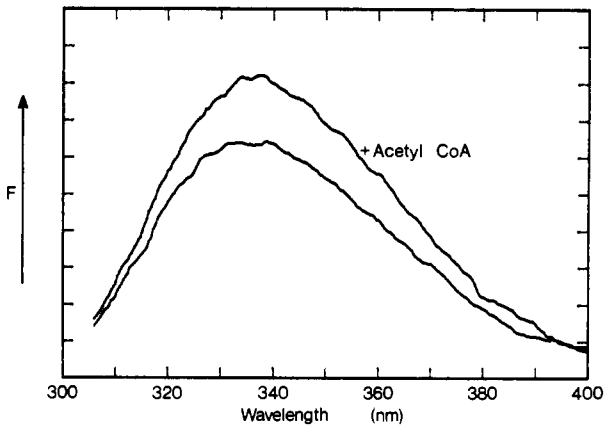


FIGURE 3: Intrinsic protein fluorescence response to the binding of acetyl-CoA. Fluorescence emission spectra are shown of wild-type CAT_{III} with and without acetyl-CoA (400 μM). The excitation wavelength was 295 nm.

Fluorescence Responses to the Binding of Acetyl-CoA. The use of 295 nm as the excitation wavelength avoids problems arising through inner filter effects from acetyl-CoA since the coenzyme does not absorb significantly above ~285 nm. Thus, the response of CAT_{III} to the binding of acetyl-CoA may be measured by direct comparison of the emission spectrum of free enzyme with that of the binary complex. The association of acetyl-CoA with wild-type CAT_{III} provokes an enhancement of intrinsic protein fluorescence of approximately 24%, as illustrated in Figure 3. The CAT variants W16Y and W86F, which have in common the presence of Trp-152, show enhancements of fluorescence intensity of 36 and 29%, respectively, on association with acetyl-CoA. (The increased percent changes compared to wild type are due to the diminution of intrinsic fluorescence from the loss of Trp-16 or Trp-86, neither of which are reporters of acetyl-CoA binding.) As predicted, the results with the W152F protein showed it to retain little or no fluorescence response to acetyl-CoA association.

Results with the doubly substituted (one-Trp) proteins paralleled those discussed above in that Trp-152 can be seen to be largely responsible for the tryptophan-related fluorescence response to CAT_{III} to the binding of acetyl-CoA. In particular, W16Y:W86F (retaining Trp-152) exhibited an enhancement of 27% on addition of the substrate, somewhat less than expected given the loss of two unresponsive tryotophan residues.

Fluorescence Responses to CoA Binding. The results observed with unesterified CoA as the ligand are somewhat unexpected in that, despite the 24% enhancement of fluorescence intensity on binding acetyl-CoA, wild-type CAT_{III} shows a very modest fluorescence enhancement on association with CoA (+7%; Figure 4a; Table IV). The singly substituted variants retaining Trp-152 (W16Y and W86F) also exhibit small enhancements, whereas proteins with the W152F substitution each show a small quench.

However, the most surprising effects were observed with the W16Y:W86F protein which retains only Trp-152, for which the addition of CoA produced a striking enhancement (28%) of intrinsic fluorescence (Figure 4b), comparable in magnitude

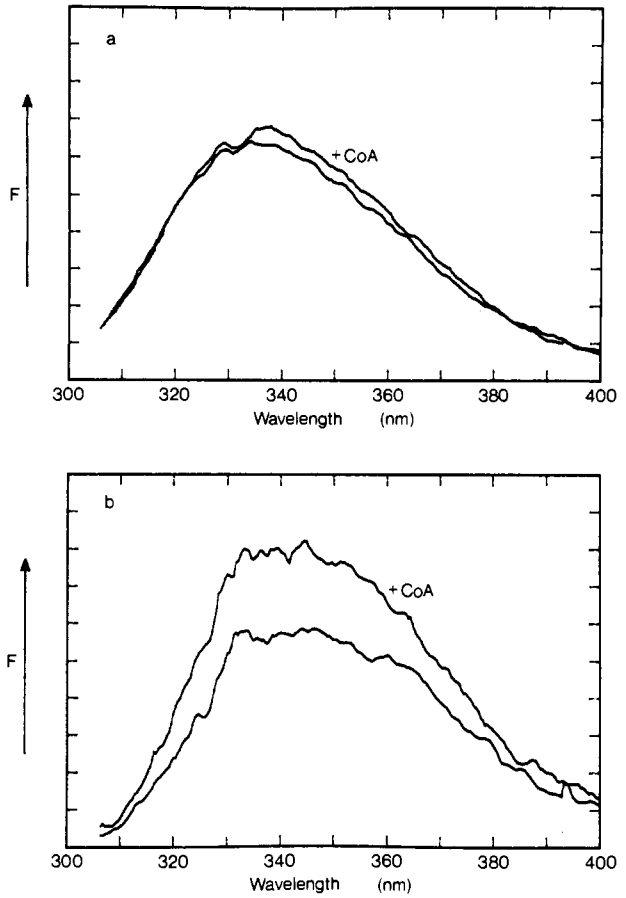


FIGURE 4: Intrinsic protein fluorescence response to the binding of CoA. Fluorescence emission spectra of (a) wild-type CAT_{III} and (b) W16Y:W86F with and without CoA (400 μM). The excitation wavelength was 295 nm.

to that (27%) obtained on addition of acetyl-CoA to that protein. Thus, the W16Y:W86F variant of CAT_{III}, for reasons which are not yet clear (see Discussion), provides an approach to monitoring the binding of either acetyl-CoA or CoA by fluorescence enhancement, a property not shared by the wild-type CAT_{III} or other variants substituted at one or two tryptophans.

Discussion

The decrease in tryotophan-mediated fluorescence of CAT_{III} which accompanies the binding of chloramphenicol (Figure 5) may be compared to the *lack* of quench seen on binding the structurally related analogue, *p*-cyano-CM. It is unlikely that the contrasting fluorescence response is due to any difference in the mode of binding of the compounds since the analogue is acetylated efficiently by CAT_{III} (Table II) and because X-ray diffraction data suggest that no structural change in CAT_{III} is induced by chloramphenicol binding (Leslie, 1990). A comparison of alternative chloramphenicol analogues showed that the fluorescence quench on binding to CAT_{III} is unique to chloramphenicol-related compounds which possess the *p*-nitro moiety (data not shown). In the absence of other substituents, the presence of the *p*-nitro group changes the electron distribution of the phenyl ring such that the aromatic absorption band is shifted from ~200–230 nm to one centered at ~280 nm. Consequently, the absorption by ligand at longer wavelengths overlaps not only with that of the protein but also with the tryptophan emission, together yielding the inner filter effect.

It seems likely that the genuine tryptophan fluorescence quench seen on the binding of chloramphenicol to CAT_{III} arises

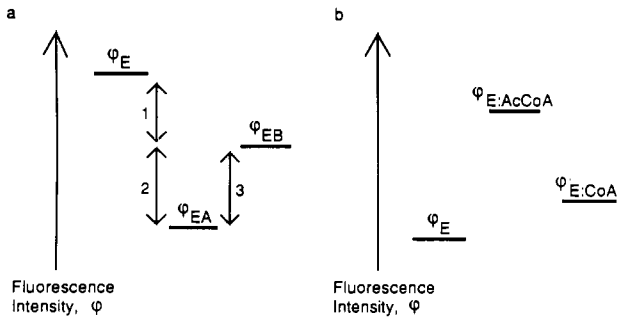


FIGURE 5: The relative fluorescence intensities of enzyme·ligand binary complexes. The symbol φ refers to observed fluorescence intensity rather than quantum yield. (a) The changes in the fluorescence intensity of wild-type CAT_{III} occurring throughout the competition binding assay for chloramphenicol. The arrows represent (1) the inner filter effect due to the presence of chloramphenicol, (2) the actual quench due to binding chloramphenicol, and (3) the dequench of fluorescence upon the displacement of bound chloramphenicol by *p*-cyano-CM. (b) The binding of acetyl-CoA to wild-type CAT_{III} produces a 24% enhancement of the intrinsic fluorescence, while an enhancement of only 7% is apparent on association of CoA.

from a nonradiative energy-transfer process. Such a phenomenon, involving neither emission nor absorption of photons, arises through a change in dipole during a transition between ground and excited states. Thus, through coupling of dipoles to chloramphenicol, the excited-state energy of Trp-86 and Trp-152 may be transferred to the bound substrate, allowing both residues to return to ground state without emission taking place. The indirectly excited chloramphenicol returns to the ground state by internal conversion.

Such resonance energy transfer may occur over distances of 10–50 Å, its efficiency depending on the degree of overlap between the emission/absorption spectra of the donor/acceptor pair and relative orientation of the ring systems (Lakowicz, 1983). Although the spectral overlap of the trytophan emission and chloramphenicol absorption pair is not large, the indole nitrogen in each case is 15 and 14 Å, respectively, from the phenyl ring of chloramphenicol in the crystal structure of the CAT·chloramphenicol binary complex, putting both residues well within the distance over which nonradiative energy transfer may occur.

The change in fluorescence intensity which accompanies the association of acetyl-CoA or CoA to CAT_{III} arises mainly from Trp-152, as demonstrated by the responses of the doubly substituted variants seen in Table IV. Both ligands perturb the environment of Trp-152 to a similar extent, resulting in a 27–28% enhancement in its emission, possibly due to the displacement of as many as 10 solvent molecules from the ligand binding site (Leslie et al., 1988), together with an increase in hydrophobicity due to the positioning of the adenine ring system of CoA very close to, and nearly coplanar with, the indole ring of Trp-152. Nonetheless, both Trp-16 and Trp-86 must also contribute to the fluorescence enhancement on formation of the binary complex with acetyl-CoA, albeit to a lesser degree. Were they not to do so, the absolute enhancement observed with wild-type CAT_{III} should be reflected in a considerably greater response than that noted in W16Y:W86F, due to the removal from the latter of noncontributory "background" fluorescence (i.e., that of Trp-16 and Trp-86).

The extent to which Trp-16 and Trp-86 respond to the binding event may be dependent on the size and nature of the substituent at the sulfur of CoA. CoAS(-H) gives ~7% fluorescence enhancement with wild-type CAT but yields 28% enhancement with Trp-152 CAT (W16Y:W86F). Binding

of CoAS(-$COCH_3$) on the other hand increases wild-type fluorescence intensity by 24%, which implies some contribution from both Trp-16 and Trp-86. The results of recent preliminary experiments using CoAS(-CH_2CH_3), the ethyl thioether analogue of acetyl-CoA, are consistent with this reasoning in that association of ethyl-CoA to wild-type CAT_{III} produces a 25% enhancement of protein fluorescence (data not shown).

The similarity of the response of Trp-152 to the binding of acetyl-CoA and CoA implies that both molecules affect their immediate environment in the same way and therefore bind in a similar manner. It is impossible to be more specific about the nature of the long-range effects on the binding of acetyl-CoA without crystallographic data for the CAT·acetyl-CoA binary complex, to compare it with the deduced CAT·CoA structure (Leslie et al., 1988).

In summary, the results obtained allow the tryotophan-related fluorescence response of wild-type CAT_{III} to chloramphenicol binding to be assigned in approximate equal proportions to Trp-86 and Trp-152 and suggest that the quenching is due to nonradiative energy transfer from each of these residues to bound chloramphenicol. Although the fluorescence enhancement on CoA binding may be assigned largely to Trp-152, it seems likely that both Trp-16 and Trp-86 are responsible for the increased fluorescence intensity changes observed on binding acetyl-CoA.

Rather few detailed studies of fluorescence with the assignment of the contribution of each residue have been reported for proteins with multiple tryptophans. Although in principle a resolution of the role of individual tryptophans may be achieved by fluorescence lifetime measurements, only site-directed mutagenesis can yield unambiguous assignments. Notable examples are those of *Bacillus stearothermophilus* lactate dehydrogenase (Waldman et al., 1987), *E. coli* succinyl-CoA synthetase (Nishimura et al., 1990), and *Bacillus amyoliquefaciens* barnase (Loewenthal et al., 1991). The site-directed mutagenesis approach to fluorescence assignment not only allows the direct and independent measurement of the contribution of each tryptophan residue to total fluorescence but also facilitates the determination of the effect on the fluorecence properties of a given tryptophan residue to the association of ligands.

Over and above theoretical considerations, the assignments of ligand-induced fluorescence changes provides a firmer foundation for the use of intrinsic fluorophores in quantitative and time-dependent studies of substrate (or product) binding to CAT and to studies of its mechanism (Ellis et al., 1991).

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