Analysis of the Binding of 1,3-Diacetylchloramphenicol to Chloramphenicol Acetyltransferase by Isotope-Edited ^{1}H NMR and Site-Directed Mutagenesis†

J. P. Derrick,‡ L.-Y. Lian,§ G. C. K. Roberts,‡,§ and W. V. Shaw*,‡

Department of Biochemistry and Biological NMR Centre, University of Leicester, University Road, Leicester, LE1 7RH, U.K.

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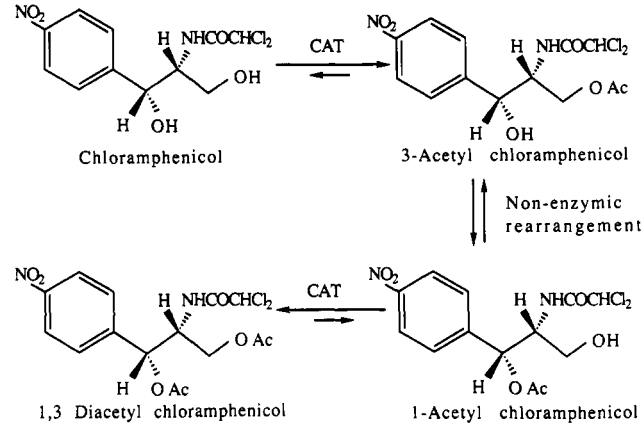
ABSTRACT: The binary complex of diacetylchloramphenicol and chloramphenicol acetyltransferase (CAT) has been studied by a combination of isotope-edited ^{1}H NMR spectroscopy and site-directed mutagenesis. One-dimensional HMQC spectra of the complex between 1,3-[2-^{13}C]diacetylchloramphenicol and the type III natural variant of CAT revealed the two methyl ^{1}H signals arising from each ^{13}C-labeled carbon atom in the acetyl groups of the bound ligand. Slow hydrolysis of the 3-acetyl group by the enzyme precluded further analysis of this binary complex. It was possible to slow down the rate of hydrolysis by use of the catalytically defective S148A mutant of CAT_{III} (Lewendon et al., 1990); in the complex of diacetylchloramphenicol with S148A CAT_{III}, the chemical shifts of the acetyl groups of the bound ligand were the same as in the wild-type complex. The acetyl signals were individually assigned by repeating the experiment using 1-[2-^{13}C], 3-[2-^{12}C]diacetylchloramphenicol, where only one signal from the bound ligand was observed. A two-dimensional ^{1}H,^{1}H NOESY experiment, with ^{13}C(ω_2) half-filter, on the 1,3-[2-^{13}C]diacetylchloramphenicol/S148A CAT_{III} complex showed a number of intermolecular NOEs from each methyl group in the ligand to residues in the chloramphenicol binding site. The 3-acetyl group showed strong NOEs to two aromatic signals which were selected for assignment. The possibility that the NOEs originated from the aromatic protons of diacetylchloramphenicol itself was eliminated by assignment of the signals from enzyme-bound diacetylchloramphenicol and chloramphenicol using perdeuterated CAT_{III}. Examination of the X-ray crystal structure of the chloramphenicol/CAT_{III} binary complex indicated four plausible candidate aromatic residues: Y25, F33, F103, and F158. Each was separately substituted with isoleucine, and the two-dimensional ^{13}C (ω_2) half-filter NOESY experiment was repeated on each mutant enzyme/diacetylchloramphenicol binary complex. The two NOEs were present in each case, except for the Y25I mutant, which yielded no cross peaks. We conclude that Tyr-25 is close to the 3-acetyl group in the 1,3-diacetylchloramphenicol/CAT_{III} binary complex; this information allows a more precise formulation of a structural model for the interaction of CAT_{III} with its ultimate product (in the forward reaction) or substrate (for the reverse reaction).

Chloramphenicol acetyltransferase (CAT;[1] EC 2.3.1.28), the primary effector of resistance of bacteria to the antibiotic chloramphenicol (Shaw, 1967; Shaw & Leslie, 1991), catalyses acetyl transfer from acetyl-CoA to the 3-hydroxyl of chloramphenicol to produce 3-acetylchloramphenicol (Scheme I). The latter can then undergo a nonenzymatic intramolecular acetyl migration to form 1-acetylchloramphenicol, which is the substrate for a second cycle of acetylation by CAT, yielding 1,3-diacetylchloramphenicol. The second acetylation is not necessary for inactivation of the antibiotic activity of chloramphenicol, since neither 3-acetylchloramphenicol nor 1,3-diacetylchloramphenicol binds to prokaryotic ribosomes (Shaw & Unowsky, 1968). The three-dimensional structure of the binary complex of chloramphenicol with the type III variant of CAT (CAT_{III}) has been determined by X-ray crystallography to 1.75-Å resolution (Leslie, 1990), but there is no direct structural information on the 1,3-diacetylchloramphenicol/CAT_{III} binary product complex since attempts to obtain satisfactory crystals for analysis by X-ray diffraction have proven unsuccessful. Thus there is no information on either the location of the acetyl groups or their effect on the conformation of the chloramphenicol moiety of the bound ligand. Such information will be particularly valuable in dissecting the mechanism of acetyl transfer in CAT_{III}, given that 1,3-diacetylchloramphenicol is the substrate for one of the reverse reactions shown in Scheme I.

Scheme I



High-field NMR offers an alternative method to determine the location of the acetyl groups, but such experiments are not trivial because of the high molecular weight of CAT_{III}, a trimer of identical subunits having a total molecular weight of 75 000.

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* Corresponding author.

‡ Department of Biochemistry.

§ Biological NMR Centre.

[1] Abbreviations: CAT, chloramphenicol acetyltransferase; CAT_{III}, type III variant of chloramphenicol acetyltransferase; HMQC, heteronuclear multiple-quantum coherence, DSS, sodium 2,2′-dimethyl-2-silapentane-5-sulfonate.

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The ^1H NMR spectra of this enzyme are too complex to analyze by conventional NMR methods. An alternative approach is to use stable isotope labeling, such as selective deuteration (Markley et al., 1968; LeMaster, 1989) or ^{13}C or ^{15}N labeling in conjunction with "isotope-editing" methods (Derrick et al., 1991; Otting & Wüthrich, 1990; Griffey & Redfield, 1987), to simplify the ^1H spectra. ^{13}C- or ^{15}N-labeled ligands can be employed to study the conformation of the bound species and its contacts with neighboring amino acid residues [e.g., Fesik et al. (1988)]. Here we demonstrate that useful structural information on ligand binding to a large protein can be obtained by isotope-edited ^1H NMR in combination with site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Preparation of 1,3-[2-^{13}C]Diacetylchloramphenicol and 1-[2-^{13}C],3-[2-^{12}C]Diacetylchloramphenicol. A 30-mg (90 μmol) sample of chloramphenicol was dissolved in 0.5 mL of dry pyridine and placed on ice; 40 μL (360 μmol) of 2-[^{13}C]-acetyl chloride (99% ^{13}C, Aldrich) was added and the reaction left to proceed on ice for 10 min. The reaction was terminated by the addition of 3 mL of deionized water and the product extracted into 10 mL of ethyl acetate. The organic phase was washed with 0.5 M HCl (2 × 5 mL) and 1 M $KHCO_3$ (2 × 5 mL) and dried with anhydrous $MgSO_4$. 1,3-Diacetylchloramphenicol was separated from chloramphenicol and the monoacetylated products of the reaction by Chromatotron preparative TLC (1-mm plate thickness) using a 95% chloroform/5% methanol solvent system. 1,3-[2-^{13}C]Diacetylchloramphenicol: colorless oil; ^1H NMR (50 mM sodium phosphate (pH* 7.5) in D_2O) δ_H (referenced to dioxane at 0 ppm) 4.51 (d, meta to NO_2), 3.90 (d, ortho to NO_2), 2.50 (s, $CHCl_2$), 2.34 (d, C-1 H), 0.90 (q, C-2 H), 0.57 (C-3 H), 0.48 (C-3 H), −1.53 (d, C1 CH_3), −1.66 (d, C3 CH_3). 1-[2-^{13}C],3-[2-^{12}C]diacetylchloramphenicol was prepared in an identical fashion, using 3-acetylchloramphenicol as starting material.

Site-Directed Mutagenesis and Expression of CAT_{III}. Oligonucleotide-directed mismatch mutagenesis was employed using the deoxyuridine selection protocol described by Künkel et al. (1987). Double mutants were prepared using single-stranded M13 DNA template, with deoxyuridine misincorporation containing the *cat* gene with the S148A mutation (Lewendon et al., 1990). The presence of the two appropriate mutations and the absence of additional mutations was confirmed by determination of the nucleotide sequence of the entire *cat* gene and adjacent 5′-noncoding regions. Wild-type CAT_{III} proteins were overexpressed in *Escherichia coli* by transfer of the *cat* gene to plasmid pUC18 (Murray et al., 1988).

Purification of CAT_{III}. Mutant CAT variants with the substitutions S148A, F103I/S148A, F158I/S148A, and F33I/S148A were purified from *E. coli* extracts by affinity chromatography (Lewendon et al., 1988). The Y25I/S148A double mutant was purified by an alternative method as described previously (Murray et al., 1991). The purity of each enzyme preparation was assessed by SDS–polyacrylamide gel electrophoresis and the protein concentration measured by the method of Lowry et al. (1951) using wild-type CAT_{III} as standard.

Preparation of Perdeuterated CAT_{III}. The plasmid pIM5 (Murray et al., 1988) was transformed using a $CaCl_2$ procedure into the *E. coli* strain MG1655 (Maniatis et al., 1982), obtained from Dr. B. J. Bachmann, (*E. coli* Genetic Stock Center, Yale University). The growth medium contained Na_2DPO_4 (6 g/L), KD_2PO_4 (3 g/L), NaCl (0.5 g/L), ND_4Cl (1.0 g/L), and $MgSO_4$ (0.5 mg/mL) in 90% D_2O (Fluorochem) and 10% sterile distilled H_2O. Succinic-d_4 acid (98% + D; Aldrich) was added to 10 g/L and the pH adjusted to 7.0 with 1 M Na_2HPO_4. The medium was sterilized by filtration through a 0.22-μm filter (Millipore) and ampicillin added to 100 μg/mL. The cells were grown from a 5% (v/v) inoculum by shaking in baffled flasks at 30 °C. After 48 h, the production of CAT_{III} was induced by raising the temperature of growth to 37 °C, and growth was allowed to proceed for a further 72–96 h. The final optical density at 600 nm was between 3 and 4. The cells were harvested and the perdeuterated CAT_{III} was purified as described previously (Lewendon et al., 1988). The final yield was 89 mg of CAT_{III} from 700 mL of deuterated medium.

Assay of CAT Activity. The rate of chloramphenicol acetylation (the forward reaction) was followed spectrophotometrically at 25 °C and steady-state kinetics were conducted as described previously (Lewendon et al., 1988).

NMR Methods. All samples of CAT_{III} for NMR were transferred into 50 mM sodium phosphate buffer in D_2O (pH* 7.5) as described by Derrick et al. (1991). For the 1-D and 2-D ^{13}C (ω_2) half-filtered NOESY experiments, the concentration of 1,3-[2-^{13}C]-diacetylchloramphenicol was equal to that of CAT subunits, approximately 2 mM 1,3-[2-^{13}C]diacetylchloramphenicol and 50 mg/mL protein. NMR experiments were recorded on Bruker AM500 and AMX600 spectrometers at 313 K. For all one- and two-dimensional experiments, the residual HOD resonance was suppressed by using low-power presaturation. The 2-D ^{13}C (ω_2) filtered $^1H,^1H$ NOESY experiment was performed as described previously (Fesik et al., 1987; Otting & Wüthrich, 1990). ^{13}C decoupling in ω_2 was carried out using the WALTZ-16 pulse sequence (Shaka et al., 1983), and frequency discrimination via TPPI (Marion & Wüthrich, 1983). Typical pulse lengths used were, on the AM500, 90°(^1H) = 11.5 μs, 90°(^{13}C) = 10 μs, 90°(^{13}C for decoupling) = 80 μs; on the AMX600, 90°(^1H) = 13 μs, 90°(^{13}C) = 10 μs, 90°(^{13}C for decoupling) = 78 μs. A spectral width of 13 ppm in each dimension and mixing times between 60 and 90 ms were used, with typically 300–512 t_1 increments each of 2K data points in the ω_2 dimension. Quadrature detection in the ω_1 dimension was obtained by using the time proportional phase incrementation method. Each 2-D experiment took about 8 h. The data were processed using a Gaussian window function in both dimensions (GB = 0.1 Hz, LB = −10 Hz) with additional base-plane correction after Fourier transformation and phase correction. All chemical shifts are referenced to dioxane at 0 ppm (3.76 ppm from DSS under the conditions of these experiments).

For the experiments using perdeuterated wild-type CAT_{III}, the protein concentration was at 59 mg/mL (2.4 mM in substrate binding sites). Diacetylchloramphenicol was added in increments to give the final concentrations indicated. In order to limit hydrolysis of diacetylchloramphenicol, each ^1H spectrum was acquired in 7 min (64 scans) and the entire titration was complete within 30 min.

RESULTS AND DISCUSSION

The ^1H NMR spectrum of CAT_{III} is particularly crowded in the aliphatic region (Figure 1A), and the methyl resonances of bound diacetylchloramphenicol cannot be unambiguously identified. Use of a heteronuclear multiple-quantum coherence method to "edit" the spectrum of the 1,3-[2-^{13}C]diacetylchloramphenicol/wild-type CAT_{III} complex will permit

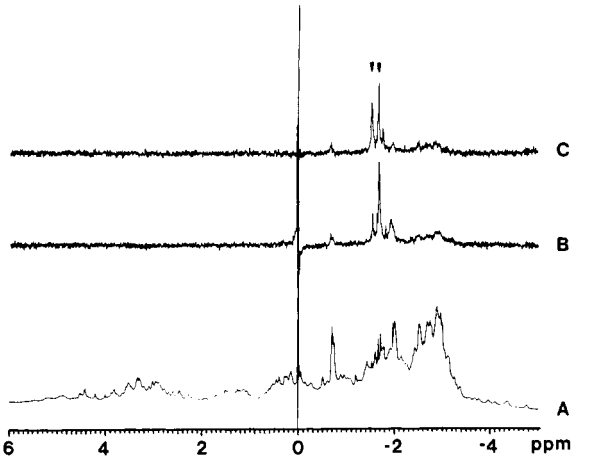

FIGURE 1: (A) 500-MHz ^{1}H NMR spectrum of wild-type CAT_{III}; (B) 500-MHz 1-D HMQC of 1,3-[2-^{13}C]diacetylchloramphenicol/wild-type CAT_{III} complex; (C) 1-D HMQC of diacetylchloramphenicol/CAT_{III} S148A complex.

selective detection of the two signals from protons directly attached to the ^{13}C nuclei in the 1-acetyl and 3-acetyl methyl groups of the bound ligand. Such an experiment with CAT_{III} is complicated by slow enzyme-catalyzed hydrolysis of the 3-acetyl group, leading to the appearance of more than two signals (Figure 1B). The instability of the ligand in the presence of wild-type CAT_{III} precluded analysis of the complex by two-dimensional methods. The problem was overcome by using a CAT_{III} mutant (S148A), which is known to be isostructural with wild-type CAT_{III} but has a k_{cat} which is 53-fold lower (Lewendon et al., 1990). (Serine 148 is postulated to stabilize the tetrahedral transition state which is formed in the course of transfer of the 3-acetyl group from CoA to chloramphenicol.) As anticipated, the 1-D HMQC spectrum from the 1,3-[2-^{13}C]-diacetylchloramphenicol/CAT_{III} S148A complex gave only two main signals, corresponding to the 1-acetyl and 3-acetyl methyl protons of the bound ligand (indicated in Figure 1C). The lifetime of this complex (8–12 h) was sufficient to permit analysis by 2-D NMR.

Assignment of the Signals from the Methyl Protons of the 1- and 3-Acetyl Groups. The specific assignment of the signals to the 1-acetyl and 3-acetyl groups was accomplished by using 1-[2-^{13}C],3-[2-^{12}C]diacetylchloramphenicol. The 1-D HMQC spectrum of this ligand revealed a single resonance from the methyl protons on the 1-acetyl group at −1.53 ppm. Similarly, a 1-D HMQC spectrum of the complex of 1-[2-^{13}C],3-[2-^{12}C]diacetylchloramphenicol with CAT_{III} S148A gave one signal from the 1-acetyl group, corresponding to the more downfield of the two resonances in Figure 1C. [It is this signal which disappears with time in the complex with wild-type CAT_{III}. Hydrolysis of the 3-acetyl group leads to an intramolecular rearrangement in which the equilibrium position is in favor of the 3-acetyl form (Scheme I). As hydrolysis proceeds, therefore, the signal from the 1-acetyl group declines.]

NOEs to the Acetyl Protons in Diacetylchloramphenicol/CAT_{III}S148A Complex. Resonances of protons close in space to the methyl groups of 1,3-diacetylchloramphenicol in its complex with the enzyme were identified by performing a ^{1}H,^{1}H NOESY experiment with ^{13}C (ω_2) half-filter on the 1,3-[2-^{13}C]diacetylchloramphenicol/CAT_{III} S148A complex, using a mixing time of 60 ms (Figure 2). This experiment allows one to observe only cross peaks corresponding to NOEs involving protons directly attached to ^{13}C. The patterns of NOEs from the 1- and 3-acetyl groups are different, reflecting

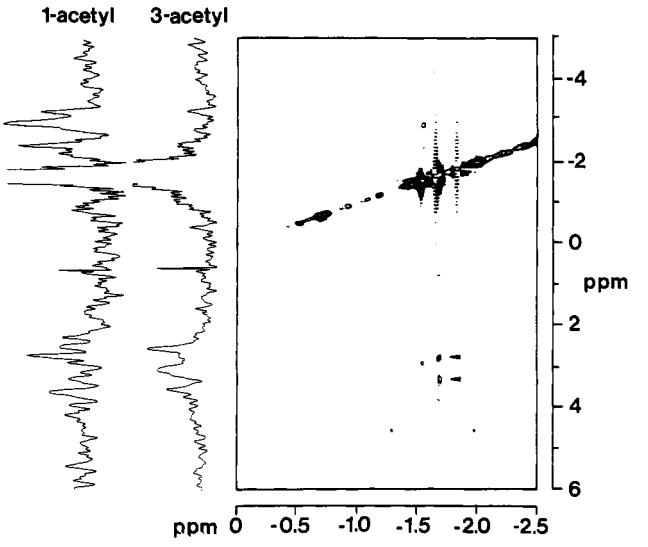

FIGURE 2: 600-MHz 2-D ^{1}H,^{1}H NOESY experiment with ^{13}C (ω_2) half-filter (τ_m = 60 ms) on the 1,3-[2-^{13}C]diacetylchloramphenicol/CAT_{III}S148A complex (columns showing 1-acetyl and 3-acetyl NOEs inset).

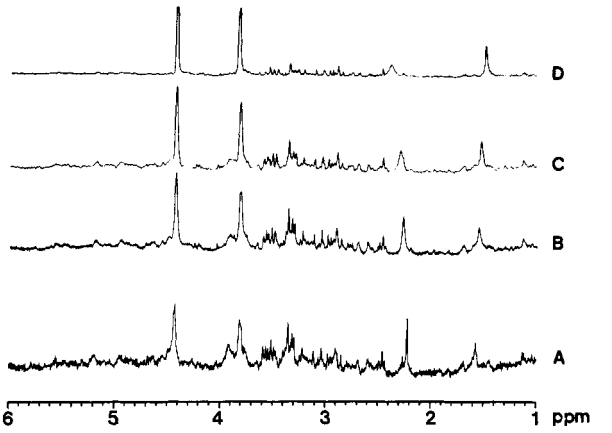

FIGURE 3: 500-MHz ^{1}H NMR spectra for the titration of chloramphenicol into perdeuterated CAT_{III}: (A) total concentration of chloramphenicol, 0.7 mM; (B) 1.2 mM chloramphenicol; (C) 1.7 mM chloramphenicol; (D) 2.7 mM chloramphenicol.

the different environments of each methyl group. The NOEs from the 1-acetyl group are weaker; probably due to the slow decay of this signal during the experiment. NOEs to protons in the aromatic region of the spectrum were detected for both the 1- and 3-acetyl groups. In particular, the 3-acetyl group shows two strong NOEs to resonances at 2.75 and 3.20 ppm (indicated in Figure 2). These two strong NOEs were selected for further study because they are well resolved and because the number of candidate aromatic residues in the active site is limited.

Assignment of these NOEs to specific residues is essential if useful structural information about the diacetylchloramphenicol/CAT_{III} S148A complex is to be obtained. However, for a protein as large as CAT_{III}, the conventional 2-D methods used for the assignment of ^{1}H NMR spectra of smaller proteins cannot be applied due to the short T_2 values and the complexity of the spectra. One obvious possibility which needs to be considered is that the NOEs originate from the aromatic ring protons of chloramphenicol itself. This possibility was explored by determining the resonance positions of those protons in bound chloramphenicol and diacetylchloramphenicol; in order to detect these resonances in the complex we used perdeuterated CAT_{III} (see Experimental Procedures). Figure 3 shows the results of an experiment in which chloramphenicol was

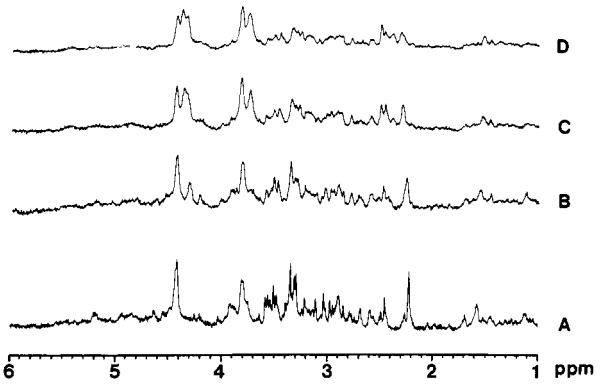

FIGURE 4: 500-MHz ^{1}H NMR spectra for the titration of diacetylchloramphenicol into perdeuterated CAT_{III}: (A) no added diacetylchloramphenicol; (B) 0.5 mM diacetylchloramphenicol; (C) 1.0 mM diacetylchloramphenicol; (D) 2.0 mM diacetylchloramphenicol.

added in increasing concentrations to a sample of perdeuterated CAT_{III}. Signals at 4.42 and 3.82 ppm, present in the absence of added chloramphenicol, originate from a small amount of chloramphenicol bound to the enzyme (0.3 molar equiv per subunit of CAT), arising from the purification procedure. As the concentration of chloramphenicol is increased, these resonances increase in intensity, with no change in chemical shift, indicating that the chemical shifts of the aromatic protons of chloramphenicol are the same in the free and bound states. By contrast, the resonance at 2.20 ppm in spectrum A (Figure 3), which arises from the dichloroacetamido group, is seen to shift downfield (and to broaden, due to exchange effects) as the chloramphenicol concentration is increased. In the case of diacetylchloramphenicol (Figure 4), the dichloroacetamido signal behaves similarly, but for the aromatic protons, two new signals appear slightly upfield of those of chloramphenicol. (The spectra are complicated by the simultaneous increase in the intensity of the chloramphenicol signals, due to slow hydrolysis of diacetylchloramphenicol during the course of the experiment.) These new signals, at 4.35 and 3.72 ppm when first detectable, indicate that the aromatic proton resonances of bound diacetylchloramphenicol are somewhat upfield of those of bound chloramphenicol. They also clearly eliminate the aromatic protons of the bound ligand as the origin of the NOEs at 2.75 and 3.20 ppm in Figure 2.

The crystal structure of the chloramphenicol/CAT_{III}S148A binary complex (Lewendon et al., 1990) was examined to identify all plausible aromatic residues which could give rise to the two NOEs observed in the experiment detailed in Figure 2. By use of the molecular modeling package QUANTA (Polygen Corp., Waltham, MA), two *O*-acetyl groups were added onto the C1 and C3 atoms of chloramphenicol and all aromatic ring protons within 3.5 Å (^{1}H–^{1}H distances) of the methyl groups were monitored. This analysis revealed four candidate aromatic residues which could give rise to the aromatic NOEs observed: Phe-33, Phe-103, Phe-158, and Tyr-25. To make the assignment of the NOE cross peaks, these residues in CAT_{III} S148A were each in turn replaced by isoleucine—a substitution which, although removing protons with chemical shifts in the aromatic region, still retains substantial hydrophobicity of the side chain in each case. The steady-state kinetic properties of these mutants are summarized in Table I. The mutations F33I and F158I, in conjunction with S148A, did not greatly alter the k_{cat} for the acetylation of chloramphenicol, relative to the value for S148A alone. The effects of the Y25I and F103I mutations were more

Table I: Steady-State Kinetic Parameters for the Acetylation of Chloramphenicol by Wild-Type and Mutant Chloramphenicol Acetyltransferases[a]

protein	k_{cat} (s^{-1})	K_m (μM) chloramphenicol	K_m (μM) acetyl-CoA
wild type	599	11.6	93
S148A	11.2	5.2	121
Y25I/S148A	0.75	40	189
F33I/S148A	6.4	34	576
F103I/S148A	0.43	21	368
F158I/S148A	10.7	9.9	163

[a] Kinetic parameters are the mean of two or more measurements and were determined as described under Experimental Procedures, with the exception of wild-type CAT_{III} and the S148A variant for which data are taken from Lewendon et al. (1990).

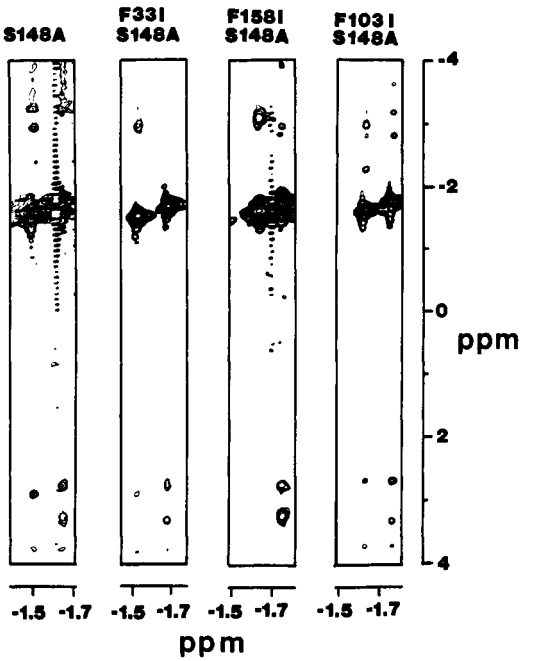

FIGURE 5: 2-D ^{1}H,^{1}H NOESY experiments with ^{13}C (ω_2) half-filter on the 1,3-[2-^{13}C]diacetylchloramphenicol/CAT_{III} mutant complexes (τ_m = 60 ms for all complexes except F103I/S148A, where τ_m = 90 ms).

pronounced, producing decreases in k_{cat} of 15- and 26-fold, respectively, and the K_m for chloramphenicol was slightly increased for all the mutants, from 1.9-fold for S148A/F158I to 7.7-fold for Y25I/S148A. The modest decrease in apparent affinity for chloramphenicol in each case reflects a decrease in the hydrophobic interactions which are known to play an important part in the binding of chloramphenicol by CAT_{III} (Murray et al., 1991). The K_m for acetyl-CoA is also increased in each mutant, although to a lesser extent than chloramphenicol.

2-D ^{1}H,^{1}H NOESY experiments with ^{13}C (ω_2) half-filter were carried out on all the mutant CAT_{III}/1,3-[2-^{13}C]diacetylchloramphenicol complexes. The two NOEs from the 3-acetyl group of diacetylchloramphenicol are present in the F33I/S148A and F158I/S148A mutants (Figure 5B and C, respectively), at the same mixing time as used in Figure 2 (60 ms). In the case of the F103I/S148A mutant the NOEs were weaker, with only one signal detectable at 60 ms (data not shown), although both were observed at a mixing time of 90 ms (Figure 5D). At longer mixing times more NOEs appeared, many of which were common to the 3-acetyl and 1-acetyl columns, due to spin diffusion between protons in the active site (a similar problem was encountered with other mutants

studied and placed an upper limit on the mixing times which could be used). The fact that the two NOEs at 2.75 and 3.20 ppm persist in all the 2-D $^1H,^1H$ NOESY experiments on the CAT_{III} mutant/diacetylchloramphenicol complexes positively rules out the aromatic ring protons of F33, F103, and F158 as the source of these NOEs. Three out of the original four candidate residues are thus excluded and the assignment must be made to the only remaining possibility, Y25.

This assignment was tested further by examination of the Y25I/S148A CAT_{III}/1,3-[2-^{13}C]diacetylchloramphenicol binary complex. The 1-D HMQC spectrum of the complex differed from all the other mutant CAT_{III} binary complexes in that both the methyl proton signals of the 1- and 3-acetyl groups were shifted downfield and were considerably broader. It is possible that this is caused by exchange broadening, due to a change in the affinity of the Y25I/S148A mutant for diacetylchloramphenicol. A 2-D $^1H,^1H$ NOESY experiment with ^{13}C (ω_2) half-filter was carried out on the Y25I/S148A CAT_{III}/1,3-[2-^{13}C]diacetylchloramphenicol complex at a mixing time of 60 ms. No NOEs to any part of the spectrum were observed from either the 1-acetyl or 3-acetyl group (results not shown). It was therefore not possible to confirm the assignment of the two NOEs to Y25 directly by observation of new NOEs to side-chain protons in the substituted isoleucine, although the changes in chemical shifts and line widths of the acetyl proton signals are consistent with the conclusion that Y25 is involved in ligand binding.

The demonstration of a spatial proximity between the 3-acetyl group and the aromatic ring protons of Y25 provides two restraints which could be used to model diacetylchloramphenicol into the active site of CAT_{III}. Clearly, more distance constraints will be needed to do this with confidence. Other lines of evidence also suggest that the conformation or orientation of diacetylchloramphenicol in the active site may differ from that of chloramphenicol. For example, from the results in Figures 3 and 4, it is clear that the chemical shifts of the protons in the aromatic ring of enzyme-bound diacetylchloramphenicol are different from those of enzyme-bound chloramphenicol, suggesting that the ring may bind differently in the two cases. Steady-state kinetic studies have established that diacetylchloramphenicol is a 100-fold poorer substrate (deduced from k_{cat} values) for the reverse reaction than is 3-acetylchloramphenicol, although the K_m for both ligands is similar (Lewendon, A., and Shaw, W. V., unpublished observations). In the case of the forward reaction, 1-acetylchloramphenicol is a much poorer substrate than chloramphenicol [k_{cat}'s are 15 and 599 s^{-1}, respectively (Murray et al., 1991)]. Such results indicate that the presence of an acetyl substituent on the 1-hydroxyl affects the rate of acetylation or deacetylation at the 3-position.

The results presented here demonstrate that isotope-aided ^1H NMR techniques can be used to obtain specific and useful structural information about ligand/protein interactions in the CAT_{III} system and suggest that comparable results could be obtained for other enzymes which are too large to analyze by conventional NMR methods. More generally, it can be seen that solution studies complement the crystallographic approach, especially when important binary or ternary complexes of enzyme and ligand(s) fail to yield crystals suitable for X-ray diffraction work. With CAT_{III} it is now possible to define two binary complexes (CAT_{III}/chloramphenicol and CAT_{III}/CoA), to model a tetrahedral intermediate and confirm its plausibility by site-directed mutagenesis (Lewendon et al., 1990), and to infer from NMR data a possible structure for the ultimate CAT_{III}/product complex, that of enzyme and diacetylchloramphenicol. Analogous studies using Fourier transform infrared spectroscopy have demonstrated a hydrogen bond between the ester carbonyl oxygen of 3-acetylchloramphenicol and the hydroxyl of Ser-148 (Derrick, J. P., Shaw, W. V., White, A., and Wharton, C. W., unpublished experiments). Such structural information should allow a more complete picture of the interplay between ligand binding transactions and catalysis, one which can be tested by pre-steady-state kinetic techniques applied to the CAT_{III} system (Ellis et al., 1991a,b).

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