Articles

Analysis of Hydrogen Bonding in Enzyme–Substrate Complexes of Chloramphenicol Acetyltransferase by Infrared Spectroscopy and Site-Directed Mutagenesis†

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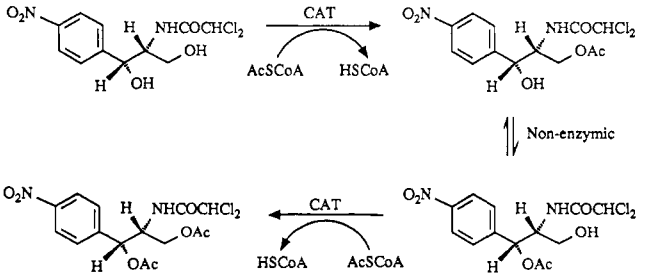
ABSTRACT: Chloramphenicol acetyltransferase (CAT) reversibly transfers an acetyl group between CoA and the 3-hydroxyl of either chloramphenicol (Cm) or 1-acetylchloramphenicol (1AcCm). The products of the forward reactions, 3-acetylchloramphenicol (3-AcCm) and 1,3-diacetylchloramphenicol (1,3Ac_2-Cm), are the substrates for the reverse reaction. The role of the 3-acetyl carbonyl group in the binding of the substrates 3AcCm and 1,3Ac_2Cm to CAT has been investigated using infrared spectroscopy. Comparison of difference spectra (3-[^{12}C=O]acetyl- minus 3-[^{13}C=O]acetyl-) obtained for the binary complexes of 3AcCm with wild-type CAT, and with a variant wherein serine-148 is replaced by alanine (S148A), reveals a large (9 cm^{-1}) down frequency shift for the 3-acetyl carbonyl stretch in the wild-type complex, indicative of a hydrogen bond between this carbonyl and the hydroxyl group of Ser-148. The carbonyl bandwidth in the wild-type complex is reduced by 33% compared to that for the complex with S148A, indicating restriction of carbonyl mobility and dispersion in the former, an observation consistent with the proposed hydrogen bond between the ester carbonyl and the hydroxyl of Ser-148. Repetition of the experiment using 1,3Ac_2Cm as the ligand reveals a frequency shift of only 3 cm^{-1} between wild-type and S148A complexes, indicating only a small change in the strength of carbonyl interaction. As the carbonyl stretch frequency (1727 cm^{-1}) for the wild-type CAT complex with 1,3Ac_2Cm is the same as that of the S148A enzyme complex with 3AcCm, it is likely that the hydrogen bond of the 3-acetyl carbonyl with Ser-148 is absent in the complex of CAT with 1,3Ac_2Cm and therefore that (a) the modes of binding of the two ligands are different and (b) an explanation is available for the relative inefficiency of interconversion of 1-acetylchloramphenicol and 1,3Ac_2Cm. More generally, the results also illustrate the value of infrared spectroscopy in the study of the binding of alternative ligands to the same active site.

Chloramphenicol acetyltransferase (CAT;[1] EC 2.3.1.28) is a common effector of prokaryotic resistance to the antibiotic chloramphenicol (Cm), a potent inhibitor of the peptidyltransferase activity of bacterial ribosomes (Shaw, 1967). The enzyme catalyzes acetyl transfer from acetyl-CoA to the 3-hydroxyl of chloramphenicol, yielding 3AcCm, which is unable to bind to the ribosome and hence is devoid of antimicrobial activity. 3AcCm can undergo nonenzymic intramolecular rearrangement to 1-acetylchloramphenicol (1AcCm), which is a substrate for a second enzymic acetylation cycle at the 3-hydroxyl to yield 1,3Ac_2Cm as the final product (Scheme 1). Since the enzymic acetylation is reversible, it is readily appreciated that 3AcCm and 1,3Ac_2Cm, the products of the forward reaction, are also substrates for acetyl transfer to CoA [reviewed in Shaw and Leslie (1991)].

Scheme 1: Enzymic and Nonenzymic Relationships between Chloramphenicol, 3-Acetylchloramphenicol, 1-Acetylchloramphenicol, and 1,3-Diacetylchloramphenicol



The structure of the binary complex between type III CAT and Cm has been determined at 1.75-Å resolution, and that

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase (type III variant); Cm, chloramphenicol; 1AcCm, 1-acetylchloramphenicol; 3AcCm, 3-acetylchloramphenicol; 3[^{12}C=O]AcCm and 3[^{13}C=O]AcCm, 3AcCm containining ^{12}C or ^{13}C in the carbonyl of the 3-acetyl group; 1,3Ac_2Cm, 1,3-diacetylchloramphenicol; 1Ac,3[^{12}C=O]AcCm and 1Ac,3[^{13}C=O]AcCm, 1,3Ac_2Cm containining ^{12}C or ^{13}C in the carbonyl of the 3-acetyl group; S148A, site-directed mutant of CAT wherein alanine replaces serine-148.

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with CoA at 2.4-Å, by X-ray crystallography (Leslie *et al.*, 1988: Leslie, 1990). The enzyme is trimeric (3 × 25 kDa) with three identical active sites located at the interfaces between adjacent subunits. Cm binds in a deep cleft between the subunits,with the majority of contacting residues provided by one subunit, whereas the catalytically essential imidazole of His-195 is provided by the adjacent monomer. His-195 forms a hydrogen bond to the 3-hydroxyl group of Cm and is thus critically disposed to abstract the hydroxyl proton and promote nucleophilic attack at the thioester carbonyl carbon of acetyl-CoA, yielding an oxyanion tetrahedral intermediate with negative charge developing on the carbonyl oxygen of the acetyl group. Modeling studies produced a plausible model for the bound tetrahedral intermediate and predicted an important role for the hydroxyl of Ser-148 in stabilizing the putative oxyanion, a proposal subsequently confirmed by site-directed mutagenesis and X-ray crystallography (Lewendon *et al.*, 1990). The importance of His-195 had been predicted on the basis of sequence alignments and chemical modification studies (Kleanthous *et al.*, 1985) and is supported by recent kinetic and mutagenic analyses (Lewendon & Shaw, 1993; Lewendon *et al.*, 1994).

As each of the acetylated Cm derivatives are devoid of antibiotic activity, the second acetylation cycle catalyzed by CAT has no obvious biological function *in vivo*. Although utilization of additional acetyl-CoA to produce 1,3Ac_2Cm appears wasteful, the reaction is common to all natural isolates of CAT for which data are available. [An exception is a recently-identified second class of bacterial chloramphenicol acetylating enzymes which are not homologous with CAT (Parent & Roy, 1992; Murray *et al.*, manuscript in preparation).] The second acetylation cycle and the binding of competitive inhibitors carrying hydroxyl, acetyl, and propionyl groups linked to the C-1 position of 3-iodo-3-deoxychloramphenicol have been studied by steady-state kinetic analysis, site-directed mutagenesis, and X-ray crystallography (Murray *et al.*, 1991). Although the structure of the CAT/Cm binary complex indicated a cavity where the 1-acetyl group might bind without reorientation of the remainder of the ligand, mutations introduced to preclude binding at this site did not yield enzymes with the expected enhanced discrimination against 1AcCm.

No structural information is available for crystalline complexes between CAT and any of the acetylated ligands (acetyl-CoA, 1AcCm, 3AcCm, or 1,3Ac_2Cm), due to the intramolecular rearrangement of the monoacetylated species and the fact that both the acetyl-CoA thioester and the 3-acetyl group of acetylated Cm are subject to slow enzymic hydrolysis when bound to CAT in the absence of an appropriate second substrate. The same factors and the complexity of ^{1}H-NMR spectra, combined with the time required for data collection for a protein as large as CAT (75 kDa), preclude analysis by NMR of the binding of acetylated substrates to the wild-type enzyme. However, Derrick *et al.* (1992) used isotope-edited ^{1}H NMR spectroscopy in combination with site-directed mutagenesis to study the complex between 1,3[2-^{13}C]Ac_2Cm and a catalytically-compromised mutant of CAT (to slow hydrolysis of the 3-acetyl moiety), demonstrating NOEs between methyl protons of the 3-acetyl group and aromatic protons of Tyr-25, a residue which is known to interact with Cm in the binary complex (Leslie, 1990; Murray et al., 1991b).

Infrared spectroscopy can provide a very effective means of demonstrating and evaluating specific interactions between enzymes and bound ligands (Wharton, 1986), and a number of systems have been successfully studied using this technique. Examples include triosephosphate isomerase (Belasco & Knowles, 1980; Komives *et al.*, 1991), β-lactamase (Fisher *et al.*, 1980), aldolase (Belasco & Knowles, 1983), citrate synthase (Kurz & Drysdale, 1987), α-chymotrypsin (White & Wharton, 1990; White *et al.*, 1992; Johal *et al.*, 1994), and phospholipase A_2 (Slaich *et al.*, 1992). Infrared spectroscopy offers considerable time resolution, making the technique especially suitable for labile systems—such as the complexes between CAT and its acetylated substrates. Furthermore, infrared spectroscopy can not only detect protein–ligand interactions but can also yield quantitative information about their nature and strength (White & Wharton, 1990). A disadvantage of the technique is that isotope editing is essential to eliminate spectral contributions arising from perturbation between free and complexed enzyme (Tonge *et al.*, 1991; White *et al.*,1992). Isotope editing also allows identification of a single specific interaction among a number of similar interactions (Slaich *et al.*, 1992). The vibrational frequencies of groups measured by infrared spectroscopy can furnish a measurement of bond strength and can further be related to hydrogen bonding and environmental polarity. Also the widths of infrared absorption bands can be related to the mobility and conformational dispersity of the absorbing group. For hydrogen bonds between the protein and bound ligand in enzyme–ligand complexes, a down frequency shift and narrowing of the infrared band for the ligand group contributing to the hydrogen bond can be expected (White & Wharton, 1990).

Ester carbonyl groups are especially amenable to study by infrared spectroscopy (Fisher *et al.*, 1980; White & Wharton, 1990). Although their stretching vibrational mode gives fairly strong and readily detected absorption at, or just above, 1700 cm^{-1}, isotopic substitution of the carbonyl by ^{13}C and/or ^{18}O is required to eliminate contributions from enzymic amide and carboxyl groups (White *et al.*, 1992; Johal *et al.*, 1994). The experiments described here use the infrared method to (a) identify a hydrogen bond between the hydroxyl group of Ser-148 of CAT and the (3-acetyl) carbonyl of 3AcCm and (b) demonstrate that this interaction is absent in the binary complex of CAT with 1,3Ac_2Cm. The implications of differences in the binding of the two ligands are discussed in the context of spectroscopic (NMR) and crystallographic studies of CAT which have called attention to unexpected binding modes for substrates or their analogues and their consequences for catalytic efficiency.

EXPERIMENTAL PROCEDURES

Preparation of 3AcCm, 1,3Ac_2Cm, 3[^{13}C=O]AcCm, and 1Ac,3[^{13}C=O]AcCm. 3AcCm and 3[^{13}C=O]AcCm: 30 mg of Cm (90 μmol) was dissolved in 0.5 mL of dry pyridine and cooled on ice; 180 μmol of acetyl chloride or 1-[^{13}C]acetyl chloride (99% ^{13}C, Aldrich) was added and the reaction allowed to proceed on ice for 10 min before being terminated by the addition of deionized water. Products were immediately extracted into ethyl acetate, washed with 0.5 M HCl and 1 M $KHCO_3$, and dried with anhydrous $MgSO_4$ as described previously (Derrick *et al.*, 1992). 3AcCm was separated from Cm and other acetylated species by preparative TLC using a Chromatotron apparatus and 1 mm silica plate developed in chloroform/methanol (95:5 v/v). 1,3Ac_2Cm and 1Ac,3-[^{13}C=O]AcCm: Diacetylated chloramphenicol derivatives were prepared by the same method using either 3AcCm or 3[^{13}C=O]AcCm as starting material. Acetylated chloramphenicol derivatives were stored in dry acetonitrile prior to use.

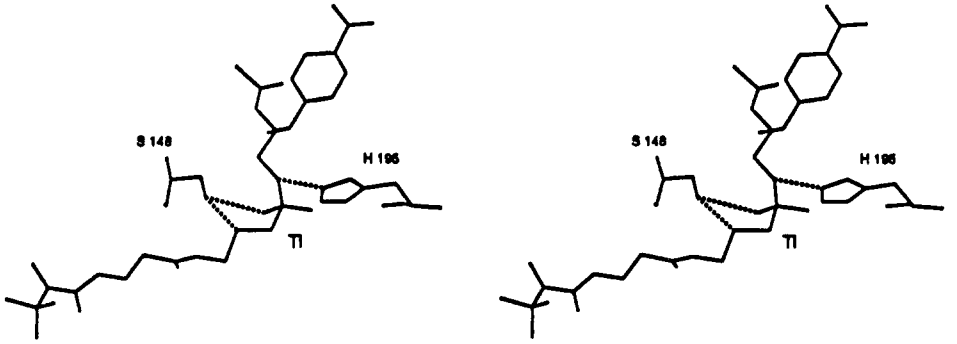

FIGURE 1: Stereoview showing the modeled oxyanion tetrahedral intermediate (TI) bound to CAT. The hydroxyl of Ser-148 makes two hydrogen bond interactions with the intermediate, to the oxyanion and to an NH group in CoA. The hydrogen bond between His-195 and the oxygen atom derived from the 3-hydroxyl group of chloramphenicol is also included to emphasize the requirement for prior proton abstraction by $N^{\epsilon 2}$ of the catalytic imidazole group in the binary complex, *en route* to the tetrahedral intermediate. For clarity, part of the intermediate beyond the first phosphate group of CoA has been deleted.

Expression, Purification, and Characterization of Wild-Type and S148A CAT and Their Interaction with Ligands. High-level expression of wild-type and mutant genes encoding CAT was achieved using previously described plasmid vectors (Murray *et al.*, 1988). CAT was purified from cell-free extracts of *Escherichia coli* by affinity chromatography (Lewendon *et al.*, 1988). Purity was assessed by SDS–polyacrylamide gel electrophoresis and concentration determined by extinction at 280 nm ($\epsilon_{0.1\%}$ = 1.314). The steady-state kinetic analysis of the interaction of 3AcCm with both wild-type and S148A CAT was carried out by the methods described by Kleanthous and Shaw (1984). Since the transacetylation reaction catalyzed by CAT proceeds in both directions by a random order of addition (ternary complex) mechanism, it is possible to determine dissociation constants (K_d) for binary complexes of CAT and substrates, in this case, those for CAT/AcCm and CAT/1,3Ac_2Cm.

Infrared Spectroscopy. CAT samples were transferred into 50 mM sodium phosphate buffer in D_2O (pH* 7.5) by repeated washing using an Amicon ultrafiltration unit. Enzyme concentration was adjusted to 50 mg/mL (2 mM monomers) and kept on ice. Acetylated ligands (33 mM stock solutions in dry acetonitrile) were added to CAT in the ratio 6:100 (~2 mM final concentration of ligand) using a Hamilton syringe. This was examined immediately to minimize hydrolysis and acetyl migration. An equivalent amount of acetonitrile was included in control experiments in the absence of enzyme. Infrared spectra were collected using a Nicolet 60SX Fourier transform infrared spectrometer (Nicolet Instruments Limited, Warwick, U.K.), at 2 cm^{-1} wavenumber resolution, using 1024 scans for each spectrum, and at a scanning rate of 4 s^{-1}. The spectrometer was connected to a stable supply of air dried to a dew point of −70 °C in order to avoid appearance of spectral bands for water vapor. The infrared cell used was a 50-μm path length transmission cell constructed by calcium fluoride windows and a Teflon spacer (Specac Limited, Orpington, Kent, U.K.). The cell was specially designed and constructed in-house with fine-bore tubing and suitable connectors. This allowed filling and emptying from outside the spectrometer, which was essential to prevent entry of water vapor and also limited the dead volume of the cell to 70 μl. The double-beam spectra were generated by ratioing of single-beam spectra collected as described above.

RESULTS

Binding of 3AcCm. Preliminary experiments comparing infrared spectra (^{12}C–^{13}C) of 3AcCm, bound to wild-type CAT and free in solution, revealed a significant down shift

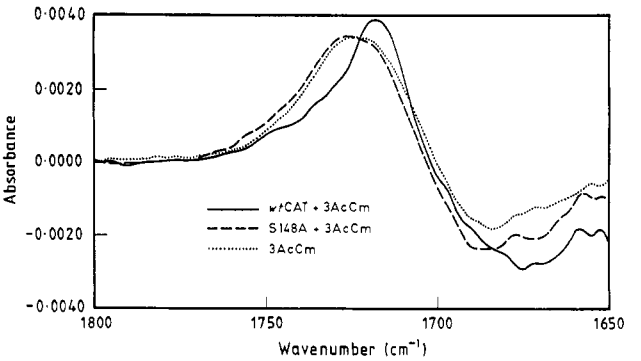

FIGURE 2: IR spectra of 3AcCm bound to *wt*CAT and S148A CAT, and unbound. Solid line: IR spectrum of *wt*CAT/3[^{12}C=O]AcCm versus *wt*CAT/3[^{13}C=O]AcCm. Dashed line: IR spectrum of S148A CAT/3[^{12}C=O]AcCm versus S148A CAT/3[^{13}C=O]AcCm. Dotted line: IR spectrum of 3[^{12}C=O]AcCm versus 3[^{13}C=O]AcCm. All spectra were collected at 2 cm^{-1} wavenumber resolution, using 1024 scans at a scanning rate of 4 s^{-1}. Path length was 50 μm. These double-beam spectra were generated by ratioing of single-beam spectra.

in the carbonyl stretch frequency of the 3-acetyl group on binding to the enzyme (data not shown), suggestive of a specific interaction between the carbonyl group and features of the substrate binding site. The hydroxyl group of Ser-148 is an obvious candidate for an interaction with the carbonyl oxygen as it is known to interact with the equivalent oxygen (an oxyanion) in the complex of CAT with the proposed tetrahedral intermediate on the reaction pathway (Figure 1). The S148A variant of CAT was prepared and used in all subsequent experiments, permitting the comparison of spectra of unbound ligands and their complexes with both wild-type and mutant CAT. The S148A enzyme is known to be isostructural with wild-type CAT, but k_{cat} for the first acetylation reaction is reduced ~50-fold due to loss of the stabilizing interaction with the oxyanion intermediate (Lewendon *et al.*, 1990). Nonetheless, as judged by the dissociation constants, from steady-state kinetic experiments, for the binary complexes of 3 AcCm with both wild-type CAT (K_d = 23 μM) and S148A CAT (K_d = 51 μM), it is clear that affinity for the ligand is not compromised for the latter protein. Furthermore, under the experimental conditions for collecting infrared spectra for both binary complexes in the experiments described below (2 mM for CAT monomers and 3 AcCm), the high affinity of both wild-type and substituted CAT ensures that more than 85% of the 3AcCm is in the bound state.

Infrared difference spectra (^{12}C–^{13}C) for 3AcCm bound to wild-type and S148A CAT and free in solution are shown in Figure 2. Associated carbonyl stretch frequency and band-

Table 1 [a]

spectrum	$\nu_{C=O}$ (cm^{-1})	$w_{C=O}$ (cm^{-1})
*wt*CAT/3[12C=O]AcCm vs *wt*CAT/3[^{13}C=O]AcCm	1718	22
S148A CAT/3[^{12}C=O]AcCm vs S148ACAT/3[^{13}C=O]AcCm	1727	33
3[^{12}C=O]AcCm vs 3[^{13}C=O]AcCm	1724	33
*wt*CAT/1Ac,3[^{12}C=O]AcCm vs *wt*CAT/1Ac,3[^{13}C=O]AcCm	1727	29
S148A CAT/1Ac,3[^{12}C=O]AcCm vs S148A CAT/1Ac,3[^{13}C=O]AcCm	1730	32
1Ac,3[^{12}C=O]AcCm vs 1Ac,3[^{13}C=O]AcCm	1727	32

[a] $\nu_{C=O}$ = frequency of ^{12}C-carbonyl band for 3-acetyl group. $\omega_{C=O}$ = full width at half-maximum height of ^{12}C-carbonyl band for 3-acetyl group.

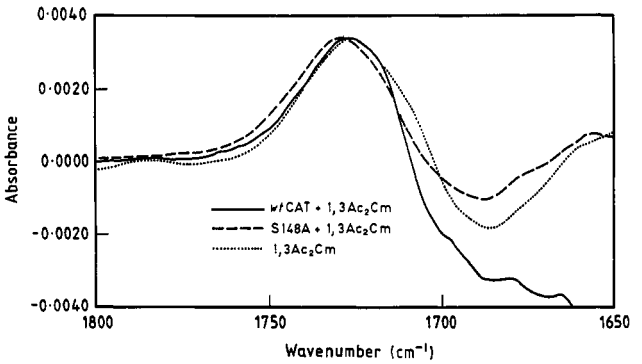

FIGURE 3: IR spectra of 1,3Ac_2Cm bound to *wt*CAT and S148A CAT, and unbound. Solid line: IR spectrum of *wt*CAT/1Ac,3-[^{12}C=O]AcCm versus *wt*CAT/1Ac,3[^{13}C=O]AcCm. Dashed line: IR spectrum of S148A CAT/1Ac,3[^{12}C=O]AcCm versus S148A CAT/1Ac,3[^{13}C=O]AcCm. Dotted line: IR spectrum of 1Ac,3-[^{12}C=O]AcCm versus 1Ac,3[^{13}C=O]AcCm. All spectra were collected at 2 cm^{-1} wavenumber resolution, using 1024 scans at a scanning rate of 4 s^{-1}. Path length was 50 μm. These double-beam spectra were generated by ratioing of single-beam spectra.

width data are summarized in Table 1. The wild-type CAT complex reveals a 9 cm^{-1} down frequency shift for the 3-acetyl carbonyl stretch when compared with the S148A complex, consistent with significantly stronger hydrogen bonding in the former. Infrared bandwidths may be directly related to the mobility of the absorbing group and, in this case, stronglysupport the proposition of hydrogen bonding between the carbonyl group and the hydroxyl of Ser-148. The carbonyl bandwidth of the wild-type complex is reduced by 33% compared to those of both the unbound ligand and the S148A complex, indicating restriction of carbonyl mobility and dispersion in the complex with wild-type CAT. Thus, it appears that hydrogen bonding to Ser-148 of CAT contributes to binding in the product complex in addition to stabilization of an intermediate during catalysis of the first acetylation cycle.

Binding of 1,3Ac_2Cm. Figure 3 shows infrared difference spectra (^{12}C–^{13}C) for 1,3Ac_2Cm free in solution and in binary complex with wild-type and S148A CAT, for which the K_d values are 32 μM and 45 μM, respectively, corresponding to binding occupancies of 88% for the wild-type enzyme and 85% for S148A CAT. In contrast to the situation described for 3AcCm, there is only a small (3 cm^{-1}) down frequency shift for the carbonyl group between the complexes with S148A and wild-type CAT (Table 1). The frequency for the wild-type complex with 1,3Ac_2Cm is identical (1727 cm^{-1}) to that of the 3AcCm/S148A CAT complex, suggesting that the hydrogen bond to Ser-148 is absent when the diacetylated ligand is bound. Bandwidth measurements again corroborate frequency data, with similar values for 1,3Ac_2Cm/wild-type CAT, 1,3Ac_2Cm/S148A CAT, and 3AcCm/S148A CAT complexes. In fact, bandwidths are the same, within experimental error, for every carbonyl population in the present study with the exception of the 3AcCm complex with wild-type CAT.

DISCUSSION

In a previous study (Murray *et al.*, 1991), substitutions were introduced into the chloramphenicol binding site of CAT by site-directed mutagenesis in an attempt to produce an enzyme variant which was selectively impaired in its capacity to acetylate 1AcCm to yield 1,3Ac_2Cm. Implicit in the design of such mutations was the assumption that both 3-acetyl acceptors (Cm and 1AcCm) are bound in an essentially identical fashion. The failure to achieve the desired kinetic properties was taken as circumstantial evidence that such an assumption might be invalid. Although the energetics of acetyl transfer *per se* (AcSR to AcOR') are the same in each case, the possibility remained that the Cm/3AcCm and 1AcCm/1,3Ac_2Cm substrate/product *pairs* might bind in different orientations, particularly as 1AcCm arises from 3AcCm via a nonenzymic rearrangement. The demonstration by infrared spectroscopy of a hydrogen bond between the hydroxyl group of Ser-148 and the 3-acetyl carbonyl of 3AcCm, and the absence of such an interaction in the binary complex of CAT with 1,3Ac_2Cm, provides direct evidence that the two ligands bind with different geometries to the catalytic center of wild-type CAT. Differences between binding of the substrates/products of the two acetylation cycles would also account for the observation of NOEs between methyl protons of the 3-acetyl group and aromatic protons of Tyr-25 when the complex between CAT and 1,3Ac_2Cm was studied by NMR spectroscopy (Derrick *et al.*, 1992). Such a result was not predicted on the basis of modeling studies of the CAT/1,-3Ac_2Cm interaction because the two acetyl groups were built onto the known structure of the CAT/Cm binary complex, assuming that the positions of the shared atoms of the two ligands were superimposable (M. J. Sutcliffe, unpublished results).

The acetylation of Cm to yield 3AcCm catalyzed by wild-type CAT is more efficient than the production of 1,3Ac_2Cm from 1AcCm, k_{cat} values being 599 s^{-1} and 15 s^{-1}, respectively (Lewendon *et al.*, 1988; Murray *et al.*, 1991a). Steady-state kinetic analysis of the two corresponding reverse reactions suggests that acetyl transfer from 1,3Ac_2Cm to CoA is similarly inefficient, compared to the situation wherein 3AcCm is the acetyl donor (A. Lewendon, personal communication). Does the detection of differences in the binding of 3AcCm and 1,3Ac_2Cm offer any clues to explain the sluggishness of the steps to and from the latter? One possibility is that the hydroxyl of Ser-148 not only fails to bind the 3-acetyl carbonyl of 1,3Ac_2Cm but is also incorrectly positioned to interact with the oxyanion tetrahedral intermediate in both the forward and reverse reactions involving 1,3Ac_2Cm. In this respect, it should be noted that deletion of the O^{γ} of Ser-148 (by the mutation S148A) results in a dimunition in k_{cat} for the acetylation of Cm which is similar in magnitude to that which occurs when 1AcCm replaces Cm as acetyl acceptor with the wild-type enzyme (k_{cat} = 11.2 s^{-1} for S148A CAT) (Lewendon *et al.*, 1990). An alternative explanation is that although the expected interaction between Ser-148 and the oxyanion of the tetrahedral intermediate is maintained in the second acetylation cycle, the 3-hydroxyl group of the acetyl acceptor is inappropriately oriented for deprotonation by the imidazole

of His-195 when 1AcCm is the substrate.

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