Ligand Interaction Energies and Molecular Recognition by Chloramphenicol Acetyltransferase†

Paul M. Cullis,*,‡ Ann Lewendon,§ William V. Shaw,§ and John A. Williams‡

Departments of Chemistry and Biochemistry, University of Leicester, Leicester LE1 7RH, U.K.

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ABSTRACT: The apparent binding energy for the interaction of the 3-hydroxyl group of chloramphenicol (CM) with the proposed general base (His-195) in chloramphenicol acetyltransferase (CAT) was determined by comparison of the dissociation constants of CM and 3-deoxyCM with CAT. The ΔG_{app} for this hydrogen bond to the N-3 of the imidazole ring is 1.5 kcal mol^{-1}. Extending the use of modified ligands, in an approach which is complementary to that of site-directed mutagenesis, the binding affinity of each of a family of 3-halo-3-deoxychloramphenicol derivatives was observed to increase in the series F < Cl < Br < I and is dominated by hydrophobic considerations. There is a linear free energy relationship between the dissociation constants for binding to CAT and an empirical hydrophobicity scale derived from reverse-phase HPLC retention times. The existence of such a relationship allows a true estimate of the total energetic contribution of interactions between the 3-hydroxyl group of CM and its contacts at the active site of CAT to be made on the basis of a regression analysis. The calculated value of ΔG_{bind} (2.7 kcal mol^{-1}) must include not only the hydrogen bond but also some favorable van der Waals interactions. The results demonstrate some of the advantages of an analysis of the energetics of ligand binding using modified ligands, in an approach that is formally analogous with and complementary to the use of site-directed mutations. The use of substrate analogues has a particular contribution to make in studies of interactions at or near the catalytic center, where enzyme mutagenesis may lead to structural changes that are difficult to detect in the absence of high-resolution structural information.

The use of site-directed mutations to assess quantitatively the contributions to binding and catalysis of individual enzyme–ligand interactions is now well established, as is the general principle that amino acid substitutions should not disrupt the protein structure (Knowles, 1987; Shaw, 1987). Nonetheless, possible changes at present are limited to the side chains of the 20 naturally occurring amino acids found in proteins and specified by the genetic code (Roesser et al., 1986). Furthermore, interactions of ligands with backbone functional groups cannot be probed by this approach. In contrast to such directed local changes in enzyme structure, "mutation" of the substrate affords a considerably wider range of chemically defined alterations to the interaction of enzyme and ligand and provides access to the same energetic data. Although varying substrate structure has been widely exploited, its use in parallel or in concert with genetic manipulation significantly amplifies the range of tools available to dissect protein–ligand interactions. Using modified substrates, we report here the results of an analysis of the interaction of the 3-substituted substrate analogues of chloramphenicol (CM) with the cognate binding site in chloramphenicol acetyltransferase (CAT), the enzymic effector of bacterial resistance to CM (Shaw, 1967).

The inactivation of CM by CAT occurs via acetylation of the 3-hydroxyl group with acetyl coenzyme A (CoA) as the acyl donor (Figure 1). Of the 11 natural variants of CAT for which amino acid sequence data are available, the type III enzyme has been studied in the greatest detail both structurally and mechanistically. CAT_{III} is a trimer of identical subunits of M_r 25 000, with the active sites located at each of the three subunit interfaces. The structure of the binary complex of CAT_{III} and CM has been refined to 1.75 Å and that of enzyme with CoA is available at 2.4-Å (Leslie et al., 1988; Leslie, 1990) resolution. CM and CoA approach the active site from opposite faces of the protein via a remarkable "tunnel" that extends through the protein.

Steady-state kinetics have revealed the mechanism to be a sequential one involving the random order of addition of substrates to form a ternary complex (Kleanthous & Shaw, 1984). The imidazole of His-195 has been identified as the general base in the acyl transfer reaction on the basis of its conservation within the CAT family (Shaw, 1983), reactivity with the affinity reagent 3-bromoacetyl chloramphenicol (Kleanthous et al., 1985), and more recently by the high-resolution structure of the binary complex (Leslie, 1990). It is apparent from the latter that whereas most of the binding interactions of CM with CAT_{III} are contributed by the face of one of the subunits forming the intersubunit cleft, His-195 arises from the polypeptide chain of the opposing subunit. The interaction of CM with CAT involves only two direct hydrogen bonds (the 3-hydroxyl of CM to N-3 of His-195 and the carbonyl oxygen of the dichloroacetamide group of CM to the hydroxyl of Tyr-25). A third hydrogen bond (the 1-hydroxyl of CM to Thr-174) occurs via a bridging water molecule. In addition there are extensive hydrophobic interactions dictated by the amphipathic nature of CM (Figure 1). The 3-hydroxyl group that is the acetyl acceptor is not only hydrogen bonded to the imidazole of His-195 but also inaccessible to solvent. Although in principle site-directed mutagenesis provides an approach to the energetics of such an interaction by substitution of His-195 with a residue that is not a hydrogen-bonded acceptor, there are limitations to the interpretation of data from such a study. More particularly, when the residue involved is central to catalysis, its substitution by other amino acids is almost certain to generate proteins with little or no catalytic activity, precluding the use of kinetics to characterize their properties. An alternative approach is to replace the 3-hydroxyl group of CM, an equal partner in the catalytically

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* To whom correspondence should be addressed.

‡ Department of Chemistry.

§ Department of Biochemistry.

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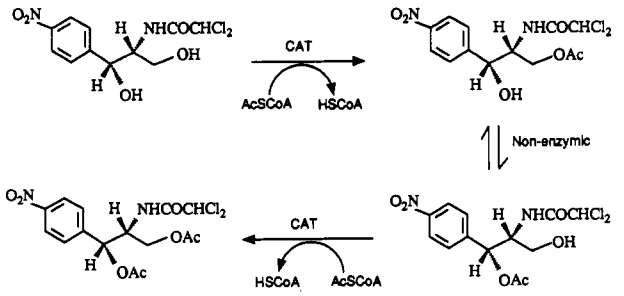


FIGURE 1: Reactions catalyzed by chloramphenicol acetyltransferase.

important interaction, with the aim of generating a competitive inhibitor. The interaction of each chemically modified ligand with the enzyme can then be studied by established steady-state kinetic methods.

Experience in enzyme mutagenesis suggests that "subtractive" substitutions are the most instructive in that they are likely to remove a partner in the interaction under study without introducing unwanted and often disruptive alternative interactions. A useful example chosen from the CAT system is the study of the role of a conserved residue (Asp-199) that lies close to the catalytic center (Lewendon et al., 1988). The substitution Asp → Asn yielded a protein (Asn-199 CAT) that has now been shown by X-ray crystallography to have sustained a major disruption of the active site due to the loss of a buried salt bridge and the establishment of a network of hydrogen bonds not present in the wild-type enzyme (Lewendon et al., 1988). Whereas the Asp → Asn substitution seemed sterically reasonable, it proved to be disruptive.

A study of the interaction of the 3-hydroxyl of CM with the N-3 of the imidazole of His-195, via an uncharged hydrogen bond, could be carried out either by the replacement of the catalytically important histidine or by changes at the C-3 of CM. Because of the presence of a novel hydrogen bond of the N-1 hydrogen of His-195 with the backbone carbonyl of the same residue and its likely importance to the geometry of the active site, we elected to alter the substrate. The desired subtractive change in the structure of the substrate involves the synthesis of 3-deoxychloramphenicol which was readily prepared from (−)-norpseudoephedrine (Rebstock et al., 1951). Replacement of the 3-hydroxyl group of CM by hydrogen could, in principle, leave a cavity in the enzyme–inhibitor complex, which could accommodate a water molecule. If so, the presence of the latter would be expected to have a direct effect on the observed thermodynamics (see discussion below). To extend this analysis, we prepared a series of non-hydrogen-bond-donating CM analogues containing 3-substituents of increasing steric requirement and differing hydrophobicities, with the expectation that such an approach could probe the environment of the 3-hydroxy group. The series of 3-halo-3-deoxychloramphenicol derivatives was readily available from chloramphenicol itself. 3-Fluoro-3-deoxychloramphenicol has been synthesized previously and shown to have antibiotic activity and to be resistant to inactivation by CAT (Nagabhushan et al., 1980; Syriopoulou et al., 1981).

Experimental Procedures

NMR spectra were recorded on a Varian EM390 90-MHz spectrometer, with TMS as internal standard. Melting points are uncorrected.

Synthesis of Chloramphenicol Analogues

3-Iodo-3-deoxychloramphenicol. Chloramphenicol was dissolved in dry pyridine, and the solution was cooled to 0 °C. *p*-Toluenesulfonyl chloride was added with stirring over 20 min. After 2 h the solution was poured into cold water and stirred for several minutes. The resulting oil was extracted with ethyl acetate, and the combined organic layers were washed with dilute HCl, aqueous $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$) and solvent evaporated. The resulting 3-(*p*-toluenesulfonyl)chloramphenicol was dissolved in acetone and heated under reflux with magnetic stirring for 6 h with sodium iodide under a nitrogen atmosphere. After the solution was cooled to 0 °C and sodium *p*-toluenesulfonate was filtered off, the solvent was evaporated and the residue dissolved in ethyl acetate. The solution was washed with 10% aqueous sodium thiosulfate and water, dried ($MgSO_4$), and evaporated to give the title compound, which was recrystallized from 67% aqueous ethanol. 3-Iodo-3-deoxychloramphenicol: mp 138–139 °C; ^{1}H NMR ($CDCl_3$) δ 3.1–3.6 (m, 2 H), 4.2–4.5 (m, 1 H), 5.3 (br s, 2 H, one of which D_2O exch), 6.0 (s, 1 H), 7.3–7.6 (m, 3 H, one of which slow D_2O exch), 8.2 (d, 2 H).

3-Chloro-3-deoxychloramphenicol and 3-Bromo-3-deoxychloramphenicol. Chloramphenicol was converted to the *threo*-2-dichloromethyl-5-(*p*-nitro-α-hydroxybenzyl)-Δ^2-oxazoline and then to the 3-halo compound by treatment with the appropriate hydrogen halide in anhydrous dioxan, according to the methods reported for the racemic compounds (Farkas & Sicher, 1953, 1954). 3-Chloro-3-deoxychloramphenicol: mp 127–129 °C; ^{1}H NMR ($CDCl_3$) δ 3.4–3.9 (m, 2 H), 4.2–4.5 (m, 1 H), 5.1–5.3 (m, 2 H, one of which D_2O exch), 5.9 (s, 1 H), 7.2–7.4 (br s, 1 H, slow D_2O exch), 7.5 (d, 2 H), 8.1 (d, 2 H). 3-Bromo-3-deoxychloramphenicol: mp 135–137 °C; ^{1}H NMR ($CDCl_3$) δ 3.3–3.8 (m, 2 H), 4.2–4.5 (m, 1 H), 5.2–5.4 (m, 2 H, one of which D_2O exch), 5.9 (s, 1 H), 7.2–7.5 (br d, 1 H, slow D_2O exch), 7.5 (d, 2 H), 8.1 (d, 2 H).

3-Deoxychloramphenicol was prepared by the method of Rebstock et al. (1951). (−)-Norpseudoephedrine was dissolved in anhydrous pyridine, and the solution was cooled to 0 °C. Acetic anhydride was added, and the solution was kept at 0 °C overnight. The solution was poured into water (200 mL) and extracted with ethyl acetate (3 × 250 mL), and the organic layer was washed (2 M HCl, aqueous $NaHCO_3$, H_2O) and dried ($MgSO_4$). The organic layer was evaporated to give (−)-norpseudoephedrine and diacetate as a colorless oil. The diacetate was cooled to −5 °C and treated with fuming nitric acid for 45 min at this temperature. The solution was poured onto ice, neutralized with solid sodium bicarbonate, and extracted with ethyl acetate. The combined organic fractions were washed with water (2 × 200 mL), dried ($MgSO_4$), and evaporated to dryness to give (−)-*p*-nitronorpseudoephedrine diacetate as a pale green oil. Acetate groups were hydrolyzed by reflux in 5% HCl for 2.5 h, and the aqueous solution was extracted with ether (2 × 50 mL). After raising the pH to 11 with 1 M NaOH, the product was extracted with ethyl acetate (3 × 100 mL), dried, and evaporated to give the free amine. The free amine was heated under reflux in dry ethanol (80 mL) containing methyl dichloroacetate (5.6 g) for 45 min. After removal of volatile materials by rotary evaporation, the faintly yellow crystalline residue was triturated with petroleum ether, then dissolved in ethyl acetate, washed with 2 mM HCl, aqueous $NaHCO_3$, and H_2O, and then dried ($MgSO_4$) and evaporated to give crude 3-deoxychloramphenicol as a waxy solid. Small quantities of the ortho analogue were removed by chromatography on silica gel, eluted with ethyl acetate–chloroform (1:4 v/v). 3-Deoxychloramphenicol: waxy solid; ^{1}H NMR ($CDCl_3$) δ 1.3 (d, 3 H), 3.7 (br s, D_2O exch), 4.2–4.5 (m, 1 H), 4.8 (d, 1 H), 5.8 (s, 1 H), 6.9 (br d, 1 H slow D_2O exch), 7.6 (d, 2 H), 8.1 (d, 2 H).

Hydrophobicity Measurements of Chloramphenicol and Analogues. HPLC was performed by using a Shimadzu LC 4A HPLC system equipped with a Shimadzu SPD-2AS UV detector set at 280 nm and a Shimadzu C-R2AX chart re-

corder. A 250-mm × 5-mm column packed with 5-mm ODS-capped silica was used (Shandon). A mobile phase consisting of 40% aqueous methanol, degassed by purging with helium, was used at a flow rate of 2 mL/min. Samples were dissolved in the mobile phase to give 1 mg/mL, and solutions (5 μL) were loaded via a Rheodyne 7125 injector.

Inhibition Studies

Purification of CAT. Wild-type CAT was purified from *Escherichia coli* extracts by affinity column chromatography on chloramphenicol–Sepharose as previously described (Lewendon et al., 1988).

Assay of CAT Activity. CAT activity was assayed spectrophotometrically at 25 °C by monitoring the release of CoASH by reaction with DTNB. One unit of enzyme activity is defined as the amount that will convert 1 μmol of chloramphenicol to product per minute.

(*i*) *Forward Reaction.* The procedure described by Lewendon et al. (1988) was used to measure rates of acetylation of chloramphenicol. The standard assay contained 50 mM Tris-HCl buffer, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.1 mM chloramphenicol, and 0.4 mM acetyl-CoA. The reaction was initiated by addition of enzyme, and the formation of CoA was monitored at 412 nm by its reaction with DTNB.

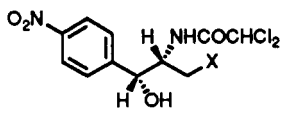
(*ii*) *Kinetic Analysis.* Linear initial rates for steady-state kinetic analyses were measured in triplicate over times during which less than 15% depletion of substrates occurred. Kinetic parameters were determined from linear slope and intercept replots from manually drawn double-reciprocal plots (Kleanthous & Shaw, 1984). Concentrations of inhibitors and chloramphenicol were varied in the standard assay during competitive inhibition studies that routinely included acetyl-CoA at fixed concentrations ≈5 times K_m. The K_i values were determined from linear slope replots derived from double-reciprocal plots as described.

Results and Discussion

As predicted, deletion of the 3-hydroxyl group of CM results in a reduced affinity. As shown in Table I, 3-deoxy-CM was found to be a competitive inhibitor with a K_i value of 48 μM, compared to a K_d for CM of 3.6 μM. Replacement of the hydroxyl group with the fluoro substituent results in a further reduction in affinity (K_i = 78 μM). Although substitution of a hydroxyl by fluorine has been widely exploited in medicinal chemistry because of their similar van der Waals radii, it should be emphasized that the latter is restricted to being a hydrogen-bond acceptor, while the former can be both a hydrogen-bond donor and acceptor. Unexpectedly, the affinity of the 3-halo-3-deoxy-CM compounds increased in the series F < Cl < Br < I. Prior to an analysis of the refined structure of the binary complex of CAT_{III}, it seemed likely that there might be a problem accommodating a substituent as bulky as the iodo group. Figure 2 shows a stereopair of the active-site cleft with chloramphenicol bound at the bottom of the picture. There is clearly a substantial cavity adjacent to the 3-hydroxyl group, which suggests that the site can be occupied by the larger 3-halo substituents. Three ordered water molecules are located in this region (marked by crosses), some or all of which may be displaced on binding a 3-substituted analogue of CM. Figure 3 shows space-filling representations of the least sterically demanding (3-deoxy-CM) and most sterically demanding (3-iodo-3-deoxy-CM) analogues of CM, depicted in the same relative conformations as CM when bound to CAT.

Quantitative analysis of the energetics of individual interactions within the ES complex probed by modification of either the enzyme or the substrate is achieved by a direct comparison of dissociation constants for the modified and unmodified

Table I



X	K_d (μM)	$C = t_x - t_0/t_0$ (capacity factor[b])
OH	3.6[a]	3.71
H	48	6.97
F	73	6.22
Cl	19	10.85
Br	7[c]	13.27
I	3.4	18.1

[a] K_d value derived from a fluorescence binding assay for chloramphenicol (Ellis and Shaw, unpublished results) and K_i values for the 3-substituted CM analogues. [b] C is the capacity factor from HPLC retention times for 3-substituted CM derivatives (t_x) relative to those for water (t_0) on a C_{18} column eluted with methanol–water (2:3), as described under Experimental Procedures. [c] The K_d for this ligand has been directly measured by fluorescence methods (Ellis and Shaw, unpublished results) and found to be equal to K_i.

complexes. Such a comparison yields an apparent binding energy associated with the interaction. For the case of a base (B) on an enzyme (E) interacting with a hydrogen-bond donor (XH) on the substrate (S), the binding stoichiometry can be expressed by eq 1. The process for a modified substrate is expressed by eq 2.

$$E\text{–}B\cdots OH_2 + S\text{–}XH\cdots HOH \rightleftharpoons [E\text{–}B\cdots HX\text{–}S] + H_2O\cdots HOH \quad (1)$$

$$E\text{–}B\cdots OH_2 + S\text{–}Y/HOH \rightleftharpoons [E\text{–}B/Y\text{–}S] + H_2O\cdots HOH \quad (2)$$

ΔG_{app} can be expresssed in terms of the various bonds that have been altered in the modification according to a simple thermodynamic cycle discussed by Fersht (1988) giving

$$\Delta G_{app} = (G_{S\text{-}Y/W} - G_{S\text{-}XH\cdot W}) - (G_{E\text{-}B/Y\text{-}S} - G_{E\text{-}B\cdot HX\text{-}S}) + \Delta G_{reorg} \quad (3)$$

where $G_{E\text{-}B\cdot HX\text{-}S}$ is the hydrogen-bond dissociation energy of EB···HX–S, the unmodified complex, $G_{E\text{-}B/Y\text{-}S}$ is the dissociation energy in the modified complex, $G_{S\text{-}XH\cdot W}$ is the energy of interaction of substrate with water, $G_{S\text{-}Y/W}$ is the energy of interaction between modified substrate and water, and ΔG_{reorg} is a reorganizational energy term that includes any conformational changes, perturbations to the binding of the rest of the ligand to the enzyme, or changes to the solvent arising from the modification of the ligand. ΔG_{bind} (the incremental binding energy in the ES complex) can be expressed in terms of the hydrogen-bond dissociation energies according to eq 1, hence

$$\Delta G_{bind} = G_{E\text{-}B\cdot HX\text{-}S} + G_{WW} - G_{E\text{-}B\cdot W} - G_{S\text{-}XH\cdot W} + \Delta G_R \quad (4)$$

where G_{WW} is the hydrogen-bond dissociation energy of $H_2O\cdots HOH$, $G_{E\text{-}B\cdot W}$ is that of EB···HOH, etc., and ΔG_R is a composite energy term corresponding to any other energetics associated with the reaction (e.g., favorable entropy associated with release of bound water). Eliminating common terms in eqs 3 and 4 gives the relationship between ΔG_{app} and ΔG_{bind}:

$$\Delta G_{app} = \Delta G_{bind} - G_{WW} + G_{E\text{-}B\cdot W} - G_{E\text{-}B/Y\text{-}S} + G_{S\text{-}Y/W} + \Delta G_{reorg} - \Delta G_R \quad (5)$$

Clearly, ΔG_{app} does not equal ΔG_{bind} except in the special case where the remaining terms in eq 5 cancel out or where a mutation allows access of solvent to the unpaired donor (Fersht, 1988).

Substitution of the 3-hydroxyl group of CM should have

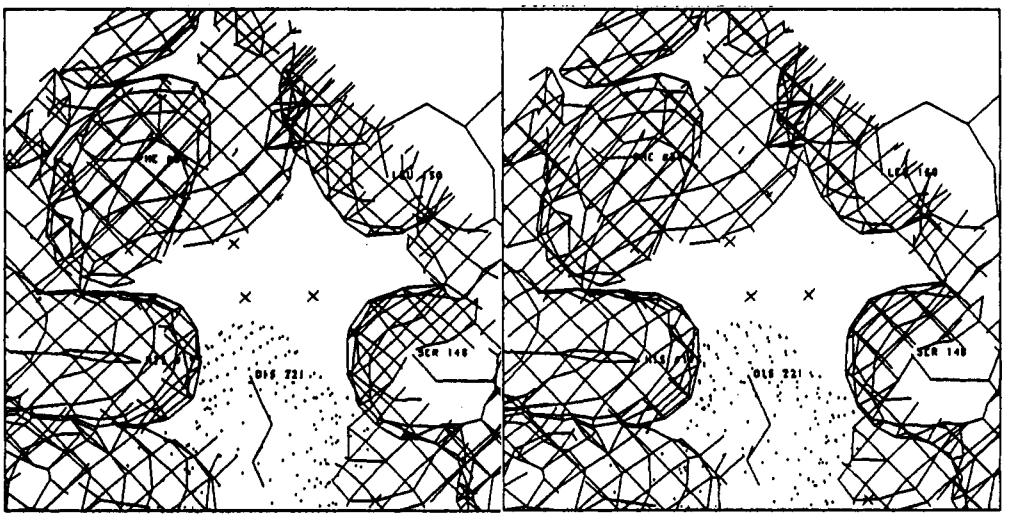


FIGURE 2: Stereoview of the active-site cleft of chloramphenicol acetyltransferase with the 3-hydroxyl group of chloramphenicol shown as a van der Waals dot surface. The three crosses represent ordered water molecules.

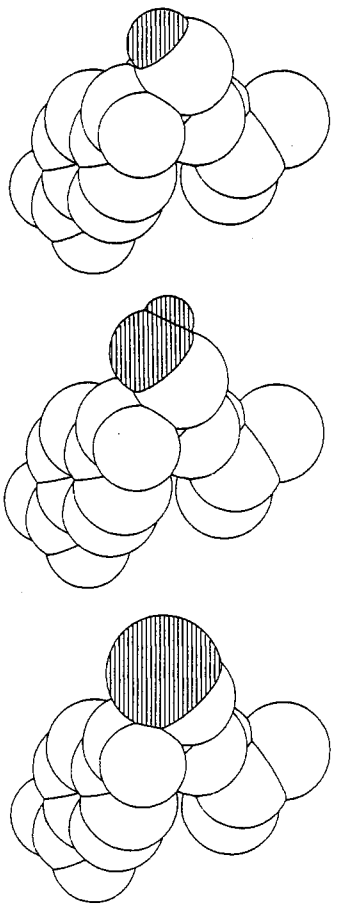


FIGURE 3: Space-filling representations of 3-deoxychloramphenicol (top), chloramphenicol (middle), and 3-iodo-3-deoxychloramphenicol (bottom), shown in the same relative conformations as found in the CAT–CM binary complex.

two primary effects on binding to CAT. First, as intended, it removes the hydrogen-bond donor to His-195. Secondly, it also directly affects the interaction of each of the resulting CM analogues with solvent water. Binding of a substrate to the active site of a protein requires the replacement of interactions with solvent water, either wholly or in part, by favorable interaction with active-site residues. Such considerations allowed a clear rationalization of the results shown in Table I. The replacement of the hydroxyl group by a halogen reduces the hydrophilic character of the ligand, reducing the energetic cost of removing it from solvent to the active site. With this in mind, the observed trend in affinities is qualitatively as expected if hydrophobicity considerations are dominant. In a quantitative analysis of this data, we have correlated the apparent binding constants to CAT with an experimentally determined parameter related to the hydrophobicity of the ligand. The hydrophobic character of ligands is frequently estimated by determining the equilibrium distribution from water into a reference phase such as an immiscible organic solvent (Leo et al., 1971) or, at the limit, the gas phase (Wolfenden et al., 1981). We have used an HPLC method to estimate hydrophobicity, wherein the retention time on a reverse-phase (C_{18}) column has been shown to be related to the partition coeffient between water and an organic phase (Minick et al., 1988; Terada, 1986). It is important to use an experimental estimate of hydrophobicity rather than estimates based upon subsituent effects, since assumptions of additivity do not hold for the solvation of polyfunctional molecules such as CM.

The binding of this range of inhibitors to CAT shows a linear free energy relationship with the HPLC-derived experimental scale of hydrophobicity (Figure 4). The data are compatible with the view that each ligand is bound in the same way and that the size of the 3-substituent does not disrupt the major binding interactions between the CM analogues and CAT_{III}. The only ligand that does not fall on this line is the substrate itself, which is bound more favorably than would be predicted from its hydrophobicity. This clearly arises from the additional hydrogen bond to the 3-hydroxyl of CM that is not available to any of the other ligands.

Comparison of the binding of CM with that of 3-deoxy-CM reveals a ΔG_{app} of 1.5 kcal mol^{-1}. The calculated value may underestimate the contribution to binding of this hydrogen bond since the absence of the hydroxyl group removes a favorable interaction with the active site, but this will be offset by the fact that 3-deoxy-CM is considerably less hydrophilic than CM. Furthermore, if the binding of 3-deoxy-CM leaves a cavity in which a water molecule remains bound, the thermodynamics will reflect the fact that the enzyme is not desolvated to the same extent as in the CAT–CM complex but with the consequent energetic cost of "burying" a water molecule. The latter two effects may be expected to offset each other. The data in Figure 4 provide an approach to making a better estimate of the energetic contribution of the hydrogen bond between CM and His-195. In the absence of the hydrogen bond between the 3-OH of CM and His-195, the parent compound would be expected to fall on the same regression line as the other ligands. It follows that the extent to which CM is displaced from this line is a direct measure

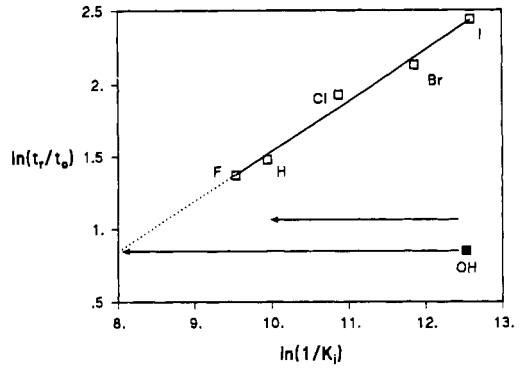


FIGURE 4: Linear free energy relationship between the binding of a range of 3-substituted chloramphenicol analogues and their relative hydrophobicities estimated on the basis of an HPLC method (see Experimental Procedures).

of the *additional* stabilization energy provided by having a hydroxyl group at the 3-position in the native enzyme–CM complex. The ΔG_{bind} derived from such an analysis is 2.7 kcal mol^{-1}, almost twice the value of the ΔG_{app} derived from simply removing the hydroxyl group. Whereas the value of ΔG_{app} is in line with the range of values reported in other systems (Fersht, 1988), that of ΔG_{bind} is rather larger than those typically found for neutral hydrogen bonds in enzyme–substrate complexes. The hydrogen bond under study is located in a comparatively hydrophobic cavity, out of contact with bulk water, and is likely therefore to be a comparatively strong one. However, as discussed above, ΔG_{app} and ΔG_{bind} are only equal in the special case where the remaining terms in eq 5 fortuitously cancel out. In addition to the removal of the hydrogen bond, removal of the hydroxyl group of the ligand may remove favorable van der Waals interactions and also affect the lowest energy conformation of the free ligand, the energetic components of which presumably appear respectively in ΔG_R and ΔG_{reorg} of eq 5.

In the case of mutation of a *charged* hydrogen bond, it has been suggested that ΔG_{app} overestimates ΔG_{bind} because the term relating to the interaction of the unpaired charged hydrogen partner with water dominates the expression equivalent to eq 5 (Fersht, 1988). In the present case ΔG_{app} appears to significantly *underestimate* ΔG_{bind}. Such will be the case where favorable van der Waals interactions with a partner in a hydrogen-bonded pair are also lost upon deletion of the hydrogen bond.

Conclusions

This study has shown that the binding of the 3-position of CM by CAT_{III} is dominated by hydrophobic effects, in that favorable interactions arise from partitioning the antibiotic from aqueous to a largely nonpolar environment. By means of altering the structure of the ligand, we have been able to estimate the contribution of the interaction of an important hydrogen bond between CM and the catalytic imidazole of CAT to the overall energy of binding. The ΔG_{app} deduced from the deletion of the 3-hydroxyl group of CM is likely to have underestimated the ΔG_{bind} by almost a factor of 2. Although ΔG_{app} is in the typical range for neutral hydrogen bonds in proteins (0.5–1.5 kcal mol^{-1}) (Fersht, 1988), ΔG_{bind} is considerably larger than normally found. The latter observation may, in part, be a reflection of the fact that this hydrogen bond is deep within a hydrophobic active site and therefore stronger than a hydrogen bond in a more polar environment. In addition, it may reflect a contribution from van der Waals interactions between the hydroxyl group and the enzyme. Surprisingly, we have demonstrated that the specificity in terms of the relative affinities of this range of related inhibitory ligands appears to be dictated by interactions with the solvent. It should be stressed, however, that, with the exception of the hydrogen bond to the 3-hydroxyl of CM, the remaining important interactions with CAT are available to all of the 3-substituted CM analogues. These other interactions must make a significant contribution to the gross specificity of CAT for dissimilar ligands.

In a similar vein the interaction of elastase with related phosphonate, phosphonamidate, and phosphinate inhibitors has been shown to be interpretable in terms of the relative hydrophobicities. Impressively, Kollman, on the basis of molecular dynamics calculations, accurately predicted the binding of the latter compound before it had been reported (Merz & Kollman, 1989). Wells and co-workers have used enzyme mutagenesis and substrate modification to probe the steric and hydrophobic effects in subtilisin and have found that, in the absence of steric effects, the binding of a series of related substrates is dominated by considerations of hydrophobicity (Estell et al., 1986). The CAT system appears to offer a particularly promising opportunity to test an hypothesis in more detail, not least because of the high-resolution structure of the CAT–CM binary complex and the very large number of analogues of CM which have been prepared and characterized.

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