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MINIREVIEW

O-Acetyltransferases for Chloramphenicol and Other Natural Products

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The range of enzymatic mechanisms by which bacteria inactivate biologically active molecules is limited by two kinds of constraint, namely, the structure-activity correlations for each class of chemical agent and the metabolic repertoire available to bacteria. In the case of chloramphenicol (Cml), which, given its size, has a wealth of vulnerable functional groups, there are in principle a number of possibilities. Since virtually all the chemical functionalities of Cml (Fig. 1) are known to contribute to its effectiveness as an inhibitor of ribosomal peptidyltransferase activity (15, 33), it is not surprising that there have been reports of enzyme-mediated resistance to Cml arising from dehalogenation, nitro group reduction, and hydrolysis of the amide bond (for a review, see reference 36) and modification of one or both of the hydroxyls of Cml by phosphorylation (26) and acetylation (36–38). Nonetheless, after more than four decades of medical and veterinary use, the preponderant enzymatic modification mechanism that has been observed to underlie Cml resistance in bacteria of clinical importance is that of O-acetylation.

CATs

Genes for chloramphenicol acetyltransferase (CAT) are widespread among most genera of gram-positive and gram-negative bacteria, and, at least in strictly biochemical terms, there is little to be gained by addressing whether a specific example (or class) of *cat* determinant is chromosomal or plasmid linked or is associated with the presence of a transposon or integron.

For the purpose of the present discussion, it may be most useful to stress the properties of selected CAT variants and note patterns of conservation of primary structure, as deduced from the 23 nonredundant nucleotide sequences currently available in databases. Such information is necessary to discern whether individual *cat* genes are likely to specify polypeptides with similar folds and a common (trimeric) quaternary structure, and are therefore members of a well-defined enzyme "family."

All CAT polypeptides are in the size range of 24 to 26 kDa and normally exist in solution as compact homotrimers. A slight qualification arises from the knowledge that polypeptides of two CAT variants which have been studied extensively associate in vivo and in vitro to give fully functional hybrids, $\alpha_2\beta$ and $\alpha\beta_2$ heterotrimers, with physical and catalytic properties predicted from a knowledge of those of α_3 and β_3, the parental species (9). Natural isolates of Cml-resistant bacteria may harbor more than one *cat* gene, and under such circumstances, it is likely that the intracellular CAT pool will include both hybrid and parental trimers.

An apparent constraint to the extent of amino acid sequence divergence between any two CAT variants can best be appreciated by comparing the primary structures of the predicted gene products of 23 *cat* determinants with known DNA sequences. Although only a few examples have been included in Fig. 2, a more comprehensive analysis reveals a lower limit of 28% identity in the most divergent of all possible pairwise comparisons. Only 23 residues (~11% of the total) appear to be conserved absolutely. These residues comprise not only amino acids with side chains that are involved in catalysis and substrate binding but also those that contribute to critical structural elements, necessary for the precise folding and stable packing of polypeptide chains. A reference point for understanding the variety in primary structures of CAT variants is the type III enzyme (CAT_{III}), for which a wealth of information is now available, including the tertiary structure at high resolution and the structural determinants for the binding of each substrate (17, 18, 36–38). Also included in Fig. 2 are the sequence of the type I variant (CAT_I), specified by many naturally occurring F-like R plasmids of gram-negative bacteria (36) as well as by transposon Tn*9* (5), and that of CAT_{II}, notable among enteric CAT variants for its particular sensitivity to inhibition by thiol-reactive reagents and its association with *Haemophilus influenzae* (29). CAT_I, on the other hand, has the remarkable properties of not only a high affinity for triphenylmethane dyes, such as crystal violet (for a review, see reference 36), but also the ability to bind a steroidal antibiotic (fusidic acid) both tightly and very specifically (6, 31). The latter property is sufficient to confer resistance to fusidate in mutant strains of *Escherichia coli* that have been selected for their sensitivity to the antibiotic prior to the introduction of the gene for CAT_I. A plausible mechanism is the effective sequestration of fusidate by high levels of CAT_I, thereby preventing access of the antibiotic to its cellular target, ribosomal elongation factor G. The structural basis for the curious binding of fusidate to CAT_I, which is competitive with respect to Cml (K_i = 1.5 μM), has been deduced by protein engineering and X-ray crystallography. Of the eight residues in the Cml binding pocket of CAT_{III} which differ from those of CAT_I, only four appear to be responsible for the former's low affinity (K_i = 279 μM) for fusidate. Replacement of each with their four counterparts in CAT_I is sufficient to convert CAT_{III} to an affinity state (K_i = 5.4 μM) which approaches that of wild-type CAT_I (31). Although the 3-hydroxyl group of the steroid A ring is approximately at the same position as that of Cml in the Cml:CAT_{III} structure, it is clear from atomic coordinates that the observed lack of acetyl transfer is due to steric factors at the active site.

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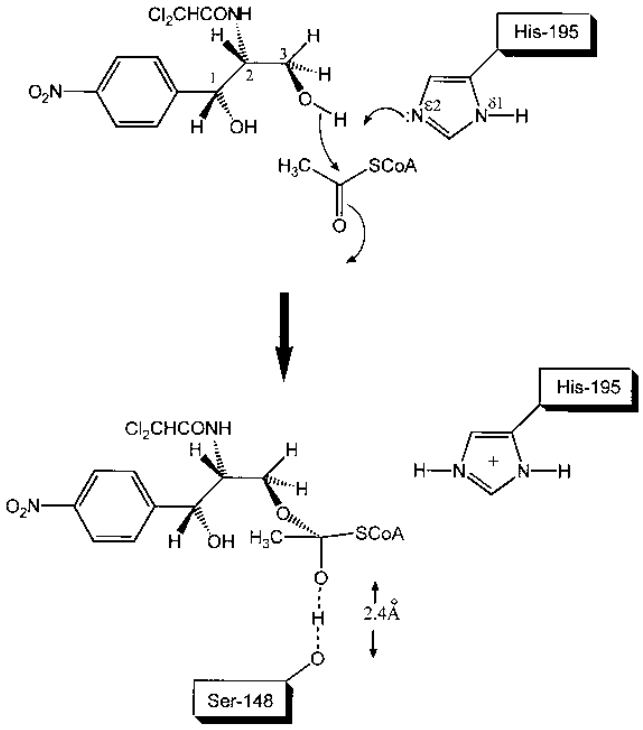

FIG. 1. Structure of Cml and the early steps in the chemical mechanism of the CAT reaction. The $N^{\varepsilon 2}$ of His-195 serves as a general base to deprotonate the primary (C-3) alcohol of the antibiotic. The resulting oxyanion of Cml then attacks the carbonyl carbon of the acetyl moiety of acetyl-CoA. The product shown is the modeled tetrahedral intermediate which shares a hydrogen atom with the side chain oxygen of Ser-148 and is notable for the inferred short distance (~2.4 Å) between the two oxygen atoms. Collapse of the tetrahedral intermediate yields the final products, 3-*O*-acetyl Cml and HS-CoA (data not shown), with the coincident regeneration of the neutral imidazole of His-195 for another cycle of acetylation.

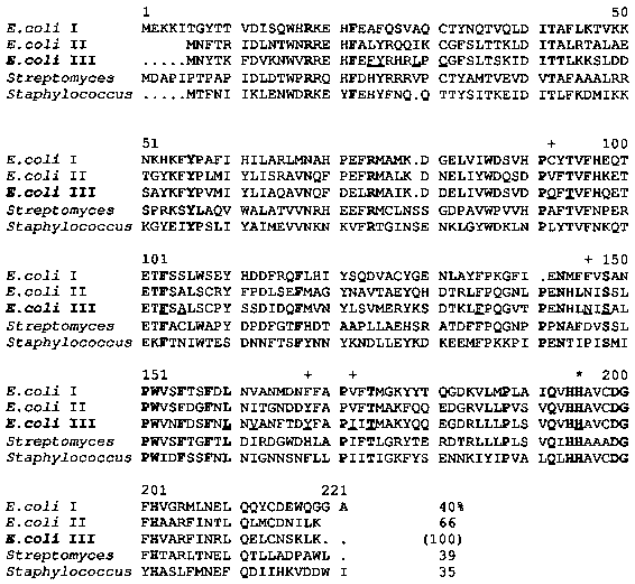

FIG. 2. Amino acid sequence alignment of selected CAT variants. The 23 residues in boldface type are conserved in all CAT sequences available in accessible databases. The amino acids of CAT_{III} that are underlined are in the Cml binding pocket (17), and by inference, so are their homologs in other CAT sequences. Substitution of the four corresponding residues in CAT_{III} with those with plus marks above the CAT_I sequence is sufficient to convert CAT_{III} to a state of high affinity for fusidic acid (see text and reference 31). References for CAT_I and CAT_{II} are 5 and 29, respectively. The CAT of *Streptomyces acrimycini* (28) and that of *S. aureus* plasmid pC221 (7) were reported elsewhere. The catalytic base in the chemical mechanism (Fig. 1) is $N^{\varepsilon 2}$ of His-195 (*). The percent sequence identity of each variant with that of CAT_{III} (27) is indicated.

The catalytic machinery of CAT_{III} comprises a number of amino acid side chains (as well as backbone atoms and ordered water molecules) which make precise contacts with one another or with a substrate molecule. Since homotrimers have rotational symmetry at the molecular threefold axis (Fig. 3a), and consequently have three identical active sites, only atoms in a single monomer need be addressed. Nonetheless, each of the structurally and functionally equivalent active sites lies deep in the interfacial clefts between subunits (Fig. 3b). Central to catalysis (22) is His-195, which arises from one face of each cleft to supply the general base ($N^{\varepsilon 2}$ in Fig. 1) to deprotonate the C-3 hydroxyl of Cml, producing an oxyanion intermediate which in turn attacks the carbonyl (C-2) carbon of acetyl coenzyme A (CoA) to yield a tetrahedral intermediate (Fig. 1). Essential to the stabilization of the latter, en route to the transition state for the reaction is a hydrogen bond between its oxygen and the hydroxyl of Ser-148, another absolutely conserved residue in all CATs. The catalytic importance of this hydrogen bond has been confirmed by site-directed mutagenesis (20). A neighboring participant in catalysis is Thr-174, which is also conserved and which is hydrogen bonded to a water molecule, which in turn probably makes two hydrogen bonds with the putative tetrahedral intermediate (Fig. 1), one to the 1-hydroxyl of Cml and the other to the oxygen at position 3 of the intermediate (20). Two additional conserved residues (Arg-18 and Asp-199) facilitate catalysis via a network (Fig. 4) of hydrogen bonds with His-195, anchoring the side chain of the latter in a novel conformation which allows it to fulfill its general base role (18–21).

Finally, it may be instructive to propose a means by which the ultimate diacetylated product of Cml appears, albeit at a much slower rate than the 3-acetyl derivative (14). The equations below, all of which are reversible, indicate the transformations involved in the conversion in vivo of the two substrates to their final products.

$$\text{Cml} + \text{acetyl-CoA} \rightarrow \text{3-acetyl-Cml} + \text{CoA} \quad (1)$$

$$\text{3-acetyl-Cml} \rightarrow \text{1-acetyl-Cml} \quad (2)$$

$$\text{1-acetyl-Cml} + \text{acetyl-CoA} \rightarrow \text{1,3-diacetyl-Cml} + \text{CoA} \quad (3)$$

Reaction 2 is nonenzymatic and slow, yielding at equilibrium a mixture of monoacetyl products. The 1-acetyl Cml so formed is available for a second round of enzymatic acetylation at the C-3 position, yielding 1,3-diacetyl Cml. Reaction 3 is ~150-fold less efficient than reaction 1, almost certainly due to an unfavorable fit of the substrate at the active site because of the bulky 1-acetyl substituent (30). Nonetheless, the sluggish final step (reaction 3) is of little microbiological significance since both monoacetyl derivatives of Cml are already devoid of significant antimicrobial activity, making the rate of reaction 1 the prime determinant of the Cml resistance phenotype.

In summary, the precise geometry and chemical properties of both substrate binding sites (for Cml and acetyl-CoA) and of the catalytic center of CAT_{III} each contribute to its extraordinary efficiency, turning over substrates at 600 s^{-1} (25°C) and with K_m (Cml) being 12 μM (~4 μg/ml), which is reassuringly close to the MIC of Cml for most bacteria of clinical importance. A derived kinetic parameter, the so-called specificity

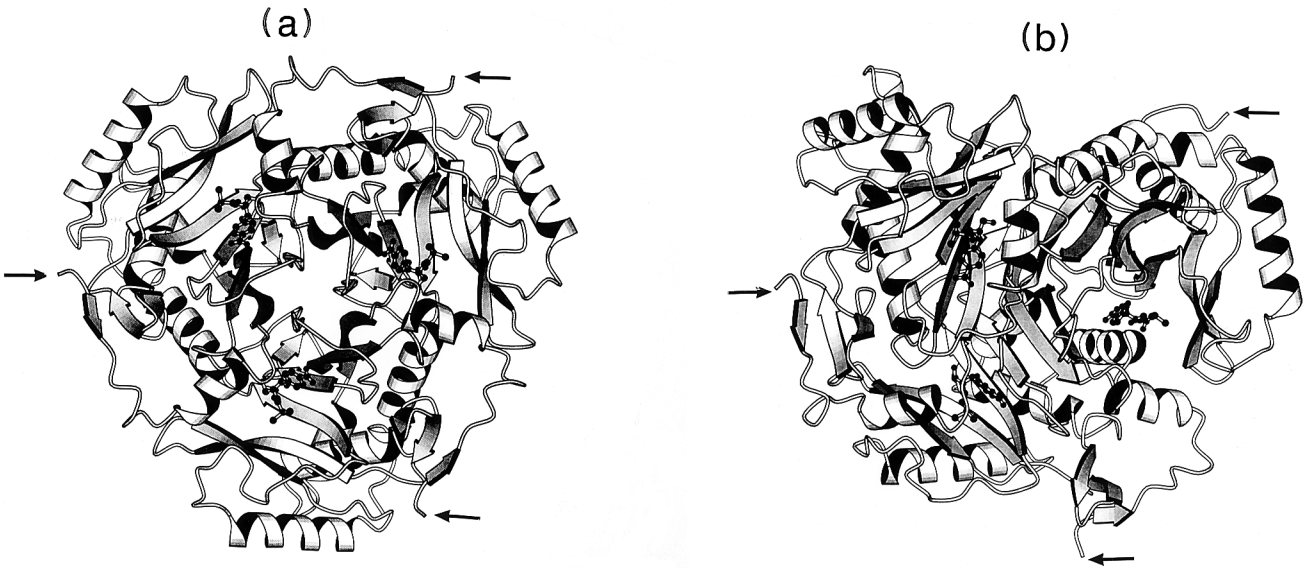

FIG. 3. The CAT_{III} trimer viewed down the molecular threefold axis (a) and with the same axis tipped down (b) to reveal one of the three identical interfacial clefts containing bound Cml (shown as a ball-and-stick structure). A small solid arrow marks the amino terminus of each of the three identical polypeptide chains. Reference 17 should be consulted for a detailed depiction and discussion of the structure of the Cml:CAT_{III} complex.

constant (k_{cat}/K_m) which combines a measure of substrate affinity with one for catalytic competence, is actually the second-order rate constant for productive collisions of an enzyme with its substrate(s). The value for CAT_{III} and Cml (5×10^7 M^{-1} s^{-1}) approaches those for well-characterized enzyme reactions which are under diffusion control (typically 1×10^8 to 1×10^9 M^{-1} s^{-1}) and hence at the limit of evolutionary development. By such criteria, CAT_{III} has evolved to a state approaching "perfection" in biological catalysis (1), wherein a fine balance has been struck between rate acceleration (k_{cat}) and specificity (and affinity) for substrate (K_m).

ANOTHER CLASS OF Cml ACETYLATING ENZYMES

The previous section highlighted the results of mechanistic and structural studies, carried out over three decades, of bacterial Cml resistance mediated by CAT. Genes encoding CAT, isolated from a diverse range of prokaryotes, have been found to be homologous to and, by implication, structurally and mechanistically equivalent to the homotrimeric enterobacterial variant (CAT_{III}) for which a high-resolution tertiary structure is available. However, during the last 5 years there have been reports of genes encoding proteins which also catalyze acetyl transfer from CoA to Cml but which appear to be unrelated structurally to the bona fide or classic CATs. It may be useful to review what is known about the structure and mechanism of this class of proteins, with emphasis on apparent similarities (and differences) between their properties and those of the CAT variants already described. We refer to enzymes of the new class as XATs (xenobiotic acetyltransferases), a departure in nomenclature which serves two functions: to distinguish such proteins from CAT and also to emphasize that enzymes of this class may use a structurally diverse range of hydroxyl-containing compounds as acyl acceptors.

Tennigkeit and Matzura (39) isolated a chromosomal Cml resistance determinant from *Agrobacterium tumefaciens* which conferred low-level (5 μg/ml) Cml resistance when expressed in *E. coli* via a multicopy replicon. Its nucleotide sequence includes an open reading frame specifying a polypeptide with

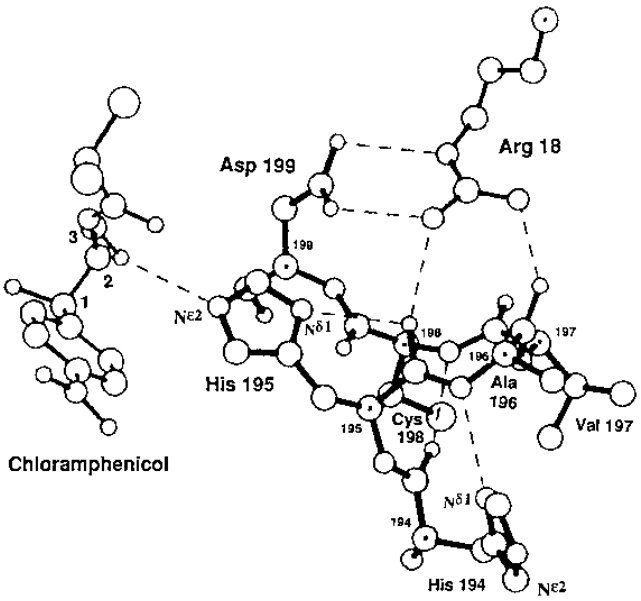

FIG. 4. Network of hydrogen bonds between the side chains of a triad of strictly conserved amino acids and atoms of neighboring residues at the active site of CAT, taken from the high-resolution structure of CAT_{III} (17). The α-carbon atoms of CAT are marked with central dots, and the relative sizes of the atoms in the diagram is Cl (of Cml) ~ S > C > N > O. The (unprotonated) $N^{\varepsilon 2}$ of His-195 is the general base in the catalytic mechanism (Fig. 1) and is hydrogen bonded to the C-3 hydroxyl of Cml. The other nitrogen ($N^{\delta 1}$) of the imidazole ring of His-195 is hydrogen bonded to the carbonyl oxygen of the same residue, an atom which is also hydrogen bonded to the guanidino group of Arg-18, the side chain of which is involved in three additional hydrogen bonds; two with the carboxyl of Asp-199 and one with the carbonyl oxygen of Ala-196. Two other plausible hydrogen bonds of possible importance for the structure and activity of the catalytic center include (a) that between the carbonyl of His-194 and the backbone –N(H) of Cys-198 and (b) that from $N^{\delta 1}$ of His-194 to the backbone –N(H) of Ala-196. Any disruption in the chemistry or geometry of this region of the structure of CAT_{III} should result in a reduction in catalytic activity and/or affinity for Cml (for reviews, see references 36 to 38). Amino acyl side chains equivalent to those of Arg-18, His-195, Asp-199, and Ser-148 (Fig. 1) occupy identical and functionally equivalent positions in the structure of the E2p (acetyltransferase) component of pyruvate dehydrogenase from *Azotobacter* (see text and references 23 and 24).

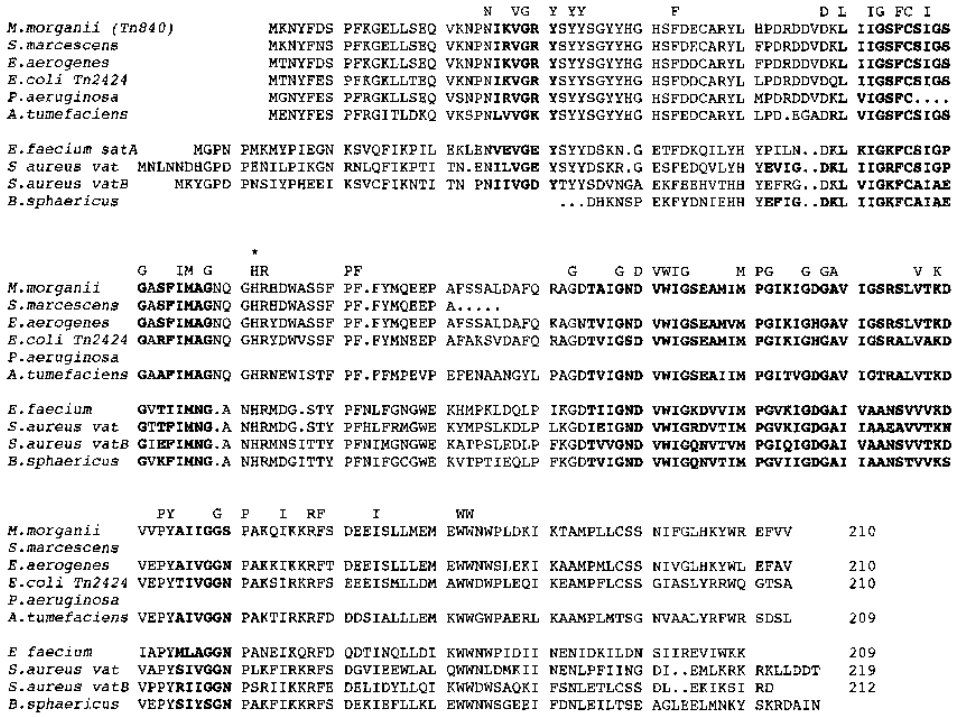

FIG. 5. Amino acid sequence alignment of XAT proteins. Strictly conserved residues are identified by single letters above the aligned sequences, and the only histidine present in all sequences is marked (*). The upper six and lower four sequences in the alignment are separated to facilitate the identification by inspection of the different patterns of conservation outside of the hexapeptide repeat segments (see text for discussion). Residues of the hexapeptide consensus repeats are indicated in boldface type. See text for discussion and literature or database references.

no homology to known CATs. Nonetheless, a radiometric Cml acetylation assay with crude extracts of *E. coli* harboring the cloned gene confirmed that the agrobacterial enzyme was indeed a CAT. Essentially identical observations were subsequently reported with respect to an *E. coli* gene associated with the multiresistance integron Tn*2424* (32), the predicted product of which is 65% identical to that of the *A. tumefaciens* protein. It was also noted (32) that an unassigned truncated reading frame in *Pseudomonas aeruginosa* (41) is homologous to the agrobacterial gene product, as are open reading frames of *Bacillus sphaericus* (25) and *Staphylococcus aureus* (3). More recently, other putative *xat* sequences have been determined, including genes from *Enterobacter aerogenes* (8) and *Morganella morganii* Tn*840* (EMBL X82455), that of *satA* (streptogramin acetyltransferase) of *Enterococcus faecium* (35), and a partial sequence from *Serratia marcescens* (40). The complete sequence of *vat* (virginiamycin acetyltransferase) from *S. aureus* was also determined (4), as was that of *vatB* from the same species (2). Although some of these genes have been postulated, on the basis of their homology with the *xat* genes of *A. tumefaciens* and Tn*2424*, to encode proteins which catalyze the acetylation of Cml, only the latter XATs and those encoded by *satA* and *vatB* (see below) have been studied biochemically.

Figure 5 shows the optimal alignment of 10 complete or partial XAT reading frames, based on information currently available in nucleic acid and protein databases. Proteins of the XAT class are encoded by the plasmid-borne *satA*, *vat*, and *vatB* genes of gram-positive cocci and are associated with resistance to antibiotics of the virginiamycin M1-streptogramin A-pristinamycin IIA class, each of which is a hydroxyl-containing macrocyclic lactone that is structurally unrelated to Cml. The known *S. aureus* genes for VATs (*vat* and *vatB*) are most likely responsible for previously reported virginiamycin M1-pristinamycin IIA acetyltransferase activity in this species (10, 16). The corresponding enzyme activity (streptogramin acetyltransferase) associated with expression of *satA* in *E. faecium* (35) was purified to homogeneity and was found to be unable to acetylate Cml (31a). The purified protein, a potent virginiamycin M1 acetyltransferase (K_m < 14 μM) is, like CAT, a homotrimer. Careful inspection of the sequence data presented in Fig. 5 reveals that the XATs encoded by *satA*, *vat*, and *vatB* (as well as the corresponding partial sequence derived from *B. sphaericus*) form a distinct subclass which differs from the other six tabulated XAT sequences in several segments of primary structure. Putting aside the segmental homologies that are due to structural elements referred to as "isoleucine patches" or "hexapeptide repeats" (highlighted in boldface type in Fig. 5 and discussed below), it may be seen that the four sequences at the bottom of Fig. 5 (*sat A*, *vat*, *vatB*, and *B. sphaericus*) have more in common with one another than with the six aligned sequences above them in Fig. 5. A plausible explanation, for which there are as yet no supporting data, is that the differences in primary structure between the two subgroups is a manifestation of their differences in acetyl acceptor specificity.

The kinetic properties of the XAT from *A. tumefaciens* suggest the interesting possibility that, notwithstanding an ability to acetylate Cml, its actual acetyl acceptor specificity may be for a novel, or at least unexpected, metabolite or natural product, one which is structurally unrelated to Cml. Very high level expression (~20% of soluble protein) of the gene yields only low-level (<20 μg/ml) Cml resistance in *E. coli* JM101, whereas under precisely comparable conditions, CAT_{III} confers resistance up to the solubility limit of the antibiotic (~2 mg/ml). The low MIC of Cml conferred by the *A. tumefaciens* XAT, which suggests that its main physiological role may not

be antibiotic resistance per se, can best be explained by the observation that the K_m of XAT for Cml is 159 μM, whereas that of CAT_{III} is 12 μM. Nonetheless, in most other respects the kinetic mechanisms are remarkably similar (31a).

The critical importance of His-195 as a general base in the catalytic mechanism of CAT (Fig. 1) has been discussed. Given the clear similarities of the CAT and XAT reactions, there was a case for considering whether analogous amino acid residues might play comparable catalytic roles in both proteins. Sequence alignment of XATs revealed a uniquely conserved histidyl residue (His-78 in the XAT of *A. tumefaciens*) which was replaced with alanine by site-directed mutagenesis. Purified Ala-78 XAT was found to be devoid of acetyltransferase activity toward Cml by a sensitive radiometric assay, suggesting the likely importance of His-78 as a catalytic residue of the agrobacterial XAT. Evidence that it plays a role precisely analogous to that of His-195 in CAT_{III} should come from high-resolution structural studies that are under way, as will clues about the specificity for the acetyl acceptor.

In summary, studies of the XAT of *A. tumefaciens* have revealed an intriguing combination of similarities and differences when it is compared with CAT. Although both enzymes are homotrimers of similar molecular mass and catalyze the same reaction, most probably by similar chemical mechanisms, it seems likely that the global tertiary structures of the XAT of *A. tumefaciens* (and related proteins in Fig. 5) and CAT are radically different. The structure of CAT_{III} determined by X-ray crystallography (17, 18) is essentially identical to that of the acetyltransferase (E2p) component of the pyruvate dehydrogenase (PDH) multienzyme complex of bacteria, a possible evolutionary antecedent of CAT (23, 24). The E2p subunit of PDH catalyzes an S-to-S transfer (rather than an S-to-O transfer, as in CATs) of acetyl from the thiol of the lipoyl arm of PDH to that of CoA, and its structure, determined by X-ray diffraction, is virtually superimposable on that of CAT_{III}, even though the only conserved residues are those contributing to the catalytic machinery (Fig. 4) common to both enzymes (23, 24). Available XAT primary structures have no homologies with naturally occurring CAT or E2p variants, and molecular modeling studies suggest that the XAT amino acid sequences are incompatible with the tertiary fold which is characteristic of CAT and E2p. Furthermore, XATs appear to be members of a large family of (mostly) prokaryotic proteins characterized by the presence of multiple repeats of a six-residue consensus sequence known as an "isoleucine patch" (11). *E. coli* alone has at least 10 genes for such proteins, some of which have as yet no ascribed function. Each of those studied thus far are trimeric, are acyltransferases, and fall into one of several apparent subfamilies, based on variation in the number and organization of the isoleucine patch domains (see below). The structure of one such protein, UDP-*N*-acetylglucosamine acyltransferase (*lpxA* gene product) of *E. coli*, was reported recently (34). Each monomer of this homotrimeric enzyme contains extensive repeats (28-fold) of the isoleucine patch, each of which is an important structural determinant of an unusual left-handed β-helical domain. In the trimer the three β-helices make important and extensive contacts along the threefold axis, providing interactions that must be important for the stability and function of the protein. While most XATs contain far fewer hexad repeats than the *lpxA* product (Fig. 5), it seems likely that important structural similarities are present.

For the sake of completeness, it should be noted that there may be a structural continuum extending from the XAT family, discussed here in the context of antibiotic resistance, to even more distant enzymes involved in the O-acylation of a number of metabolites and natural products and typified by the gene products of *lacA* (galactoside acetyltransferase), *cysE* (L-serine acetyltransferase), and *nodL*. This triad of microbial genes, to which attention was first directed by Downie (12), consists of homologous acetyltransferases, which have subsequently been shown to share significant primary structural features with the XATs (32, 35). An important example, the rhizobial NodL protein, a lipo-oligosaccharide *O*-acetyltransferase responsible for signaling specificity in legume root nodulation (and consequent nitrogen fixation), is not only trimeric but also shares structural features that give it a crystallographic signature very similar to that of *lpxA* (13). Although the evidence remains largely circumstantial, there may now be sufficient data to support the hypothesis that there is in fact a much larger extended family of trimeric natural product acetyltransferases than had been imagined, one in which the XATs involved in antibiotic resistance represent a well-conserved branch.

SPECULATIONS

In addition to issues pertaining to the structure and function of XATs and their functional relationship to CATs, the discovery of a new family of antibiotic resistance determinants yields more questions than answers. How common are XATs in the microbial world? Do all XAT variants that acetylate Cml confer on their hosts the same low-level resistance phenotype as does the XAT of *A. tumefaciens*, and do each of the enzymes have a similarly low affinity (high K_m) for Cml? If so, why do they persist in the microbial biosphere when high-level resistance to Cml is easily afforded by acquisition of a bona fide gene for CAT? Might the low-Cml-affinity XATs be "locked in" that phenotype because to improve might mean the sacrifice of an acetylation function, as yet undetected, that is more central to the physiology of the host? Have those which are able to acetylate Cml evolved from XATs which efficiently acetylate streptogramin A, or vice versa? If so, might there be bifunctional XAT variants conferring resistance to both antibiotics? Are there XATs, either undiscovered or uncharacterized, which confer resistance to hydroxyl-containing antibiotics other than chloramphenicol or streptogramin A? If novel synthetic antimicrobial agents with essential hydroxyl groups find their way into general use, what may be the prospects for the emergence of XATs able to acetylate (and inactivate) them? Might there be proteins with structures similar to that of XAT which confer resistance to one or more antibiotics by enzyme mechanisms other than acyl transfer? These and other questions are certain to be resolved as more *xat* genes are characterized and their protein products are studied rigorously with respect to both structure and enzymatic activity.

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