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MINIREVIEW

Extended-Spectrum and Inhibitor-Resistant TEM-Type β-Lactamases: Mutations, Specificity, and Three-Dimensional Structure

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INTRODUCTION

In the last decade the usefulness of the oximino-β-lactams and monobactams has been compromised by the increasing presence of clinically derived extended-spectrum β-lactamases (10, 16, 40, 41, 53, 65, 74). Especially alarming are studies that describe isolates resistant to mechanism-based β-lactamase inhibitors of the clavulanate type (3, 4, 6, 62, 88). With the atomic-level detail that X-ray crystallography is now providing about the tertiary structures of the class A (or group 2) β-lactamases, it is becoming possible to rationalize how a particular amino acid change might alter the substrate profile and catalytic parameters. The intent of this minireview, therefore, is to help microbiologists and molecular geneticists visualize the relationship between β-lactamase mutations and three-dimensional structure. Although extended-spectrum β-lactamases from both chromosomal and plasmid genes are known (40, 67, 85), the focus here is on the better-characterized plasmid-mediated TEM and SHV-type β-lactamases of class A.

CRYSTALLOGRAPHIC STRUCTURES

The term "structure," as used here, means the three-dimensional tertiary structure established at the atomic-level by X-ray crystallography. Structures to 0.2-nm resolution or better are published for four class A β-lactamases: from *Staphylococcus aureus* PC1 (29), from *Bacillus licheniformis* 749/C (49, 64), from *Streptomyces albus* G (19, 55), and from the TEM-1 plasmid in *Escherichia coli* (42, 84). The only X-ray structure of a spontaneous variant is that of the P54 β-lactamase of *S. aureus* PC1, in which the Asp residue at position 179 (Asp-179) is replaced by asparagine (30). Structures of two class A β-lactamases altered by site-directed mutagenesis at Glu-166 have been mapped (50, 84). The atomic coordinates for all but the *S. albus* G β-lactamase are available from the Protein Data Bank at the Brookhaven National Laboratory, Upton, N.Y. Numbering of amino acids follows a consensus numbering scheme (2), with the reactive serine at position 70. The class A structures studied to date have remarkably similar folding (Fig. 1), with corresponding backbone atoms within 0.15 to 0.20 nm. The 10 or 12 critical residues within the β-lactam binding site match even more closely to within 0.05 nm, so that the structure of a β-lactamase that has not yet been established experimentally can be "modeled" from the structure of any one of the homologous structures (5, 43).

Structures of two class C (or group 1) β-lactamases from the *ampC* gene of *Citrobacter freundii* 1203 and *Enterobacter cloacae* P99 are established (60, 68). Ancestrally related to β-lactamase structures, especially the class C structures, is the crystallographic structure of a penicillin target D-Ala–D-Ala carboxypeptidase/transpeptidase (DD-peptidase) from *Streptomyces* sp. strain R61 (46).

β-Lactamase crystals, like most crystalline enzymes, will react with substrates and inhibitors. The high water content of crystals, usually 50% by volume, allows small ligands easy access to enzyme molecules in the crystal lattice. Crystallographic mapping can show bound moieties as well as fixed water molecules. The first visualizations of an intermediate in β-lactam hydrolysis were the acylated complexes of several β-lactams with the slowly reacting DD-peptidase (44, 45, 47). Recently, a mapping of complexes of cephalothin and cefotaxime with the DD-peptidase has provided clues about their complexes with β-lactamases (52). The experimental mapping of β-lactamase intermediates, however, is more difficult to achieve and requires stabilization of the intermediates at cryogenic temperatures. The structures of a clavulanate acyl intermediate (13) and a phosphonate analog (14) bound to the *S. aureus* PC1 β-lactamase, an acylated penicillin G bound to a TEM-1 E166N mutant (84), and for the class C ampC β-lactamases, an aztreonam acyl complex of the *C. freundii* enzyme (68) and a phosphonate complex of the *E. cloacae* P99 enzyme (59) have been reported.

STRUCTURE OF THE β-LACTAM BINDING SITE

Class A β-lactamases, whether plasmid derived or chromosomal, have in common an α-helical domain and a β-sheet of five antiparallel strands surrounded by α-helices (Fig. 1). The reactive Ser-70 is at the N terminus of α-helix H2. A so-called oxyanion pocket exists between the N terminus of H2 and the B3 edge of the β-sheet (Fig. 2a). The purpose of the pocket is to polarize the β-lactam's carbonyl group, which is strongly attracted to this pocket by the hydrogen bonding of backbone NH amide groups at positions 70 and 237. The chemical mechanism of the acylation of Ser-70 and subsequent deacylation by a water molecule is under debate (14, 23, 48, 56, 84).

In parallel with X-ray crystallography, site-directed mutagenesis and kinetics studies have demonstrated the primary catalytic roles of Ser-70 and Glu-166 (1, 18, 21, 37, 58, 78) and the supplementary roles of Lys-73, Ser-130, and Lys-234 (20, 26, 38, 43). An excellent review (63) has discussed the biochemical functions of these conserved residues in detail (Fig. 2b), and because none has been found to be altered in clinical

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FIG. 1. Tertiary structure of a class A TEM-type β-lactamase. Cefotaxime is modeled in the β-lactam binding site near Ser-70. The H2 α-helix (blue), the loop at position 104, (purple), the omega loop (positions 162 to 179; green), and β-strands B3 to B4 (positions 233 to 249; orange) are highlighted. The locations of seven of the natural mutations discussed in the text are shown. The diagram was drawn with MOLSCRIPT (51).

isolates, further analysis of them will be excluded from this minireview.

β-Lactamase is a hydrolase. The possible effect of an amino acid substitution on the position of the hydrolytic water molecule in the catalytic site must be considered, but it is usually overlooked when amino acid sequences are compared. In all the X-ray structures the water molecule is strongly held between Ser-70, Glu-166, and Asn-170 (Fig. 2b), and in this

FIG. 2. (a) Close view of the β-lactam binding site in the orientation shown in Fig. 1. Hydrogen bonding between the β-lactam and the backbone groups of residue 237 is indicated by dashed lines. The two NH amide groups of the oxyanion pocket bind the CO carbonyl group of the β-lactam. (In the consensus sequence alignment [2], residue 240 is adjacent to residue 238.) (b) Crystallographic structure of side chains in the β-lactam binding site of the TEM-1 β-lactamase (42). The orientation and color scheme used in Fig. 1 are used here. The presumed hydrolytic water molecule is activated by three hydrogen bonds. (The hydrogen atoms on the water molecule and side chains are omitted.) Comparison of this plasmid β-lactamase with the chromosomal *B. licheniformis* β-lactamase (49) and *S. aureus* PC1 β-lactamase (29) shows the atomic positions of conserved residues (Ser-70, Lys-73, Ser-130, Arg-164, Glu-166, Asp-179, Lys-234, Gly-236, Arg-244) overlap closely within 0.05 nm.

activated form it is thought to be the agent which deprotonates the Ser-70 hydroxyl group prior to the formation of the tetrahedral intermediate in the acylation step. It must remain properly positioned in order to attack the ester bond of the acyl intermediate in the deacylation step. X-ray analysis of two catalytically impaired Glu-166 mutants (E166A and E166N), prepared by site-directed mutagenesis, showed that this water molecule is significantly displaced relative to its position in the wild type (50, 84).

STRUCTURAL DESCRIPTION OF POINT MUTATIONS

From the three-dimensional structures of parental forms, the expected conformational effects of mutations can be described and, it is hoped, correlated with observed changes in β-lactam specificity and catalysis. Fifteen natural and spontaneous amino acid mutations in the TEM- and SHV-type enzymes are considered in numerical order.

Residue 39 (Gln and Gln in parental TEM-1 and SHV-1, respectively). (i) Environment. Located at the end of the N-terminal α-helix, residue 39 is very far (2.0 nm) from the β-lactam binding site and is totally exposed on the right front surface of the enzyme (Fig. 1).

(ii) Change. The polar glutamine residue of the parental form is replaced by a lysine side chain in TEM-2 and seven variants (Table 1). No SHV variants exhibit changes here.

(iii) Result. Because the side chain makes no contact with the β-lactam or the protein side chains around it, it is not surprising that catalytic differences have not been detected in the TEM-1 and TEM-2 pair. Of all the amino acid variations discussed in this minireview, this one is the most obscure.

Residue 43 (Arg and Arg). (i) Environment. Residue 43 is at the beginning of the B1 β-strand; its side chain projects behind the β-sheet and donates two hydrogen bonds to the side chain of Glu-64 and to the main chain CO of residue 65.

(ii) Change. The recently reported SHV-7 variant was found with a serine at position 43 and is resistant to cefotaxime, ceftazidime, and aztreonam (9).

(iii) Result. The result of a change at position 43 is unclear. The hydrogen bonding from Arg-43 to the segment at positions 64 to 69 running behind the β-sheet may be important in maintaining the position of Ser-70. The shorter serine side chain may not be able to reach the segment, in which case Ser-70 may be shifted in some unknown way so as to optimize catalysis. If serine can span the distance to donate one hydrogen bond, the right end of the β-sheet may be pulled inward, tilting the left end of the β-sheet outward to make room for the cephalosporins.

Residue 69 (Met and Met). (i) Environment. The buried side chain at position 69 is important not because of its proximity to the reactive Ser-70 (the two side chains do not touch) but, rather, because it forms the back wall of the oxyanion pocket (Fig. 2). The side chain at position 69 lies behind β-strand B3, and by steric contact, it influences the positioning of β-strand residues 237 and 238 relative to Ser-70. In parental β-lactamases, the size of the side chain at position 69 is inversely correlated with the size of the side chain at position 238 (see the section on residue 238 below), but this correlation breaks down in many extended-spectrum β-lactamases (32).

(ii) Changes. By a random sequence generation and selection approach, it was found that TEM enzymes with leucine, isoleucine, and valine at position 69 demonstrate clavulanate resistance (69). Isoleucine appeared in a spontaneous laboratory-derived mutant of the SHV-type OHIO-1 β-lactamase designated M4 (6). M4 had elevated K_i values for clavulanate, sulbactam, and tazobactam. Site-directed mutagenesis produced a less-resistant leucine mutant (17). The first clinical isolate of an inhibitor-resistant TEM variant was reported in 1993 (4), and it was found to contain two changes, an isoleucine for a methionine at position 69 and a threonine for a methionine at position 182. The next year four TEM variants with leucine or valine at position 69 were reported (93) (Table 1). Two of these mutants also contain aspartic acid at position 276.

(iii) Result. The hydrophobicity of isoleucine is more than twice that of methionine. If modeled in its predominant rotomeric conformation about the Cα-Cβ bond, the β-methyl group of isoleucine would extend into the oxyanion pocket (see Fig. 4). These thermodynamic and steric factors may produce a deformation of the oxyanion pocket such that β-lactam binding would be impaired (7). According to this argument, the less hydrophobic Leu-69 mutant without a branched side chain (17) should, and indeed does, exhibit less of an increase in K_i. Because small β-lactams such as clavulanic acid and sulbactam have no C-6 substituents and must therefore rely primarily on attractive interactions with the oxyanion hole and Arg-244 (see the section on residue 244), it is not surprising that inhibitor resistance exists in natural variants such as TEM-33 to TEM-36 (Table 1) (93) containing changes at residue 69, 244, or 276 (see the section on residue 276).

TABLE 1. Natural mutations in the TEM- and SHV-type β-lactamases[a]

β-Lactamase	Amino acid at sequence position:										
	39	69	104	164	205	237	238	240	244	265	276
TEM-1	Q	M	E	R	Q	A	G	E	R	T	N
TEM-2	K										
TEM-3	K		K				S				
TEM-4			K				S			M	
TEM-5				S		T		K			
TEM-6			K	H							
TEM-7	K			S							
TEM-8	K		K	S			S				
TEM-9			K	S						M	
TEM-10				S				K			
TEM-11	K			H			?				
TEM-12				S							
TEM-13	K									M	
TEM-15			K				S				
TEM-24	K		K	S		T		K			
TEM-25							S			M	
TEM-26			K	S							
TEM-27				H				K		M	
TEM-30									S		
TEM-31									C		
TEM-33		L									
TEM-34		V									
TEM-35		L									D
TEM-36		V									D
SHV-1	Q	M	D	R	R	A	G	E	R	L	N
SHV-2							S				
SHV-3					L		S				
SHV-4					L		S	K			
SHV-5							S	K			
SHV-7[b]							S	K			

[a] Data were adapted from previously published reports (40, 93). A, Ala; C, Cys; D, Asp; E, Glu; G, Gly; H, His; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val. A consensus numbering is used (2). TEM-14, -16, -17, -18, and -19 are not listed because new sequences do not correspond to results obtained by oligotyping, and TEM-17 was found to correspond to TEM-15 with different silent mutations (15).

[b] Other changes occur at position 8 (I to F) in the leader and at position 43 (R to S) (9).

Residue 104 (Glu and Asp). (i) Environment. The hydrophilic side chain of residue 104 is exposed on the left side of the entrance to the binding site (Fig. 1 and 2). In substrate-free β-lactamases, its side chain or main chain CO group is hydrogen bonded to Asn-132, and it may therefore stabilize the catalytically important Ser-130 in the conserved Ser-Asp-Asn (SDN) loop from positions 130 to 132 (70). In addition, the side chain of residue 104 could easily contact the larger acylamido substituents of β-lactams.

(ii) Change. Lysine has been reported in eight TEM enzymes. This single substitution is second in frequency only to the multiple changes at position 164.

(iii) Result. The long lysine side chain could extend out to interact with the carboxylic acid group in the substituents of ceftazidime, aztreonam, or ceftibuten. This electrostatic attraction should increase initial binding, possibly lowering the K_m. However, a synthesized TEM-1 mutant with a change from glutamate to lysine at position 104 (E104K) showed little change in K_m for the first two of these β-lactams (80), so that the charge-charge interaction may involve an intermediate rather than the initial Michaelis species. Reduction of the K_m is observed in multiple mutants containing an R164S change. In the TEM-5–TEM-24 and TEM-12–TEM-26 pairs, components of which differ at position 104, V_{max} values are higher for the one containing Lys-104 (40). Recent mutagenesis work has shown that, besides forming a charge-charge bonding with substrate, the E104K change may perturb the SDN loop and its interaction with substrate (73, 90).

Residue 164 (Arg and Arg). (i) Environment. The arginine at position 164 is below the binding site in the omega loop (positions 162 to 179) that contains the catalytic Glu-166 (Fig. 3a). The guanidinium side chain is strongly linked by electrostatic attraction and hydrogen bonds to conserved Asp-179 across the neck of the loop (29, 49).

(ii) Change. Changes at residue 164 are the most common changes observed in TEM variants. Another hydrogen-bonding amino acid, either serine or histidine, appears, but with one less hydrogen bond donor. Random mutagenesis and selection for ceftazidime resistance, however, produce TEM-1 mutants having glycine in this position (72).

(iii) Result. A reduction in the number of hydrogen bonds or elimination of the electrostatic attraction will weaken the linkage across the neck of the omega loop. This change allows more flexibility in the loop, which in turn opens more space for bulky β-lactam substituents. Of equal importance is the likelihood that the carboxylic acid side chain of Glu-166 will fluctuate about its native position, where it must function as a general acid-base (21, 91). The R164S TEM-1 mutant prepared by site-directed mutagenesis showed improved k_{cat}/K_m for cefotaxime, ceftazidime, and aztreonam (80). A reversal of clavulanate resistance was found in an R244S mutant containing a second R164S mutation (35), a surprising result in view of the implication of Arg-244 in the clavulanate mechanism.

Residue 179 (Asp and Asp). (i) Environment. The highly conserved acidic residue 179 serves a structural role by linking to Arg-164 to close the omega loop. Both electrostatic and hydrogen bonding exist between the two residues.

(ii) Change. No clinical variants are reported. A very early β-lactamase variant (P54) from a laboratory isolate of *S. aureus* PC1 (66) has asparagine at position 179.

(iii) Result. Weakening of the linkage between residues 164 and 179 and increased movement of the loop will expand the binding site and destabilize Glu-166 and its attached hydrolytic water molecule. The X-ray crystal structure of the D179N mutant of the PC1 β-lactamase shows a floppy, disordered omega loop, as expected (30). Accordingly, the k_{cat} of the mutant is reduced twofold and K_m is about threefold lower for penicillin G. Random mutagenesis of 179 in TEM-1 produced three *E. coli* transformants (179N, G, and Y) with increased levels of resistance to ceftazidime (87).

Residue 182 (Met and Thr). (i) Environment. Residue 182 is quite far from the binding site (Fig. 3a), but like residue 43, it touches a β-strand (62–65) leading to Met-69 behind the oxyanion pocket.

(ii) Change. A threonine is found in a clinical isolate of a TEM β-lactamase resistant to clavulanate-type inhibitors. However, the enzyme contains a second change at position 69 that was shown to be the dominant factor in the resistance (4).

(iii) Result. The result of a change at position 182 is not clear. Perhaps the required structural change at position 69 is facilitated by the introduction of a branched β-carbon atom at position 182. Threonine is found in all SHV enzymes and in most other wild-type β-lactamases (2).

Residue 205 (Gln and Arg). (i) Environment. On the upper surface of the enzyme near the N-terminal end of helix H9, the exposed amino acid at position 205 is very far (2.2 nm) from the binding site. However, the C-terminal end of this helix forms the upper edge of the binding site (Fig. 1).

(ii) Change. In SHV-type enzymes a radical change is observed whereby the hydrophilic arginine is replaced in SHV-3 and SHV-4 by a hydrophobic leucine.

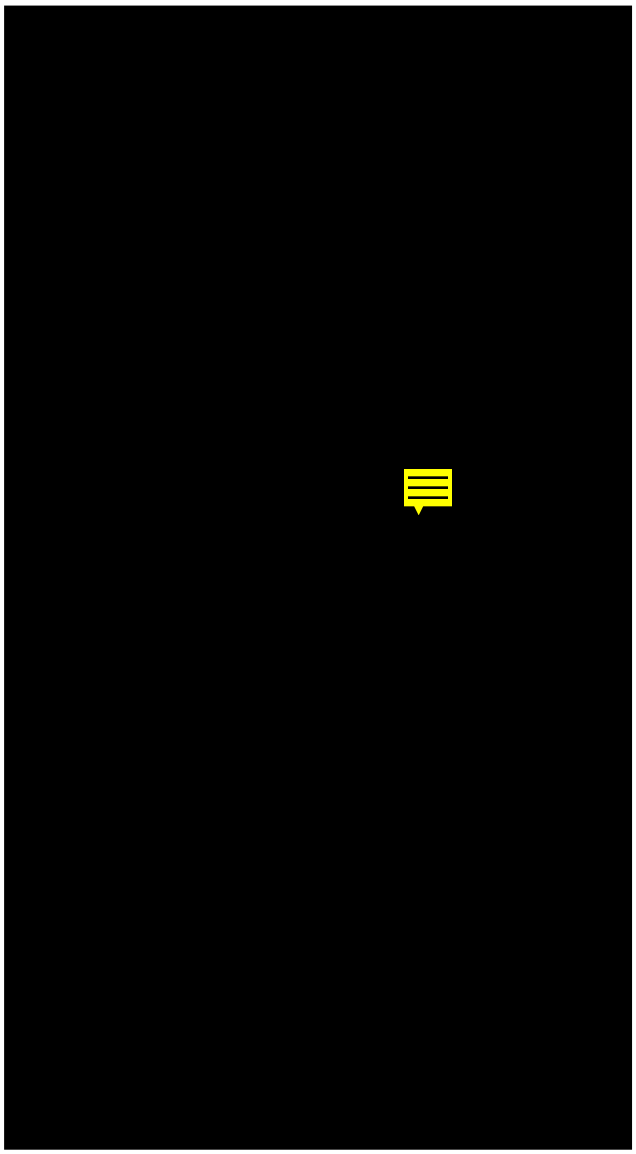
(iii) Result. Thermodynamically driven burial of a hydrophobic side chain might tilt the axis of helix H9. The C-terminal end of the helix, containing residues 216 to 218 (VAG), might move up and away from the β-lactam binding site, thereby accommodating larger C-3 substituents of cephalosporins.

Residue 237 (Ala and Ala). (i) Environment. The side chain at residue 237 is on the outer, exposed side of the B3 β-strand that forms the right edge of the binding site (Fig. 2a and b). Its backbone NH and CO groups bind the CO of the β-lactam ring and NH of the C-6 (C-7) acylamido linkage, respectively.

(ii) Changes. TEM-5 and TEM-24 contain threonine. One of the first chemically induced mutants of TEM-2 was found to contain threonine here (27), and site-saturation mutagenesis of TEM-1 showed that asparagine as well as threonine appeared (28). In both experiments, increased catalytic efficiency on cephems over penams was observed.

(iii) Result. A clear indication of the utility of threonine at position 237 comes from a crystallographic mapping of the binding of cefotaxime to the structurally homologous DD-peptidase (52). Cefotaxime, with its branched oximino substituent, was found tilted out of the binding site and unable to form the expected hydrogen bond to the backbone CO group at position 237. Instead, cefotaxime's NH donates a hydrogen bond to the side chain OH group of threonine, which exists at this position in the DD-peptidase. In TEM-5 and TEM-24 β-lactamases (11, 40, 79), the replacement of Ala-237 with a hydrogen bond acceptor such as threonine should, and does, enhance the binding of this type of expanded-spectrum β-lactam (Fig. 3a and b). Accordingly, the wild-type *Proteus vulgaris* enzyme, which is rather unique in containing Ser-237, shows reduced catalytic efficiency against oximinocephalosporins such as cefuroxime when Ala-237 is introduced by site-directed mutagenesis (85).

Residue 238 (Gly and Gly). (i) Environment. Unlike residue 237, the side chain at position 238 is on the inner side of the B3 β-strand (Fig. 2). Because it lies very close to the side chain of residue 69, the parental sequences show a correlation between the sizes of each (32). For example, the wild-type β-lactamase having the largest side chain at position 238 (valine) is the only



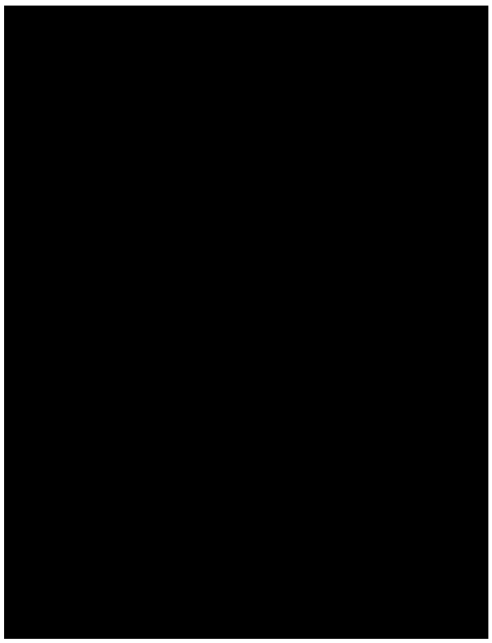


FIG. 3. (a) Residues discussed in the text, viewed as described for Fig. 1 and 2. The linkage between Arg-164 and Asp-179 in the neck of the omega loop (green) is shown. A similar multiple linkage exists between Arg-244 and Asp-176. The rotational conformation of the branched substituent of cefotaxime and the hydrogen bonding of its acylamido NH group to the side chain OH group of Thr-237 were observed in the X-ray structure of its acyl complex with a DD-peptidase (52). (b) Left-side view of cefotaxime in the binding site after a 90° rotation of panel a about a vertical axis. (c) View similar to that in panel b, but with ceftazidime modeled in the binding site. Note the rotation of the acylamido substituent relative to that in cefotaxime. This ceftazidime conformation is like that observed in the X-ray structure of an acyl complex with the *C. freundii* β-lactamase (32, 68). Possible electrostatic interactions of the carboxylic acid group with Lys-104 and Lys-240 are indicated.

one having a glycine at position 69 (*Streptomyces cacaoi* ULg). This size correlation breaks down in the extended-spectrum β-lactamases.

(ii) Changes. Serine occurs in five TEM variants and in all SHV mutants. (Both sets of variants contain methionine at position 69). Random mutagenesis of TEM-1 showed that only serine arose here in ceftazidime-resistant mutants (89). SHV-type OHIO-1 variants selected for altered specificity were found with larger cysteine and valine residues, as well as serine (77).

(iii) Result. If side chains of both residues 69 and 238 are large, crowding of the two may displace the B3 β-strand outward so that the lower portion of the binding site becomes slightly expanded. Cephalosporins with rigid, branched acylamido substituents are now better able to form hydrogen bonds with the more accessible backbone groups of residue 237 (Fig. 2a), and they should exhibit lower K_m values. Nevertheless, V_{max} or k_{cat} values may be reduced because the oxyanion pocket is deformed or, more likely, the distance between the hydrogen-bonded β-lactam and the reactive Ser-70 is longer than optimum. Any movement of the B3 β-strand may also displace Arg-244 because its side chain lies immediately above B3. Some have suggested that hydrogen bonding between the OH of Ser-238 and the oximino nitrogen atom of cefotaxime may occur (53, 75), but kinetics (57) and modeling (32) show that this is unlikely, and a non-hydrogen-bonding Val-238 mutant of an SHV β-lactamase has a better binding constant for cefotaxime (77).

Residue 240 (Glu and Glu). (i) Environment. At the end of β-strand B3, the exposed, hydrophilic residue 240 can interact with acylamide substituents of cephalosporins. This side chain would be too far from smaller ligands such as clavulanic acid or sulbactam, and changes here are not seen or expected in inhibitor-resistant variants.

(ii) Change. Lysine is observed in four TEM and three SHV mutants. Changes here and at residue 238 are the only changes common to both sets of β-lactamase mutants.

(iii) Result. The lysine side chain is able to form an electrostatic bond with the carboxylic acid group on oximino substituents. Lys-240 is therefore found in variants able to hydrolyze

ceftazidime and aztreonam (32, 53). TEM-24, with lysines at both positions 104 and 240, has the highest V_{max} for ceftazidime (40). Because mutations here will have a negligible effect on the structural or catalytic integrity of the enzyme, other hydrophilic residues are expected. Thus, several TEM mutants made from a random library and selected for increased ceftazidime resistance were found to have arginine at this position (89).

Residue 242 (Gly and Gly). (i) Environment. At the beginning of β-strand B4, position 242 is conserved as a glycine because the backbone here has a high-energy $L3_{10}$ conformation in which larger side chains are not favored (76).

(ii) Change. An SHV-type OHIO mutant, TAX-1, obtained by selection, contains cysteine at residue 242 (77). Currently, no natural SHV or TEM isolates have this alteration.

(iii) Result. Conformational stress because of the introduction of a side chain at residue 242 can be relieved by transfer of the stress along the backbone from residues 240 to 242. Modeling shows that the lower part of the less hindered B3 β-strand will move outward (7). This shift in B3 is similar to that proposed above to result from the crowding of large side chains at position 238 against Met-69. Movement of the β-strand by either mechanism will facilitate the binding of cephalosporins with rigid acylamido groups, as found for cefotaxime in the OHIO mutants (7, 77). The monobactam aztreonam remains resistant to binding by TAX-1 (K_m > 1,000 μM), possibly because the oximino carboxylic acid group is repelled by both Asp-104 and Glu-240.

Residue 244 (Arg and Arg). (i) Environment. Residue 244 is a conserved residue on the B4 β-strand (but see reference 39). Its long side chain reaches over the adjacent β-strand to the upper right edge of the binding site. It is anchored in place by two hydrogen bonds to Asn-276. Via a well-ordered, bridging water molecule it may interact with the C-3 (C-4) carboxylic acid group of β-lactams (33, 43, 84, 92). However, some believe there are no direct interactions with the acid group (17) and that the role of Arg-244 is to destabilize the enzyme-product complex and optimize the turnover rate (42).

(ii) Changes. Independent of a crystallographic-based prediction of the involvement of Arg-244 with mechanism-based inhibitors (64), chemical mutagenesis in the presence of clavulanate produced a clavulanate-resistant TEM form containing Cys-244 (62). Soon after, resistant clinical isolates with cysteine or serine were reported (3, 88).

(iii) Result. A structure-based analysis of inactivation by clavulanic acid (33) has shown that Arg-244 happens to be in an excellent position to induce a water molecule to provide the proton needed for formation of an acyclic intermediate (Fig. 4). (Sulbactam and tazobactam are thought to use a different mechanism not so dependent on Arg-244 [34].) The shorter, uncharged side chains of serine or cysteine would be unable to activate the water. An unexpected finding that Ser-164 reverses clavulanate resistance conferred by a Ser-244 double mutation (35) is perhaps due in part to structural changes resulting from a weakening of the 164-179 linkage.

Residue 265 (Thr and Leu). (i) Environment. Quite distant from the binding site (1.3 nm), the side chain at position 265 is on the outer face of the β-sheet, yet it is buried by β-strand neighbors and the C-terminal α-helix (Fig. 1 and 3a).

(ii) Change. In five TEM β-lactamases methionine is seen.

(iii) Result. No catalytic changes are observed in TEM-13, a TEM-2 variant with only this mutation (40, 41), nor were changes seen in T265M TEM-1 mutants prepared by site-directed mutagenesis (31). Genetic pathways which could have produced this neutral mutation have been discussed (31).

FIG. 4. Modeled clavulanate binding and the indirect involvement of Arg-244 and Asn-276. The larger sphere is a hydrogen-bonded water molecule seen in the X-ray structure of the native enzyme and is thought to be the source of a proton in the inactivation process (33). The β-methyl group of Ile-69 is seen behind the oxyanion pocket, the NH groups of which hydrogen bond to the β-lactam CO group.

Residue 276 (Asn and Asn). (i) Environment. On the C-terminal α-helix, the partially exposed side chain at residue 276 is hydrophilic in all wild-type class A β-lactamases (Fig. 3a). In TEM and SHV the carbonyl group of asparagine accepts two hydrogen bonds from Arg-244 that orient the guanidinium group (17, 42).

(ii) Change. Two inhibitor-resistant variants (TEM-35 and TEM-36) have recently been detected. In these variants aspartic acid replaces asparagine (93).

(iii) Result. The hydrogen bonding to Arg-244 can be maintained in the N276D mutants. Such bonding orients the guanidinium group for binding β-lactams (via a water molecule) and adventitiously assists the inactivating reactions of clavulanic acid (33) (Fig. 4). Thus, an OHIO SHV-type Gly-276 mutant made by site-directed mutagenesis exhibited decreased affinity and catalytic efficiency for β-lactam substrates, as well as a 10-fold higher K_i for clavulanate (8). In the natural TEM N276D mutants, the added electrostatic component of the binding with Arg-244 will reduce the positive charge centered on the guanidinium group. This factor, and the possible disruption of the oxyanion pocket by the accompanying mutation at position 69 (Table 1), may explain the inhibitor resistance of these new variants.

CLASS C β-LACTAMASES

Few data on natural variants of the *ampC*-type enzymes are available (40, 67). Although the larger class C (group 1) amino acid sequences are difficult to align with class A amino acid sequences, X-ray analysis shows that important match points in the class A and class C enzymes are Ser-70 and Ser-64, X104 and X120, Ser-130 and Tyr-150, and X237 and X′318, respectively (60, 68). In the class C enzymes no amino acids are spatially equivalent to Arg-164 and Glu-166 of class A, because polypeptide folding is significantly different in this "omega loop" region (60). A clinically derived *E. cloacae* GC1 enzyme with extended specificity to oximino-β-lactams was found to contain a tripeptide insertion in this loop region (67). Further comparison of class A and class C crystallographic structures indicates that many of the class A mutations described above may be unnecessary in the class C β-lactamases because these

enzymes have a larger binding site more accommodating to cephalosporins with rigid substituents.

ALTERED PENICILLIN-BINDING PROTEINS

X-ray structures of membrane-bound penicillin-binding proteins may soon appear, because water-soluble forms are being crystallized (12, 22, 36, 86). In the meantime, given the similarity of the β-lactamase structures with the X-ray structure of the penicillin-inhibited DD-peptidase (25, 46), one might speculate on the structural basis of mutations discovered in the low-affinity penicillin-binding proteins (24, 81, 82). A crystallographic mapping of the binding of cefotaxime to the DD-peptidase (52) shows that a hydrogen bond exists between the acylated cefotaxime and the side chain hydroxyl group of Thr-301 (equivalent to residue 237 in class A numbering). Significantly, in PBP 2x of a laboratory isolate of *Streptococcus pneumoniae* resistant to cefotaxime (54), this threonine has mutated to a non-hydrogen-bonding alanine residue, accounting for the lower affinity for cefotaxime. The reverse mutation, alanine to threonine in TEM-5 and TEM-24 β-lactamases (11, 79), makes hydrogen bonding possible and, as expected, results in a higher affinity for cefotaxime and ceftazidime!

OUTLOOK

Questions remain. For example, why is the activity against penicillins and classical cephalosporins generally reduced in the extended-spectrum or inhibitor-resistant variants relative to the activity in the parental forms (63, 75)? One can see that nature pays a high cost in general to craft a specialized enzyme to destroy the particular β-lactam with which it must cope, and often including, if necessary, a parental plasmid to deal with the classical β-lactams (61). Why is it that the most frequent changes in the TEM enzymes (at positions 104 and 164) have not appeared in any of the SHV variants? Why do analogous substitutions in the TEM-1 and SHV-1 β-lactamases sometimes fail to elicite the same phenotypic response (89)? Despite 68% identity in TEM and SHV sequences, perhaps the three-dimensional structures of the catalytic sites of the two β-lactamases are not as similar as has been assumed.

There is no doubt that unanticipated changes in both TEM and SHV families, including new combinations of the changes discussed above, will reduce the efficacies of current and forthcoming β-lactams and β-lactamase inhibitors. What may be in store is forecast by random-replacement mutagenesis (71, 72) and in vitro DNA shuffling (83) of the TEM-1 β-lactamase gene, from which numerous functional, extended-spectrum variants that have yet to be uncovered in the clinic have been produced.

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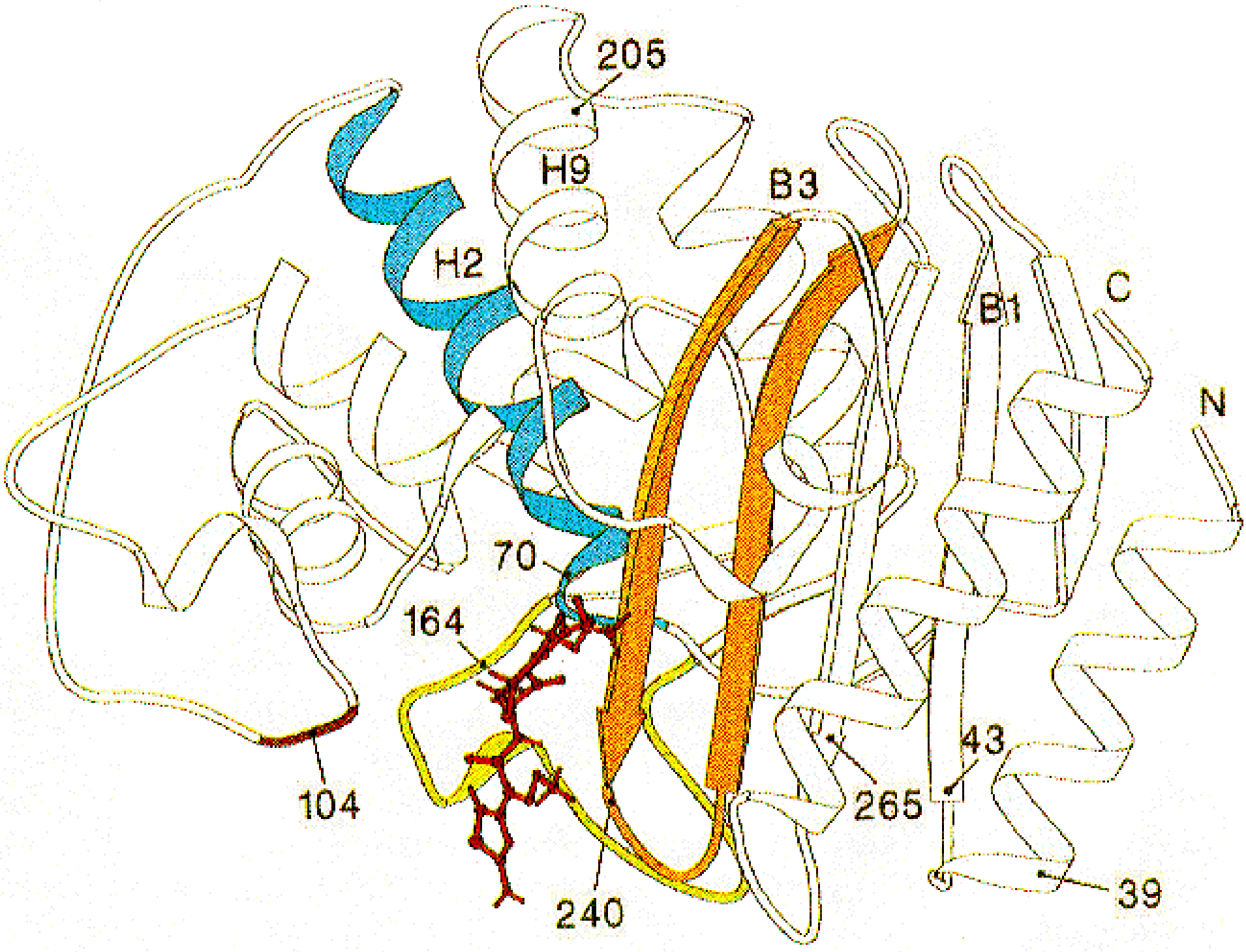
205
H9
B3
H2
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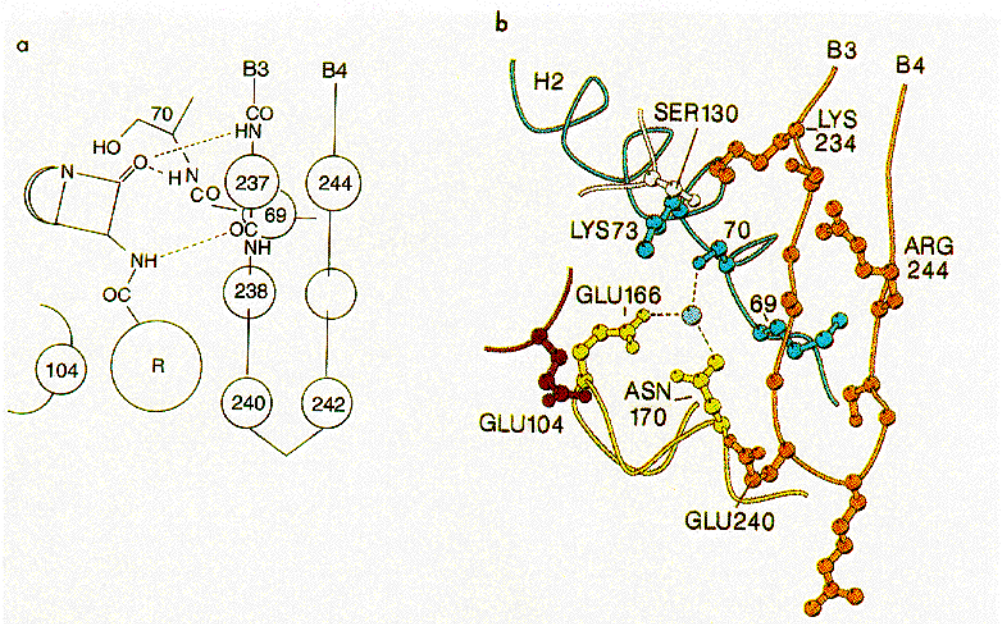
39
240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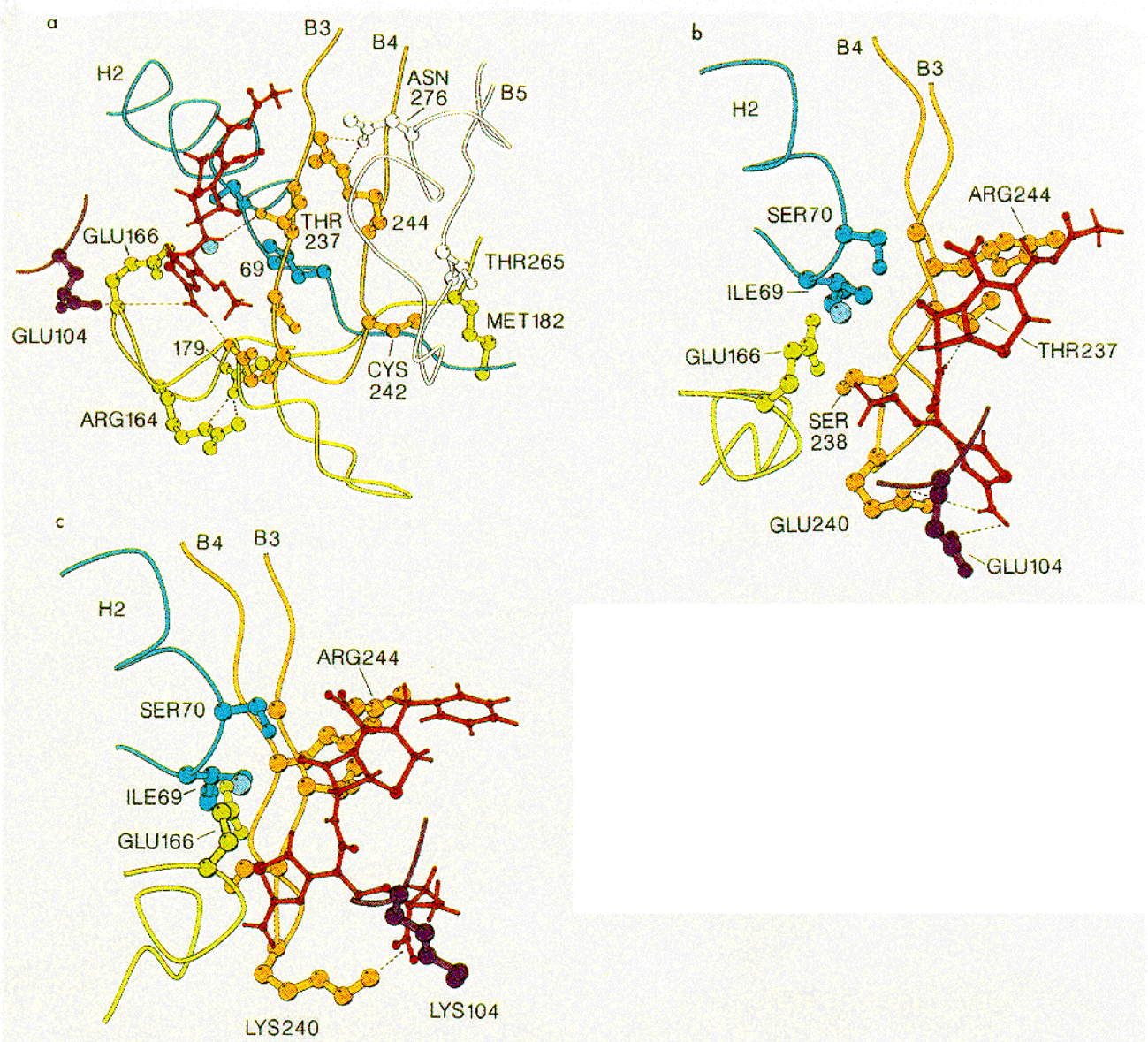
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ARG164
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B3
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SER70
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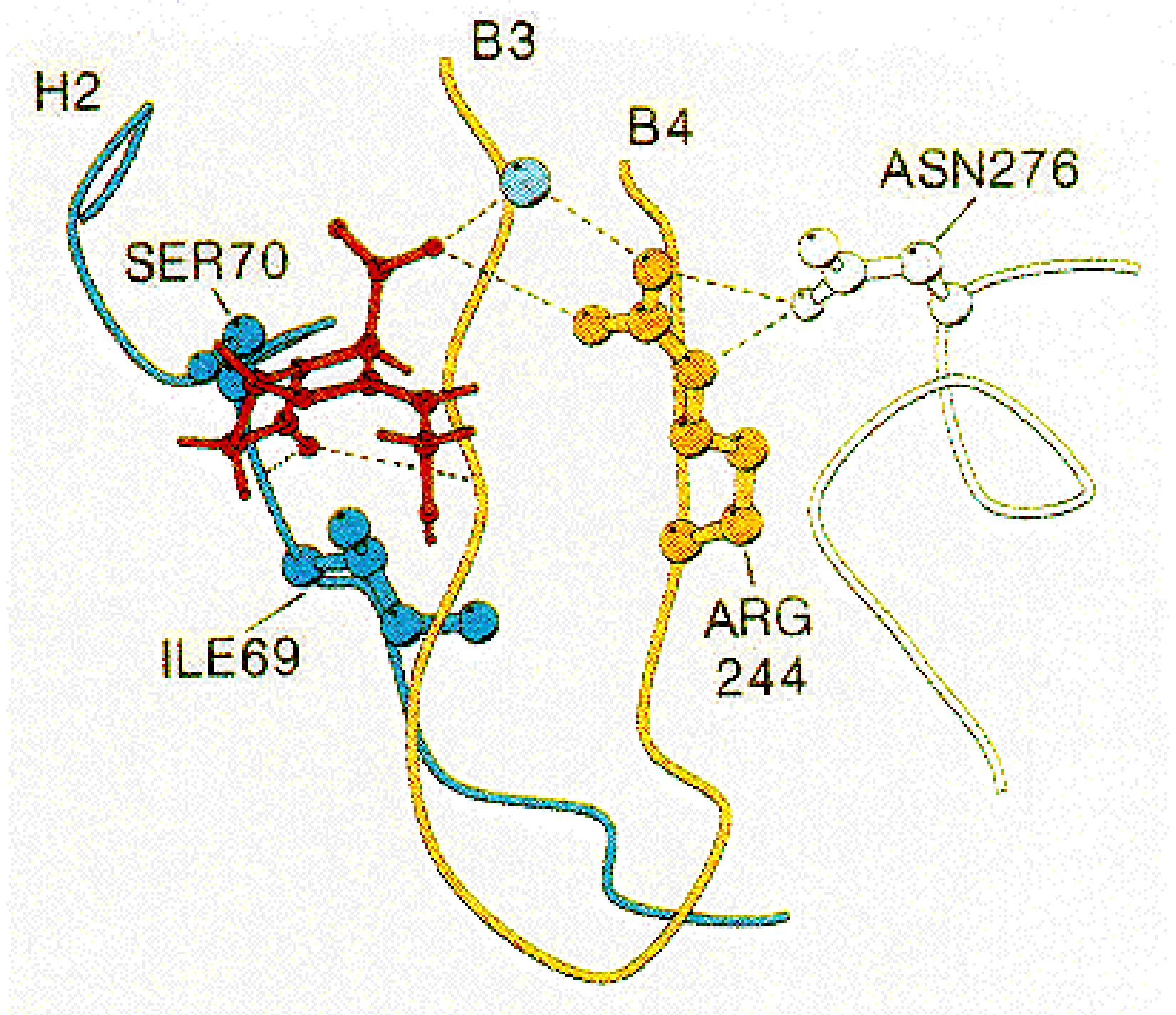
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