

## Bacterial Active-Site Serine Penicillin-Interactive Proteins and Domains: Mechanism, Structure, and Evolution

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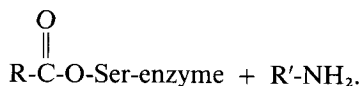
The bacterial active-site serine penicillin-hydrolyzing and penicillin-binding proteins or domains operate by a serine-ester-linked acyl enzyme mechanism similar to that of the peptidases of the trypsin and subtilisin families. On the basis of known primary and tertiary structures, predictive studies support the view that these proteins or domains form a superfamily of evolutionarily related enzymes. Although—depending on the evolutionary distance—they may have very different amino acid sequences and distinct functional characteristics and specificities, they all would have conserved the same unprecedented type of polypeptide scaffolding. When obtained, complete structural information should provide the necessary tools for the rational design of novel types of inactivators of these important enzyme targets.

### The Acyl Enzyme Mechanism

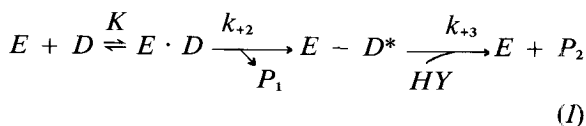
The catalyzed rupture of a peptide bond in a sus-

ceptible carbonyl donor  $\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-\text{NH}-\text{R}'$  by the active-site serine peptidases of the trypsin and subtilisin families is accomplished by transfer of the elec-

trophilic acyl group  $\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-$  to the enzyme active-site serine and then to an exogenous acceptor. Central to this mechanism is the formation of a serine-ester-linked acyl enzyme with concomitant generation of a leaving group:



The general reaction catalyzed is:



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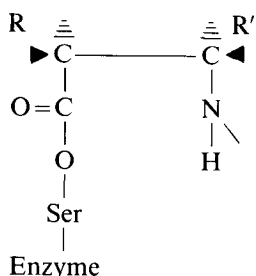
with  $E$  representing the enzyme;  $D$ , the carbonyl donor;  $HY$ , the exogenous acceptor;  $E \cdot D$ , the Michaelis complex;  $E - D^*$ , the acyl enzyme;  $K$ , the dissociation constant;  $k_{+2}$  and  $k_{+3}$ , the first-order rate constants;  $P_1$ , the leaving group  $\text{R}'-\text{NH}_2$  or first reaction product; and  $P_2$ , the second reaction product

$\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-\text{Y}$ . In addition,  $k_{+2}/K$  is the second-order rate constant of enzyme acylation by the carbonyl donor. As a result of this double transfer, the nature of  $P_2$  depends on the type of the acceptor  $HY$ . Attack of the acyl enzyme  $E - D^*$  by water, an alcohol, or an amino compound causes hydrolysis, alcoholysis, or aminolysis (i.e., transpeptidation) of the carbonyl donor, respectively. Competition at the level of the acyl enzyme between the exogenous acceptors and the endogenously released leaving group governs the fate of the carbonyl donor substrate.

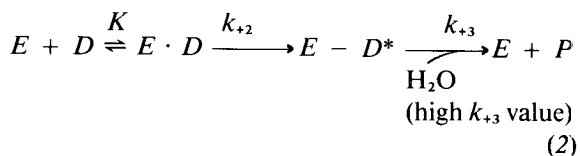
Bacteria possess proteins that interact with penicillin and other  $\beta$ -lactam antibiotics via formation of a serine-ester-linked acyl (penicilloyl, cephalosporoyl) enzyme. A common feature is that a negative charge must occur at the 3' position in the penicillins and the 4' position in the cephalosporins (this group is usually a carboxylate, but a sulfate is found in the monobactams), and the acyl side chain borne by the  $\beta$ -lactam ring must be on the  $\beta$  face in a *cis* position relative to the thiazolidine (dihydrothiazine) ring in the penicillins (cephalosporins). As a result of these requirements, the endocyclic amide bond in the  $\beta$ -lactam ring is equivalent to a C-terminal *D*-alanyl-*D*-alanine peptide bond. Another common feature is that the value of the second-order rate con-

stant of protein acylation is high, though it may vary considerably (from  $10^2$  to  $10^6$   $M^{-1}s^{-1}$ ), depending on the protein and the  $\beta$ -lactam compound considered.

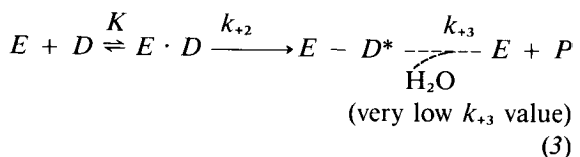
The reaction between the penicillin-interactive proteins and the  $\beta$ -lactam antibiotics is mechanistically similar to an active-site serine peptidase-catalyzed acyl transfer [1, 2]. However, because the scissile amide bond in the  $\beta$ -lactam carbonyl donor is endocyclic, acylation of the active-site serine generates a leaving group that remains part of the acyl enzyme:



Though the enzyme active site remains occupied by a nondiffusible leaving group, reformation of an intact  $\beta$ -lactam ring does not occur. However, as a result of this occupancy, only water (and, under non-physiologic conditions, powerful nucleophiles such as hydroxylamine) can attack the acyl enzyme. Depending on the susceptibility of the acyl enzyme, the penicillin-interactive proteins fall into two major categories. With the  $\beta$ -lactamases, water is an excellent attacking nucleophile; thus reaction 1 becomes:



where  $D$  is the cyclic,  $\beta$ -lactam carbonyl donor substrate and  $P$  the hydrolyzed  $\beta$ -lactam molecule. With the so-called penicillin-binding proteins (PBPs), the acyl enzyme is almost completely hydrolytically inert. Constant  $k_{+3}$  has a very small absolute value; reaction 1 becomes:



As a corollary, the  $\beta$ -lactam carbonyl donor  $D$  is a mechanism-based inactivator.

In the absence of  $\beta$ -lactam antibiotics, the PBPs play important or essential roles in wall peptidoglycan metabolism [1]. They catalyze—according to reaction 1—transfer of the electrophilic group R-D-alanyl from R-D-alanyl-D-alanine-terminated peptidoglycan precursors to an acceptor via formation of a serine-ester-linked acyl (R-D-alanyl) enzyme and generation of a leaving group D-alanine. Depending on whether the acceptor is the  $\omega$ -amino group of another peptide or water, the catalyzed reaction (transpeptidation vs. carboxypeptidation) leads to an increased or a decreased level of peptidoglycan cross-linking. Penicillin binding to the active-site serine abolishes DD-peptidase activity. Details on PBP-catalyzed acyl-transfer reactions with noncyclic (ester and amide) and cyclic ( $\beta$ -lactam) carbonyl donors have been published elsewhere [3–5].

The above distinction between  $\beta$ -lactamases and PBPs in terms of lability vs. stability of the acyl enzymes formed by reaction with  $\beta$ -lactam antibiotics is an oversimplification [1, 2].  $\beta$ -Lactamases may also give rise to long-lived acyl enzymes, and long-lived acyl enzymes may undergo various types of intramolecular rearrangements. Rupture of the bond between C-5 and C-6 in an acyl (benzylpenicilloyl) PBP causes release of the leaving group and generates a new and labile acyl enzyme that may undergo partitioning between alternate nucleophiles. Rupture of the bond between S-1 and C-5 in the acyl enzymes formed by reaction with the penam sulfones and 6- $\beta$ -bromo(iodo)penicillinate (or rupture of the bond between O-1 and C-5 in clavulanate) is an integral part of the  $\beta$ -lactamase inactivation processes. The established common feature is that of a branched pathway where  $\beta$  elimination leads to a hydrolytically inert acyl enzyme and/or further modification of some residues of the enzyme active site. A third type of rearrangement, observed with the cephalosporins that have, for example, a 3' acetoxy substituent, is elimination of this group from the acyl enzyme, with formation of an exocyclic methylene. This elimination has important consequences with respect to cephalosporin turnover by the  $\beta$ -lactamases and inactivation of the DD-peptidases, since the modified acyl enzymes are more hydrolytically inert than their parents.

In complete analogy with the bacterial PBPs, the peptidases of the trypsin and subtilisin families also behave as  $\beta$ -lactam compound-binding proteins. The

latter enzymes, however, differ from the DD-peptidases and  $\beta$ -lactamases in having endopeptidase rather than carboxypeptidase activity and in preferring L to D configuration. Given these substrate requirements, cephalosporins have been modified and converted – at the expense of their antibacterial activity – into mechanism-based inactivators of trypsin, chymotrypsin, elastase, plasmin, or thrombin [6]. Long-lived acyl (cephalosporoyl) LL-peptidases (bearing a 3' acetoxy substituent on the dihydrothiazine ring) also undergo the type of intramolecular rearrangement just described.

### Major Groups of Active-Site Serine Penicillin-Interactive Proteins

On the basis of various criteria, one may distinguish four major groups of active-site serine penicillin-interactive proteins.

**Group 1.** The low-molecular-weight PBPs ( $\sim 25,000$ – $35,000$ ) are DD-peptidases that can operate on well-defined, simple peptide or depsipeptide carbonyl donor analogues such as  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$  or  $\text{Ac}_2\text{-L-Lys-D-Ala-D-lactate}$  (reaction 1), a property that has greatly facilitated their isolation and characterization [1]. The efficacy with which they perform transpeptidation vs. hydrolysis depends on the efficacy with which the leaving group D-Ala, water, and an exogenous peptide  $\text{H}_2\text{N-X}$  attack the acyl ( $\text{Ac}_2\text{-L-Lys-D-alanyl}$ ) enzyme. When, in particular, the relative acceptor activity is  $\text{H}_2\text{O} \ll \text{D-Ala} \ll \text{H}_2\text{N-X}$ , hydrolysis – in the absence of an amino acceptor – is negligible because the released D-Ala successfully competes with  $\text{H}_2\text{O}$  and is re-utilized in a transfer reaction that maintains the concentration of the carbonyl donor at a constant level [4]. In the presence of an amino acceptor of high acceptor activity (and related to wall peptidoglycan), the carbonyl donor is quantitatively converted into the transpeptidated product  $\text{Ac}_2\text{-L-Lys-D-Ala-NH-X}$ ; i.e., the PBP functions as a strict transpeptidase.

The low-molecular-weight PBPs/DD-peptidases are inserted into the plasma membrane by a non-cleaved, carboxy terminal signal-like peptide segment, while the bulk of the polypeptide chain is on the periplasmic side of the membrane. Catalytically active, water-soluble derivatives can be obtained by proteolytic treatment of isolated membranes [7, 8]. Replacement by genetic engineering of the carboxy terminal 21-amino-acid region of *Escherichia coli* PBP5 by a shorter, 9-amino-acid sequence (possess-

ing two arginine residues and one lysine residue) causes excretion of the PBP in the periplasm [9]. *Streptomyces* R61 spontaneously excretes a low-molecular-weight PBP in the medium during growth. The 406-amino-acid PBP precursor possesses both a cleavable peptide signal and a cleavable 26-amino-acid C-terminal extension [10]. Should it not be removed during maturation, this C-terminal extension might function as a stop transfer sequence through which the enzyme would become membrane bound.

**Group 2.** The high-molecular-weight PBPs (60,000–90,000), including PBP3, 2, 1B, and 1A of *E. coli*, are bifunctional. They possess a carboxy terminal, penicillin-binding domain that catalyzes the penicillin-sensitive peptidoglycan transpeptidase reaction (peptide cross-linking) and an amino terminal domain that is assumed to catalyze a penicillin-insensitive peptidoglycan transglycosylase reaction (glycan chain elongation). These PBPs lack activity on the aforementioned peptide and depsipeptide analogues. Their bifunctional nature is revealed by the reaction products that they generate upon incubation with the lipid-linked precursor *N*-acetylglucosaminyl-*N*-acetylmuramyl (D-alanyl-D-alanine-terminated pentapeptide) diphosphoryl-undecaprinol. A gene fusion that removes the amino terminal 240-amino-acid region of PBP3 and links the carboxy terminal 349-amino-acid region to the amino terminal of the  $\beta$ -galactosidase results in a truncated polypeptide that still binds penicillin [11]. PBP1B and PBP3 are held in the plasma membrane at their amino termini, with essentially all the protein in the periplasmic space [12]. It is assumed that the same membrane organization applies to PBPs 1A and 2.

**Group 3.** The  $\beta$ -lactamases of class D (i.e., the 25,000-molecular-weight oxa-2  $\beta$ -lactamase of *Salmonella typhimurium*), of class A ( $\sim 28,000$ ), and of class C ( $\sim 35,000$ ) are defensive enzymes that protect the DD-peptidase active site of the penicillin-binding proteins or domains against inactivation by the  $\beta$ -lactam antibiotics. They are devoid of DD-peptidase activity, yet the  $\beta$ -lactamases of class C catalyze acyl transfer reactions from the same depsipeptide carbonyl donors as those utilized by the low-molecular-weight PBPs. The  $\beta$ -lactamases are secretory proteins; they are excreted in the growth medium (gram-positive) or accumulate in the periplasmic space (gram-negative). In  $\beta$ -lactamase III of *Bacillus cereus* 569/H and that of *Bacillus licheniformis*, however, there is a diacylglyceride that is thioether-linked to the amino terminal cysteine

residue and a fatty acid that is amide-linked to the same cysteine [13]. This hydrophobic moiety functions as the anchor of the protein to the plasma membrane. In fact, these  $\beta$ -lactamases have membrane-bound and secretory forms. The membrane-bound forms contain a single cysteine residue (NH<sub>2</sub>-terminal), while the hydrophilic exoforms lack cysteine.

**Group 4.** Some  $\beta$ -lactamases are constitutive. Others are inducible; i.e., they are produced in maximal amounts only in the presence of penicillin or another  $\beta$ -lactam antibiotic. The specific inducibility of a  $\beta$ -lactamase requires several regulatory genes, some of which code for the synthesis of a receptor and various regulatory products while others act as binding sites for these regulatory macromolecules. The locus *blaR1*, required for the induction of  $\beta$ -lactamase synthesis in *B. licheniformis* 749 on exposure to cephalosporin C, encodes a 601-amino-acid *BlaR1* protein [14]. The amino acid sequence, as deduced from gene sequencing, permits prediction of the two-dimensional membrane topology of the protein. Its amino terminal 354-amino-acid region would consist of four definite transmembrane segments, while the carboxy terminal 247-amino-acid region would protrude from the outer face of the membrane. This carboxy terminal region has the characteristics of an active-site serine penicillin-interactive protein (see below).

### Structure and Evolutionary Relation

The active-site serine penicillin-interactive proteins and domains fulfill many different functions and catalyze many different reactions, yet all of these proteins and domains bind the same  $\beta$ -lactam ligands and operate by the same acyl enzyme mechanism. Recent advances have shown that, in all likelihood, the proteins and domains of this group form a superfamily of evolutionarily related enzymes distinct from the trypsin and subtilisin families. This unified view has evolved from a body of data obtained in various research areas.

**Structural studies.** The water-soluble, low-molecular-weight PBP/DD-peptidase of *Streptomyces* R61, on the one hand, and the  $\beta$ -lactamases of class A (from *B. licheniformis*, *B. cereus*, *Staphylococcus aureus*, and *Streptomyces albus* G), on the other, have different sizes (349 vs. 273–290 amino acid residues) and at first sight lack relatedness in the primary structure. Nevertheless, these enzymes are similar in terms of the spatial arrangement of second-

ary structures. They are two-region proteins. One region has an "all- $\alpha$ " type structure. The other has a central core consisting of a five-stranded  $\beta$  sheet protected by  $\alpha$  helices on both faces [15–18].

The *S. aureus* and *S. albus* G  $\beta$ -lactamases are the first penicillin-interactive proteins for which connectivity between the secondary structures has been elucidated [17, 18]. These two  $\beta$ -lactamases and the *Streptomyces* R61 PBP/DD-peptidase not only have a similar three-dimensional structure but also possess at least three "boxes" (consisting of strict identities or highly homologous residues) that occur at the same critical positions in the three-dimensional structures. The tetrad Ser\*-Xaa-Xaa-Lys (where Ser\* is the active-site serine) at position  $\sim 60$ –70 in the primary structure occurs at the amino end of one of the helices of the "all- $\alpha$ " region, so that after one turn of the helix, the side chain of the lysine residue is brought back within the active-site area. The triad His-Thr-Gly (in the *Streptomyces* PBP) or Lys-Thr-Gly (in the two  $\beta$ -lactamases) is at position  $\sim 60$ –70 upstream from the carboxy terminus of the proteins. This triad occurs on the innermost strand of the five-stranded  $\beta$  sheet and forms the other side of the cavity, with the histidine imidazole ring or the lysine  $\epsilon$ -amino group also pointing to the active site. Finally, in a somewhat central position in the primary structures (but closer to the carboxy terminus than the amino terminus), there is a negatively charged residue: Asp 225 in the *Streptomyces* PBP, Glu 150 in the *Streptomyces*  $\beta$ -lactamase, and Glu 166 in the staphylococcal  $\beta$ -lactamase. This carboxylate group occurs on a loop that connects two helices, at the entrance of the active site. Remarkably, these three boxes provide the PBP and  $\beta$ -lactamase active sites with positively and negatively charged side chains that, in concert with the active-site serine, are probably important for catalysis and/or binding of the carbonyl donor.

**Predictive studies.** The primary structures of several  $\beta$ -lactamases of classes A and C, the oxa-2  $\beta$ -lactamase of class D, several low-molecular-weight PBPs, the penicillin-binding domains of the *E. coli* high-molecular-weight PBPs, and the receptor domain of the *BlaR1* protein were compared pair-wise by the Goad-Kanehisa procedure [19]. (All of the penicillin-interactive domains are assumed to start 60 residues upstream from the active-site serine residue and to terminate 60 residues downstream from the conserved His-Thr-Gly, Lys-Thr-Gly, or Lys-Ser-Gly triad.) No gaps or insertions were introduced in

the amino acid sequences, and the significance of the comparison between pairs of sequences was assessed and expressed by a standard deviation (SD) unit; an SD value of  $\geq 5$  indicated statistically significant homology. These studies led to the following conclusions: The penicillin-interactive proteins and domains form a family tree in which highly homologous groups are linked to each other through particular pairs of proteins or domains characterized by SD values of 4.3–8. This family tree, however, does not include the oxa-2  $\beta$ -lactamase or the receptor region of *BlaR1*.

The correlation was then reexamined by a forced-alignment procedure. The tetrad Ser\*-Xaa-Xaa-Lys and the triad His-Thr-Gly, Lys-Thr-Gly, or Lys-Ser-Gly served as calibration marks. (The substitutions His/Lys and Thr/Ser occur with high frequencies in homologous proteins.) The *Streptomyces* R61 PBP/DD-peptidase served as template. This reference sequence was kept unbroken while the deletions and/or insertions required for an optimal match were introduced in each of the sequences under comparison. This editing, the cost of which ranged from 6% to 24% of amino acids eliminated from the original sequences, led to the following conclusions: First, the portions of the sequences effectively aligned, including those of the oxa-2  $\beta$ -lactamase and the penicillin receptor of *BlaR1*, are significantly homologous with those of the *Streptomyces* R61 PBP/DD-peptidase. Second, all of the penicillin-interactive proteins and domains possess seven conserved boxes. Two of them are the calibration marks used for the alignments. A third is the negatively charged residue Asp or Glu known to be part of the active-site environment of the class A  $\beta$ -lactamases and the *Streptomyces* R61 PBP. Amino acid replacements in five of the boxes affect or abolish the activity of several  $\beta$ -lactamases, the *E. coli* low-molecular-weight PBP5, and the *E. coli* high-molecular-weight PBP3.

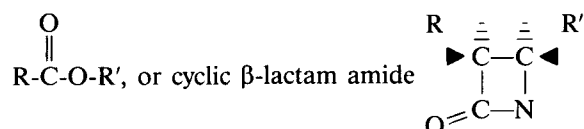
On the basis of the principle that homologous proteins have a similar three-dimensional structure, all of the active-site serine penicillin-interactive proteins and domains must have the same unprecedented type of polypeptide scaffolding. Depending on the evolutionary distance, however, the proteins and domains may have very different amino acid sequences and distinct functional characteristics and specificities.

The  $\beta$ -lactamases are dispensable enzymes, while the PBPs/DD-peptidases fulfill important or essential functions. A possible mechanism that may ex-

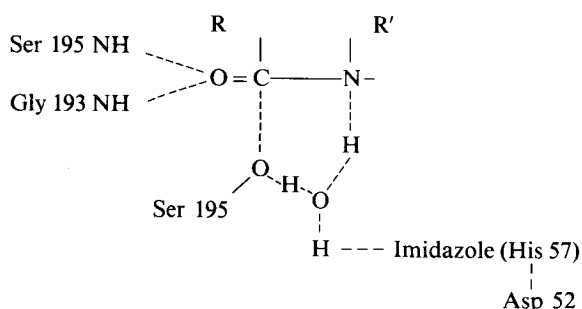
plain the emergence of active-site serine  $\beta$ -lactamases is the excretion of one or several low-molecular-weight PBPs by bacteria exposed to  $\beta$ -lactam antibiotics. Further improvement of this detoxication mechanism results in the conversion of these water-soluble PBPs into  $\beta$ -lactam-hydrolyzing enzymes. Given that (1) soil bacteria (in particular *Streptomyces* species) were probably exposed to penicillin or other  $\beta$ -lactam antibiotics long before the antibiotic era, (2) *Streptomyces* are the only bacteria known to spontaneously manufacture exoforms of the PBPs, and (3) the water-soluble PBP of *Streptomyces* R61 actually serves as a bridge between the  $\beta$ -lactamases of class A and class C in the aforementioned family tree [19], it follows that low-molecular-weight *Streptomyces* PBPs are likely ancestors of the  $\beta$ -lactamases or at least some of them. Experiments aimed at reproducing by protein engineering this proposed evolutionary transition from PBPs to  $\beta$ -lactamases are in progress.

#### Reaction Pathways and Drug Design

Following binding of a peptide  $R-C(=O)-NH-R'$ , ester



carbonyl donor to an enzyme active site, rupture of the scissile bond is carried out by reagents that fulfill, within the active site, the required functions of an electrophile (polarization of the C=O bond) and a proton abstractor-donor (attack of the carbonyl carbon atom and protonation of the nitrogen or oxygen atom). Fine structural studies carried out on chymotrypsin (and other active-site serine LL-endo-peptidases) have revealed that the backbone NH of Ser 195 and Gly 193 creates within the active site an anion hole (electrophile) that polarizes the C=O bond of the bound carbonyl donor. These studies have also attributed to the diad His 57-Asp 102 a role of seemingly prime importance in proton abstraction and donation. At variance with this view, theoretical chemistry (G. Dive, J. Lamotte-Brasseur, and J. M. Ghuysen, unpublished data) shows that proton shuttle during enzyme acylation (and probably enzyme deacylation) is a concerted operation that takes place within a six-membered ring involving a water molecule:



In this proposed mechanism, water is the real proton carrier. Very small changes in the geometry of the whole interacting system have profound effects on the fate and characteristics of the reaction. Optimal geometry is imparted by interaction between the R and R' substituents and several enzyme-binding sites and by the diad His 57–Asp 102, whose main function appears to be that of an orienting device. Although this diad is conserved in the peptidases of the trypsin and subtilisin families, peptidases may exist where the same role is fulfilled by other residues. Remarkably, there is no conserved histidine in the penicillin-interactive proteins and domains.

Understanding how the penicillin-interactive proteins and domains interact with their ligands and unraveling the connection between structure and function is a project that can now be conducted to real effect. Precise structural data are being produced with  $\beta$ -lactamases of classes A and C and with the naturally occurring or genetically engineered, water-soluble PBPs of *Streptomyces* R61 and *E. coli* (PBP5). Efforts are being made to extend these studies to the more essential high-molecular-weight PBPs and/or (in order to overcome the inherent difficulty of obtaining membrane-bound proteins in crystal forms that are suitable for X-ray analysis) to water-soluble derivatives of these PBPs. When refined to high resolution (2 Å or better), these data will allow us to describe with precision the atomic positions within the enzyme molecules. Moreover, the data, when combined with the observed effects of site-directed mutagenesis and construction of chimeric proteins, will permit us to map out the structural features that are important for binding and activity. Using the details of protein structure thus revealed, we can employ the methods of theoretical chemistry to increase our understanding, at the most fundamental level, of the behavior and energy states of the interacting partners (enzyme active site plus ligand). Defining the reaction fields in which the chemistry at the active sites takes place as well as

the dynamics and topology of the reaction pathways will allow us to predict the outcome of the reactions. As already mentioned, the importance of this approach rests upon the fact that minute changes in the atomic configuration of the active site and/or the ligand with which it is reacting profoundly modify the characteristics of the reaction and, in fact, may prevent it completely. An understanding of the effects of these small changes is the scientific foundation for the design of new types of inactivators of the penicillin-interactive proteins and domains.

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