Elucidation of the Role of Arginine-244 in the Turnover Processes of Class A β-Lactamases†

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ABSTRACT: The highly conserved arginine-244 of β-lactamases has been postulated to play a role in their initial recognition of substrates, presumably through ion pairing interactions [Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P., & Frère, J. M. (1990) *Proteins: Struct., Funct., Genet. 7*, 156–171]. However, in the Michaelis enzyme–substrate complex, no direct function has been attributed to this residue. Two mutants with substitutions of this residue in the TEM-1 β-lactamase (lysine-244 and serine-244) have been prepared to explore whether the guanidinium group of arginine-244 plays a critical role in the turnover processes. The mutant enzymes are effective catalysts for the hydrolysis of both penicillins and cephalosporins, and the lysine mutant enzyme behaves virtually identically to the wild-type β-lactamase. Comparative kinetic characterization of the serine mutant and wild-type enzymes attributed apparent binding energies of 1.3–2.3 kcal/mol for the penicillins and 0.3–1.0 kcal/mol for the cephalosporins to the transition-state species by arginine-244. Furthermore, it was shown that arginine-244 also contributes equally well to ground-state binding stabilization. These results were interpreted to indicate the involvement of a long hydrogen bond between arginine-244 and the substrate carboxylate, both in the ground and transition states. A reassessed picture for substrate anchoring involving interactions of the substrate carboxylate with the side chains of Ser-130, Ser-235, and Arg-244 is proposed to accommodate these observations.

The most common and important mechanism of acquired high-level bacterial resistance to β-lactam antibiotics, such as penicillins and cephalosporins, is the production of β-lactamases that inactivate β-lactams by promoting their hydrolytic fragmentation (Bush & Sykes, 1984). The catalytic mechanism of β-lactamases has been the subject of investigation for the past two decades. Nevertheless, the role of specific amino acid residues in the enzyme-catalyzed process is not fully understood. The recent availability of high-resolution crystal structures for the class A *Staphylococcus aureus* PC1 (Herzberg & Moult, 1987) and *Bacillus licheniformis* 749/C β-lactamases (Moews et al., 1990; Knox & Moews, 1991) should stimulate studies of the details of the catalytic processes by these enzymes.

Arginine-244 is a highly conserved residue in class A β-lactamases (Ambler et al., 1991), but it has attracted little attention as an important amino acid for the mechanism of action. No structural role can be attributed to the Arg-244 side chain, which is positioned in the B4 strand near the substrate-binding pocket, so a mechanistic function is likely to account for the evolutionary advantage for the conservation of this residue. Moews et al. (1990) speculated that the C_3 carboxylate of a penicillin substrate may be drawn initially to the Arg-244 side-chain guanidinium, since this residue is exposed near the active site opening. Subsequently, the substrate carboxylate is believed to undergo repositioning to give the Michaelis complex involving the substrate carboxylate and the Lys-234 side chain, which is further stabilized by additional hydrogen bonding with the side chain of the serine or threonine that is found at position 235 in class A β-lactamases. In addition to the role envisioned for Arg-244 by Moews et al., we wondered if this residue could play a more central function in the recognition of cephalosporins. Whereas the aforementioned interactions appeared plausible for the binding of penicillins, according to the crystal structure of the enzyme, the possibility of such interaction with a cephalosporin C_4 carboxylate is less obvious. A major difference between the two kinds of substrates is that the penicillin carboxylate is attached to a stereogenic sp^3 carbon in the thiazolidine ring, whereas the cephalosporin carboxylate is attached to an sp^2 carbon and is hence planar. The stereogenicity at the C_3 of a penicillin and the lack thereof for the C_4 of cephalosporins mandate consideration of different interactions for effective active site binding by the two classes of substrate.

Studies of kinetics of mutant enzymes with single amino acid substitutions provide an exceptionally sensitive tool for assessing the contribution of specific interactions in the catalytic process (Fersht et al., 1984; Knowles, 1987; Gerlt, 1987). We have used site-directed mutagenesis to explore the function of Arg-244 in the turnover processes of the TEM-1 β-lactamase. By this methodology, we have introduced lysine and serine residues individually at position 244 of the TEM-1 β-lactamase. Substitution by lysine should result in a conservative change that retains (at pH < 10) the charge and the hydrogen bonding ability of arginine found in the wild-type enzyme. However, substitution with serine would remove the charge and is expected to eliminate the possibility of hydrogen bonding. In fact, computer modeling of the Ser-244 mutant enzyme indicated the possibility of two new hydrogen bonds between the Ser-244 side chain and the Gly-242 main-chain carbonyl and the Ser-265 β-hydroxyl, placing the Ser-244

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hydroxyl at 9.5 Å from the substrate carboxylate. It is noteworthy that the choice of serine, rather than alanine, was based on our knowledge that this site is exposed to solvent and that the presence of a hydrophilic residue (e.g., serine) would be less disruptive of the local protein structure than the relatively hydrophobic alanine.

Materials and Methods

Ampicillin, benzylpenicillin, carbenicillin, and cephaloridine were purchased from Sigma. PADAC[1] is a Calbiochem product. Nitrocefin was obtained from Becton Dickinson Microbiological Systems. Oligonucleotides were synthesized and purified by the Molecular Biology Core Facility, Department of Biochemistry, Wayne State University. T4 DNA polymerase and T4 DNA ligase were obtained from GIBCO.

Mutagenesis of the TEM-1 β-Lactamase. Amino acid substitution at position 244 of the TEM-1 β-lactamase was performed by oligodeoxynucleotide-directed mutagenesis according to the procedure of Kunkel (1985), using the commercially available MutaGene Kit (Bio-Rad). The prototype plasmid (phagemid pTZ18U) used in the MutaGene Kit carries the complete transcriptional unit of TEM-1 β-lactamase gene as a selection marker. Therefore, we used pTZ18U for oligodeoxynuclotide-directed mutagenesis of the TEM-1 β-lactamase gene without subcloning. The phosphorylated oligodeoxynucleotide primers were annealed to single-stranded U-phage DNA containing the TEM-1 β-lactamase gene. The mutagenic primers were extended by T4 DNA polymerase, and the newly synthesized strands were sealed by T4 DNA ligase to obtain biologically active circular double-stranded DNA molecules. The double-stranded circular DNA carrying the required mutation was introduced into *Escherichia coli* MV1190, and ampicillin-resistant phenotypes were selected. Single-strand phage DNA was prepared from six randomly selected putative mutant colonies from each mutation group, and 35% of the nucleotide sequence of the structural gene surrounding position 244 was determined by the dideoxy chain termination method of Sanger et al. (1977) to confirm the presence of the required mutation.

β-Lactamase Purification. The protocol for purification of β-lactamases was a variation of a reported method (Fisher et al., 1980). The periplasmic β-lactamase was liberated by osmotic shock, as described before (Schwinghamer, 1980). The osmotic shock fluids (200 mL) were made 10 mM in tris-HCl, and the pH was adjusted to 7.0. The solution was applied to a DEAE-cellulose column (2.5 × 10 cm), and the enzyme was batch eluted with 150 mL of 100 mM tris-HCl buffer, 3 mM β-mercaptoethanol, pH 7.0. The eluate was concentrated by ultrafiltration (Amicon) with a concomitant change of buffer to 10 mM tris-HCl, 3 mM β-mercaptoethanol, pH 7.0. The solution was applied to a second DEAE-cellulose column (2.5 × 14 cm), and the activity was eluted using a linear gradient of 10–150 mM tris-HCl, 3 mM β-mercaptoethanol, pH 7.0. The fractions with activity were pooled and concentrated in an Amicon ultrafiltration device. The protein solution was subsequently purified on a Sephadex G-100 column (2 × 100 cm), as described previously (Fisher et al., 1980). Active fractions were combined, and the solution was concentrated by ultrafiltration to approximately 10 μM protein concentration. Protein concentration was determined from the absorbance at 281 nm (ϵ = 29 400 M^{-1} cm^{-1}). The purified protein solutions were stored at 4 °C without change in activity for at least 8–10 months. The two mutant enzymes and the wild-type TEM-1 β-lactamase migrated identically on reducing SDS–polyacrylamide electrophoretic gels.

Enzyme Kinetics. Kinetic measurements were carried out on Perkin-Elmer Lambda 3B or Hewlett-Packard 452 diode array instruments. The spectrophotometric assay of β-lactamase activity was carried out in 50 mM sodium phosphate, pH 7.0 (unless otherwise noted), in the presence of a high ionic strength (500 mM NaCl). Initial rates were determined from the first 5–10% of the reactions with six to seven substrate concentrations bracketing the K_m. The values for the kinetic parameters (K_m and k_{cat}) were determined from the Lineweaver–Burk plots. The assay of enzymic activity for each substrate concentration was carried out in quadruplicate. The change in the extinction coefficients and the wavelengths for each substrate at pH 7.0 were as follows: ampicillin (235 nm) $\Delta\epsilon$ = 820 M^{-1} cm^{-1}; benzylpenicillin (240 nm) $\Delta\epsilon$ = 529 M^{-1} cm^{-1}; carbenicillin (240 nm) $\Delta\epsilon$ = 400 M^{-1} cm^{-1}; nitrocefin (482 nm) $\Delta\epsilon$ = 17 400 M^{-1} cm^{-1}; PADAC (466 nm) $\Delta\epsilon$ = 9590 M^{-1}; cephaloridine (267 nm) $\Delta\epsilon$ = 1000 M^{-1} cm^{-1}. The values of $\Delta\epsilon$ at 240 nm for benzylpenicillin were 572, 557, 541, 529, 524, and 517 M^{-1} cm^{-1} at pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0, respectively.

Circular Dichroic Measurements. Circular dichroic spectra were recorded on a Jasco Model J-600 spectropolarimeter. The protein concentration was 6.7 μM for the wild-type β-lactamase and the Arg-244–Lys and the Arg-244–Ser mutant enzymes. The buffer was 10 mM sodium phosphate, pH 7.0, and a path length of 1 mm was used in the measurements.

Measurement of the Rates of Deacylation of Cefoxitin from Wild-Type and Mutant Enzymes. The method of Fisher et al. (1980) was followed with the wild-type enzyme. A 210-μL solution of the enzyme (0.69 μM) and cefoxitin (16.2 mM) in 100 mM sodium phosphate, pH 7.0, was incubated at room temperature for 20 min. A 3-μL portion of the mixture was added to a cuvette containing benzylpenicillin (1.0 mL of 2.0 mM), and its hydrolysis was followed at 240 nm for 50 min. A similar experiment was carried out with the serine mutant enzyme, except the concentration of cefoxitin was raised to 32.4 mM. The first-order rate constant for regeneration of enzymic activity (due to deacylation of enzyme–cefoxitin intermediate) was determined from the progress curve for hydrolysis of benzylpenicillin according to the method of Glick et al. (1978).

Determination of Dissociation Constants. The individual β-lactams were treated as inhibitors of PADAC hydrolysis by the TEM-1 β-lactamases, as monitored at either 610 nm ($\Delta\epsilon$ = 1240 M^{-1} cm^{-1}) or 620 nm ($\Delta\epsilon$ = 508 M^{-1} cm^{-1}). The concentration of PADAC was maintained at $5K_m$, whereas the "inhibitor" concentration was kept below its corresponding K_m. Analyses of the rate constants were carried out according to the method of Dixon (1953).

Results and Discussion

The wild-type and mutant enzymes were each purified to homogeneity. The far-UV circular dichroic (CD) spectra of enzymes at pH 7.0 were superimposable (Figure 1), suggesting that the lysine and serine substitutions at position 244 did not induce a change in the secondary structural elements of the enzyme. Furthermore, no variations in the spectra were noted within the pH range of 4–9 (data not shown), indicating that the changes in the kinetic parameters are not due to perturbation of structure of the mutant proteins at the extremes of pH.

[1] Abbreviations: tris, tris(hydroxymethyl)aminomethane; DEAE-cellulose, (diethylamino)ethyl-cellulose; SDS, sodium dodecyl sulfate; PADAC; [2-[[*p*-(dimethylamino)phenyl]azo]pyridino]cephalosporin. The three-letter notation for amino acids conforms with suggestions cited by the IUPAC–IUB Commission on Biochemical Nomenclature [(1968) *J. Biol. Chem. 243*, 3557–3559].

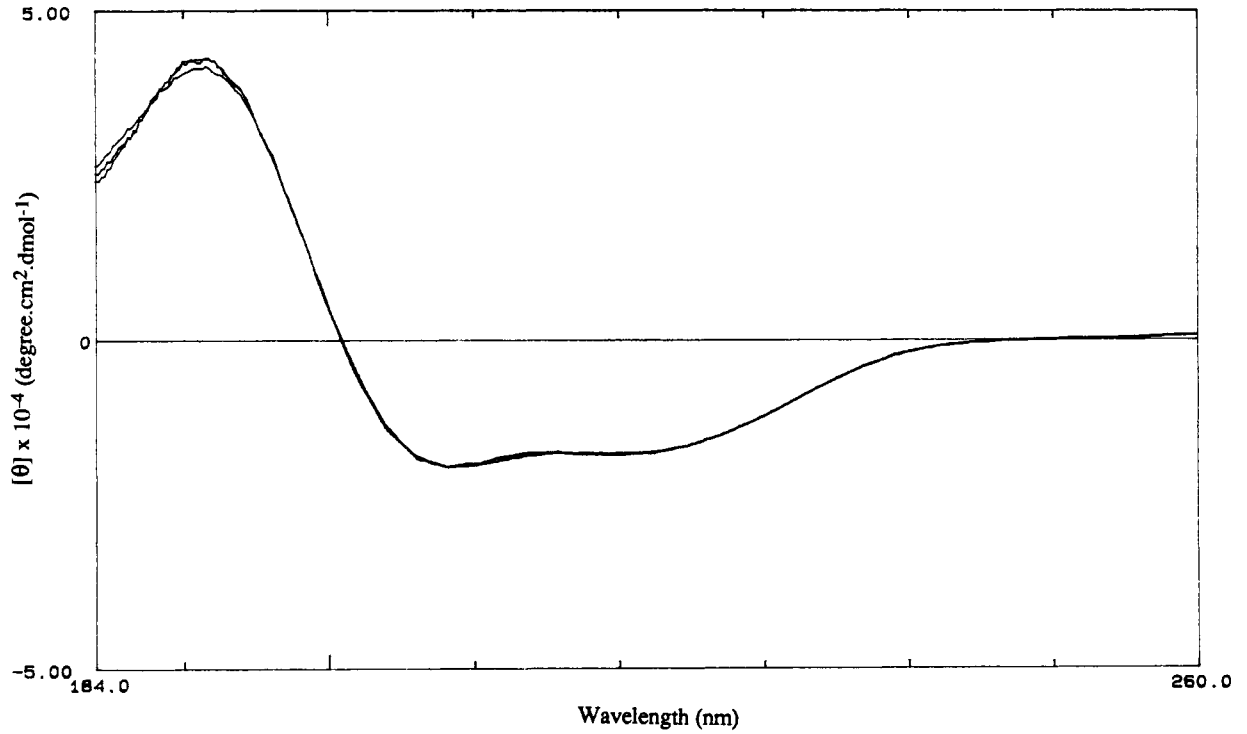


FIGURE 1: Far-ultraviolet circular dichroic spectrum of the wild-type TEM-1 β-lactamase at pH 7.0. The Arg-244–Lys and Arg-244–Ser mutant enzymes at pH 7.0 were superimposed.

Table I: Kinetic Parameters for the Hydrolytic Action of Wild-Type and Mutant TEM-1 β-Lactamases (50 mM Sodium Phosphate, 0.5 M NaCl, pH 7.0)

	wild type				Arg-244–Lys			Arg-244–Ser			
substrates	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	K_S (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	K_S (mM)
ampicillin	1532	0.02	7.7×10^7	0.06[a]	1891	0.03	6.3×10^7	1370	0.86	1.6×10^6	5.3[a]
benzylpenicillin	1998	0.02	1.0×10^8	ND[b]	2214	0.03	7.4×10^7	1895	0.16	1.2×10^7	ND
carbenicillin	657	0.05	1.3×10^7	0.08[a]	701	0.06	1.2×10^7	588	0.77	7.6×10^5	2.1[a]
nitrocefin	920	0.22	4.2×10^6	ND	890	0.29	3.1×10^6	650	0.38	1.7×10^6	ND
PADAC	651	0.31	2.1×10^6	ND	689	0.26	2.6×10^6	434	1.15	3.8×10^5	ND
cephaloridine	1506	0.93	1.6×10^6	4.2	1615	1.00	1.6×10^6	1461	1.44	1.0×10^6	8.9

[a] These values should be considered estimates. [b] Not determined. Standard deviation is ±10%.

The mutant enzymes are effective catalysts in the hydrolysis of both penicillins and cephalosporins. Table I summarizes the kinetic parameters for the turnover of three penicillins (ampicillin, benzylpenicillin, and carbenicillin) and three cephalosporins (nitrocefin, PADAC, and cephaloridine) by the wild-type and mutant enzymes. The K_m values of the penicillin substrates are much lower than those of cephalosporins, and the corresponding k_{cat}/K_m ratios for the penicillins are much higher than for cephalosporins. The Lys-244 mutant enzyme has essentially the same kinetic properties as the wild-type enzyme, as was expected. The catalytic constants (k_{cat}) for the serine mutant enzyme are lowered somewhat (by approximately 5–35%) compared to the corresponding wild-type numbers, and the K_m values are higher for the serine mutant enzyme in each case. It is noteworthy that the effect on K_m is more pronounced for the penicillins (8–43-fold) than for the cephalosporins 1.5–3.7-fold), so that the K_m values for both classes of substrates are more similar to each other than to the wild-type enzyme. These observations indicate that penicillins rely more on interaction with Arg-244. The dissociation constants (K_s) for ampicillin, carbenicillin, and cephaloridine were determined for the serine mutant and wild-type enzymes. The increase in the values for the dissociation constants for the serine mutant enzyme indicate an effect on its ground-state substrate binding.

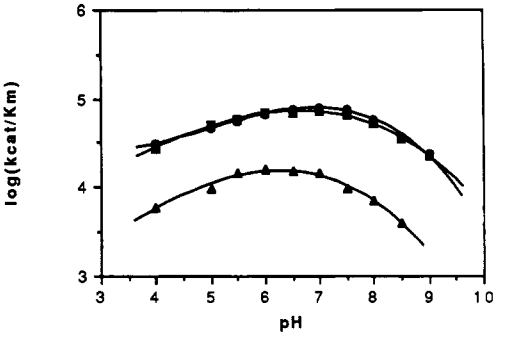


FIGURE 2: The pH dependence of log (k_{cat}/K_m) for the hydrolysis of benzylpenicillin catalyzed by the wild-type TEM-1 β-lactamase (●) and the Arg-244–Lys (■) and Arg-244–Ser (▲) mutant enzymes. Conditions are described in the text.

The pH dependence of k_{cat}/K_m for the turnover of benzylpenicillin by each enzyme is shown in Figure 2. The results with the lysine mutant enzyme appear virtually identical to those with the wild-type TEM-1. However, the pH optimum for the serine mutant enzyme has shifted to 6.2 from 6.8 for the wild-type enzyme, and the catalytic efficiency is reduced by 5-fold at the pH optimum. As stated earlier, a significant difference between the serine mutant and wild-type enzymes is reflected in the values for K_m. Whereas the K_m for ben-

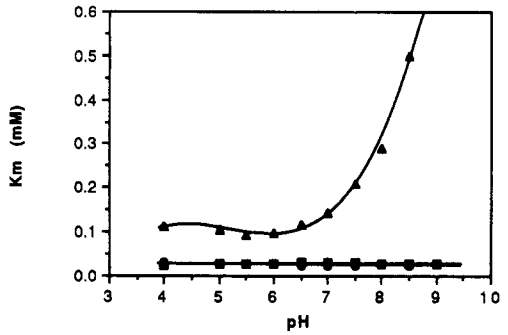


FIGURE 3: The pH dependence of K_m for the hydrolysis of benzylpenicillin catalyzed by the wild-type TEM-1 β-lactamase (●) and the Arg-244–Lys (■) and Arg-244–Ser (▲) mutant enzymes. Conditions are described in the text.

zylpenicillin did not change for the wild-type enzyme and the lysine mutant as a function of pH, the K_m increased for the serine-244 enzyme at pH > 7.0 (Figure 3).

Although the quantitative differences between the wild-type and Ser-244 enzymes presented in Table I, Figure 2, and Figure 3 reflect perturbation of the active site by the Ser-244 substitution, they do not necessarily imply that the enzyme mechanism has been altered. In fact, we have the following evidence for similarity in mechanism between the wild-type and Ser-244 enzymes. The hallmark of catalysis by class A β-lactamases is the transient and reversible acylation of Ser-70 by substrates. Both the wild-type and the Ser-244 mutant enzymes are acylated in the active site by the poor substrate cefoxitin, followed by gradual deacylation and regeneration of activity. An initial exponential phase for deacylation led into a steady-state rate for hydrolysis of benzylpenicillin which was identical to the rate of benzylpenicillin hydrolysis by the enzymes in the absence of cefoxitin. The progress curves for regeneration of activity were used to determine the deacylation rates of cefoxitin at 1.44×10^{-3} s^{-1} for the wild-type enzyme [4.8×10^{-3} s^{-1} was reported for the very similar TEM-2 β-lactamase by Fisher et al. (1980)] and 1.35×10^{-3} s^{-1} for the serine mutant enzyme. Therefore, it would appear that modification at residue 244 does not alter the overall catalytic behavior of the TEM-1 β-lactamase; the active site acylation and deacylation with cefoxitin take place analogously with both the wild-type and the serine mutant enzymes. In our attempt to demonstrate the existence of a double-displacement mechanism (i.e., acylation and deacylation) for the serine mutant enzyme, we did not add nucleophiles to the medium, as surrogates for water, to speed up the deacylation process; such experiments have not been successful with class A β-lactamases (Fisher et al., 1980; Virden et al., 1990).

A change in the apparent binding energy to the transition state of 1.3–2.3 kcal/mol and 0.3–1.0 kcal/mol for the penicillins and the cephalosporins, respectively, was evaluated from the determinations of k_{cat}/K_m for the wild-type and the serine mutant enzymes in Table I. The results calculated for representative substrates, ampicillin, carbenicillin, and cephaloridine are presented in Table II. For each substrate, the change in transition-state binding energy was very similar to that estimated for the ground-state binding (Table II). From this similarity of results, it would appear that arginine-244 contributes almost equally to both substrate and transition-state binding.

It has been difficult to set an upper limit for the length of a hydrogen bond (Baker & Hubbard, 1984). From estimates of van der Waals radii, an upper limit of 3.4 Å (distance between the heteroatoms) has been suggested (Olovsson & Jönsson, 1976). A span of 4.0 Å is measured for the closest

Table II: Apparent Binding Energies of the Arginine-244 Side Chain to Transition State and Substrate (50 mM Sodium Phosphate, 0.5 M NaCl, pH 7.0)

	binding energy with transition state[a] (kcal/mol)	binding energy with substrate[b] (kcal/mol)
ampicillin	−2.3	−2.6[c]
carbenicillin	−1.7	−1.9[c]
cephaloridine	−0.3	−0.4

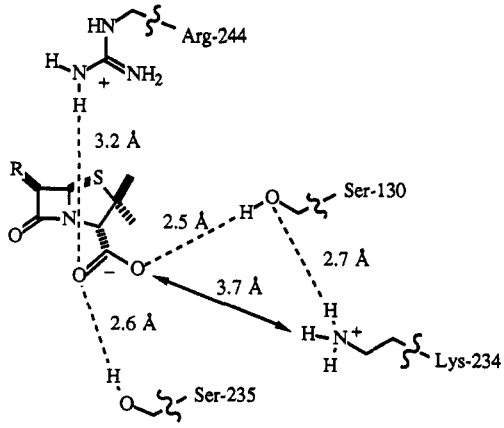
[a] Calculated from $\Delta\Delta G = RT \ln [(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$, where mut refers to the serine-244 β-lactamase and wt denotes the wild-type enzyme. [b] Calculated from $\Delta\Delta G = -RT \ln [(K_s)_{mut}/(K_s)_{wt}]$. [c] These values should be considered estimates. Standard deviation is ±10%.

distance between one of the N_η of the Arg-244 guanidinium and one of the carboxylate oxygens of benzylpenicillin, as it is modeled into the active site of the *B. licheniformis* β-lactamase (Moews et al., 1990). This distance is somewhat longer than expected and may reflect the fact that the crystals were grown at pH 5.5, whereas our experiments were carried out at pH 7.0. Typical energies for hydrogen bonds in proteins are approximately in the range of 3–5 kcal/mol. Thus, our kinetic measurements are consistent with the existence of a long hydrogen bond between residue 244 and the substrate (Fersht et al., 1985), which appears to be stronger with penicillins than with cephalosporins. This finding was not anticipated from the crystal structure, and it requires that active site binding of the substrate be reassessed.

It has been proposed by Herzberg and Moult (1987) and Moews et al. (1990) that concomitant hydrogen bonding by the side chains of Ser/Thr-235 and Lys-234 with the substrate carboxylate are two necessary interactions for the anchoring of the substrate in the active site. Mutagenesis at residue 234 by Ellerby et al. showed a large effect on the catalytic ability of the *B. licheniformis* enzyme, which they attributed to the disruption of one hydrogen bonding interaction between Lys-234 and the substrate carboxylate (Ellerby et al., 1990). However, recent refinements of the *B. licheniformis* enzyme and analysis of hydration patterns indicate that the side chain of Lys-234 is calculated to have zero surface accessibility (Knox & Moews, 1991). The side-chain amino group of Lys-234 is found tethered to a structurally conserved water molecule, W634,[2] which is believed to be entrapped in the protein in the course of the folding process. Knox and Moews (1991) assert that W634 would not be displaced by the substrate carboxylate. These authors claim, however, that W692 is the water molecule displaced by the substrate carboxylate on active site binding. We find it significant that this water molecule is bonded in the active site to the side-chain functions of Ser/Thr-235 and Arg-244 in the absence of substrate. Our finding that substrates interact in both the ground and transition states with Arg-244 is consistent with this scenario for displacement of W692 by the substrate carboxylate to allow for such interactions. It should be stated, however, that the highly conserved Lys-234 does appear to play a significant structural role in the protein. Its side chain forms a strong hydrogen bond (2.7 Å) to the Ser-130 β-hydroxyl, thereby bridging the two domains of the enzyme at the junction of which the active site cavity is sequestered. Therefore, substitutions with amino acids possessing shorter side chains than lysine would disrupt this hydrogen bond, which should affect the positioning of the B3 strand relative to the H2 helix. These observations are borne out by the mutagenesis at position 130,

[2] Numbering of the structurally conserved water molecules is according to Knox and Moews (1991).

Scheme I



which showed large effects on the catalytic ability of the enzyme, which in turn were attributed to the involvement of Ser-130 in maintaining the structure of the active site (Jacob et al., 1990). A recent report by L'Enfant et al. (1991) shows that the Lys-234–Arg mutant TEM β-lactamase retains—not unexpectedly—much of the wild-type activity. Similarly, a change of lysine for histidine at this position in the *Streptomyces albus* G enzyme showed considerable activity (Brannigan et al., 1991). Whereas we have not had access to the crystal structure of the *S. albus* G β-lactamase, we believe that a long hydrogen bond between Ser-130 and His-234 is still conceivable for this mutant enzyme. In both studies by L'Enfant et al. and Brannigan et al., effects of mutations were manifested in the values of k_{cat}/K_m for substrates, which led the authors to implicate a catalytic, rather than structural, role for Lys-234. It is our view that this is an overinterpretation of the kinetic data. The active sites of enzymes evolve to bind the transition states for the reactions that they catalyze. A violent mutation that disrupts the active site topology would by necessity manifest itself in reduced k_{cat}/K_m values for substrates.

In the light of these observations and our findings which suggest the existence of a long hydrogen bond between the Arg-244 side chain and the substrate in both the ground state and the transition state of the enzyme-substrate complex, a revised mechanism for active site binding may be envisioned. We propose that substrate binding in the active site involves hydrogen bonding interactions of the substrate carboxylate oxygens with the side chains of Arg-244, Ser-235, and Ser-130. We carried out graphics analysis on the crystal structure reported by Moews et al. (1990). To realize substrate interactions with Arg-244 side chain, torsional rotation of the substrate carboxylate was carried out. Subsequently, we maintained the separation between the β-lactam carbonyl and the Ser-70 hydroxyl oxygen (i.e., optimal orientation and proximity for the acylation of the active site Ser-70) and rotated the substrate by a few degrees to optimize distance to Arg-244, Ser/Thr-235, and Ser-130. The final picture along with the appropriate distances (between the heteroatoms) is depicted in Scheme I. The measured separation of 3.2 Å is in accord with our kinetic results indicative of a long hydrogen bond between the substrate and Arg-244. In this model, the separation between the substrate (penicillin G) and Lys-234 is 3.7 Å. This excessively long distance makes hydrogen bonding impossible and further supports the assertion by Knox and Moews (1991) that this residue is inaccessible. Arginine-244, an amino acid exposed at the fringes of the active site, seems to be involved in initial substrate recognition, as postulated by Moews et al. (1990); however, the binding energy of Arg-244 is further used to lower the activation energy for the hydrolytic reaction.

The presence of arginine at position 244 is noted in the vast majority of class A β-lactamases (Ambler et al., 1991). The exceptions are β-lactamases from *Klebsiella oxytoca* (Thr-244), *Streptomyces aureofaciens* (Ala-244), *Streptomyces albus*, *Streptomyces lavendulae*, and *Streptomyces fradiae* (the latter three show asparagine at position 244). The side-chain functions of both Thr-244 and Asn-244 can, in principle, donate a hydrogen bond to the substrate carboxylate in the β-lactamases specified above, thus assuming the role that is served by Arg-244 in the TEM-1 β-lactamase. The fact that in these β-lactamases amino acids possessing shorter side chains can serve the same function as arginine serves in the majority of class A β-lactamases reflects the differences in the respective active site topologies. However, *S. aureofaciens* β-lactamase must rely on alternative interactions with substrates, since alanine cannot serve the same function via its side chain. We find it revealing that a recent graphics analysis of the crystal structure of the *S. albus* G β-lactamase by Jacob-Dubuisson et al. (1991) suggests that the Arg-220 side chain of this enzyme occupies the same space as that of Arg-244 in the majority of class A enzymes. An arginine is found at position 220 of the *S. lavendulae* and *S. fradiae* enzymes as well. Hence, an alternative for these three enzymes is that Arg-220 can serve the same function that we attribute to Arg-244 for the majority of class A enzymes.

Acknowledgments

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Conformational Similarities between One-Chain and Two-Chain Tissue Plasminogen Activator (t-PA): Implications to the Activation Mechanism on One-Chain t-PA†

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ABSTRACT: Tissue plasminogen activator (t-PA) is an exceptional serine protease, because unlike most other serine protease zymogens single-chain tissue plasminogen activator (sct-PA) possesses a substantial amount of proteolytic activity. The unusual reaction of sct-PA afforded the opportunity to directly compare the active site environment of sct-PA and two-chain tissue plasminogen activator (tct-PA) in solution through the application of a series of nitroxide spin labels and fluorophores. These labels, which have been previously shown to covalently label the catalytic serine of other serine proteases, inactivated both sct-PA and tct-PA. The labels can be divided into two classes: those which form tetrahedral complexes (sulfonates) and those which form trigonal complexes (anthranilates). Those which formed tetrahedral complexes were found to be insensitive to structural differences between sct-PA and tct-PA at the active site. In contrast, those which formed trigonal complexes could differentiate and monitor the sct-PA to tct-PA conversion by fluorescence spectroscopy. Models of the structure of sct-PA and tct-PA were constructed on the basis of the known X-ray structures of other serine protease zymogen and active enzyme forms. One of the nitroxide spin labels was modeled into the sct-PA and tct-PA structures in two possible orientations, both of which could be sensitive to structural differences between sct-PA and tct-PA. These models formed the structural rationale used to explain the results obtained with the "tetrahedral" and "trigonal" probes, as well as to offer a possible explanation for the unique reactivity of sct-PA.

The enzymes of the mammalian serine protease family, i.e., trypsin, chymotrypsin, and elastase, are highly homologous in structure and catalytic mechanism. In addition, most of these enzymes are biosynthesized as a single-chain proenzyme or zymogen which is almost entirely devoid of enzymatic activity. The inactive zymogen form of all of these proteins requires activation via limited proteolysis in order to yield significant amounts of enzyme activity. The activation of trypsinogen and chymotrypsinogen results in an amplification of activity which has been estimated to be around 10^7–10^8 (Kerr et al., 1975).

Tissue plasminogen activator (t-PA)[1] is an atypical serine protease because the zymogen form, sct-PA, has significant enzymatic activity. This has been most clearly shown by the analysis of variants of t-PA in which the arginyl residue at

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[1] Abbreviations: t-PA, tissue plasminogen activator; tct-PA, two-chain tissue plasminogen activator; sct-PA, single-chain tissue plasminogen activator; S-2288, H-D-Ile-Pro-Arg-nitroanilide; BPTI, bovine pancreatic trypsin inhibitor; PSTI, pancreatic secretory trypsin inhibitor; DFP, diisopropyl fluorophosphate; ESR, electron spin resonance; *p*-V (*p*-CO-5NH), 4-(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*p*-(fluorosulfonyl)-benzamide; *m*-IV (*m*-CO-6NH), 4-(2,2,6,6-tetramethylpiperidine-1-oxyl)-*m*-(fluorosulfonyl)benzamide; *m*-V (*m*-CO-5NH), 3-(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*m*-(fluorosulfonyl)benzamide; *m*-VII (*m*-NCO-6NH), *N*-[*m*-(fluorosulfonyl)phenyl]-4-*N*-(2,2,6,6-tetramethylpiperidine-1-oxyl)urea; R275E-sct-PA, single-chain t-PA in which Arg 275 has been replaced by glutamate; S-2251, H-D-Val-Leu-Lys-*p*-nitroanilide; MMTS, methyl methanethiolsulfonate.

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