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Structure of the wild-type TEM-1 β-lactamase at 1.55 Å and the mutant enzyme Ser70Ala at 2.1 Å suggest the mode of noncovalent catalysis for the mutant enzyme

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One of the best-studied examples of a class A β-lactamase is *Escherichia coli* TEM-1 β-lactamase. In this class of enzymes, the active-site serine residue takes on the role of a nucleophile and carries out β-lactam hydrolysis. Here, the structures of the wild-type and the S70G enzyme determined to 1.55 and 2.1 Å, respectively, are presented. In contrast to the previously reported 1.8 Å structure, the active site of the wild-type enzyme (1.55 Å) structure does not contain sulfate and Ser70 appears to be in the deprotonated form. The X-ray crystal structure of the S70G mutant has an altered Ser130 side-chain conformation that influences the positions of water molecules in the active site. This change allows an additional water molecule to be positioned similarly to the serine hydroxyl in the wild-type enzyme. The structure of the mutant enzyme suggests that this water molecule can assume the role of an active-site nucleophile and carry out noncovalent catalysis. The drop in activity in the mutant enzyme is comparable to the drop observed in an analogous mutation of the nucleophilic serine in alkaline phosphatase, suggesting common chemical principles in the utilization of nucleophilic serine in the active site of different enzymes.

1. Introduction

Active-site serine β-lactamases have been divided into three classes (A, C and D) according to primary sequence (Ambler, 1980). These enzymes hydrolyze the amide bond in the four-membered ring of β-lactam antibiotics, conferring resistance to bacterial hosts. TEM-1 β-lactamase from *Escherichia coli* belongs to class A. Class A enzymes have a broad substrate-specificity and have evolved primarily for rapid hydrolysis (deacylation) of β-lactams based on the precise positioning of a nucleophilic water molecule near the covalent acyl-enzyme intermediate (Knox *et al.*, 1996; Matagne & Frere, 1995). The X-ray crystal structure (Fig. 1) of this enzyme has been solved by Strynadka *et al.* (1992) and then by Jelsch *et al.* (1993). The X-ray crystal structures have also been determined for other class A enzymes including *Staphylococcus aureus* PC1 (Herzberg, 1991), *Streptomyces albus* G (Dideberg *et al.*, 1987) and *Bacillus licheniformis* 749/C (Knox & Moews, 1991). A structural comparison between TEM-1 and PC1 has revealed differences in the relative orientations of the two domains and in active-site regions that can influence catalytic efficiencies and substrate specificities (Jelsch *et al.*, 1993). Perturbations of this kind may also explain the diverse catalytic properties of the class A enzymes.

In the double in-line displacement mechanism of TEM-1 β-lactamase, a covalent acyl-enzyme intermediate is formed with the serine nucleophile (Cohen & Pratt, 1980; Joris, 1984;

Knott-Hunziker *et al.*, 1982). In the acylation step, Ser70 is deprotonated for nucleophilic attack on the carbonyl C atom of the β-lactam ring either through its strong hydrogen bond to Lys73 or a water-mediated interaction to Glu166 (Matagne & Frere, 1995; Matagne *et al.*, 1998). The studies of Damblon *et al.* (1996) in combination with high-resolution structural data (Minasov *et al.*, 2002) suggest Glu166 as the primary general base in the reaction. The close proximity of the Lys73 amine group suggests that it must also participate in the deprotonation step of Ser70. This proton is back-donated to the leaving N atom of the β-lactam ring through a hydrogen-bond network in the active site with the participation of Ser130. Deacylation of this acyl-seryl intermediate is accomplished by an activated water hydrogen bonded to Glu166 (Adachi *et al.*, 1991). The backbone amide groups of Ser70 and Ala237 form the oxyanion hole which polarizes the carbonyl groups for nucleophilic attack in both acylation and deacylation and stabilizes the tetrahedral transition states in both steps (Fig. 2).

The requirement of the serine nucleophile, Ser70, for catalysis in the TEM-1 β-lactamase, as well as other enzymes belonging to class A, has been investigated using site-specific mutagenesis. In *Staph. aureus* PC1 β-lactamase, Ser70 has been replaced with an alanine (Chen *et al.*, 1996). The X-ray crystal structure has been determined to 2.1 Å resolution and kinetic properties indicated a significant but not complete loss of activity. The results seem to be similar but different from the S70G mutants. Additionally, in *Strep. albus* β-lactamase Ser70 has been systematically mutated to alanine and cysteine and the kinetic properties of the S70A and S70C enzymes showed similar trends (Jacob *et al.*, 1991). In TEM-1 β-lactamase, the same mutants were evaluated kinetically (S70C and S70G) and the results were not fully consistent (Mazzella *et al.*, 1991; Sigal *et al.*, 1984; Toth *et al.*, 1988). The S70G mutant in TEM-1 β-lactamase has been constructed using two different glycine codons, GGC and GGA (Toth *et al.*, 1988). The former codon differs from the active-site serine codon (AGC) at only the first position and results in 0.1% wild-type contamination in the S70G enzyme. A tolerated G/U mismatch between the codon for glycine and the anticodon for the $tRNA_{GCU}Ser$ results in the misincorporation of a wild-type serine with low frequency.

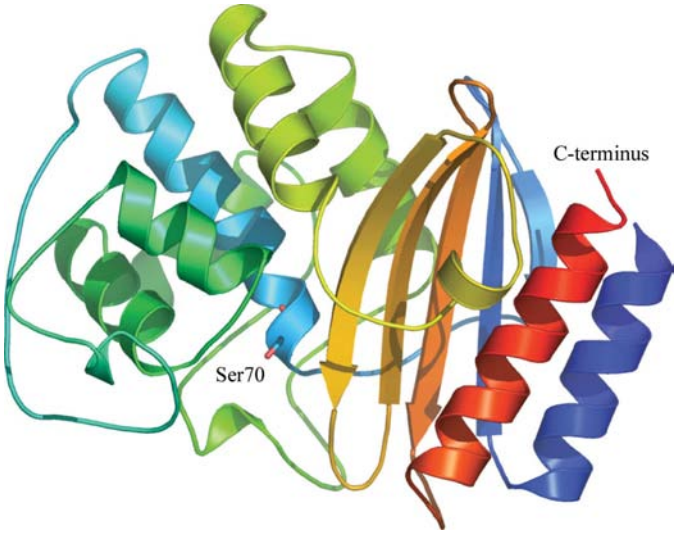

Figure 1
Ribbon diagram of TEM1 β-lactamase from *E. coli*. The active-site Ser70 is shown and labeled.

In this work, Ser70 in TEM-1 β-lactamase has been changed to a glycine to yield mutant enzyme S70G. Although the S70A, S70G and S70C enzymes have been studied from β-lactamases from several organisms, their kinetic properties have been quite different, which can be attributed to the different experimental conditions (Chen *et al.*, 1996; Jacob *et al.*, 1991; Mazzella *et al.*, 1991; Sigal *et al.*, 1982, 1984; Toth *et al.*, 1988; Knap & Pratt, 1991). This study was designed to provide independent evaluation of the active-site mutants and to shed additional light onto the mode of non-covalent catalysis. Each mutant enzyme was purified and kinetically characterized under identical conditions. In order to study the structural determinants of the residual activity, we have crystallized and refined the structures of the wild-type enzyme to 1.55 Å resolution as well as the S70G enzyme to 2.1 Å resolution. Both structures provided new insights into the details of covalent *versus* non-covalent catalysis as carried out by the wild-type enzyme, and S70G and S70A mutant enzymes, respectively.

2. Materials and methods

2.1. Materials

Agar, dibasic sodium phosphate, potassium chloride, sodium acetate, ampicillin, penicillin G and PEG 4000 were purchased from Sigma Chemical Co. Tris, electrophoresis-grade agarose and sucrose were purchased from ICN Biomedicals. Tryptone and yeast extract were obtained from Difco Laboratories. The T7 DNA sequencing kit from Amersham was used for DNA sequencing. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. Oligonucleotides required for site-specific mutagenesis and sequencing primers were purchased from Operon Technologies. DNA fragments isolated from agarose gels were purified with the gel-extraction kit from Qiagen. The MutaGene *in vitro* mutagenesis kit from Bio-Rad was used for site-specific mutagenesis. Q Sepharose Fast Flow, Source 15Q and Phenyl Sepharose chromatography resins were purchased from Amersham Pharmacia Biotech. The SE100 gel-filtration column was from BioRad.

2.2. Strains and plasmids

The *E. coli* strain MV1190 [Δ(*lac-pro*AB), *supE*, *thi*, Δ(*sri-recA*)306::Tn10(tetr)/F′ *traD*36, *proAB*, *lacI*q, *lacZ*ΔM15], pUC119 and the M13 phage M13K07 were obtained from J. Messing. The repair-deficient strain HB2154 [F′, *ara*, *thi*, Δ*pro-lac*, *mutL*::Tn10(tetr)/F′ *proAB*, *lacI*q, *lacZ*ΔM15] was used for site-directed mutagenesis (Carter *et al.*, 1985). The *E. coli* strain XL1-Blue [*supE44*, *hsdR*17, *recA1*, *endA1*, *gyrA46*, *thi*, *relA1*, *lac*/F′ *proAB*, *lacI*q, *lacZ*ΔM15, Tn10(tetr)] was purchased from Stratagene. Plasmid pUC4K containing the

kanamycin-resistance gene encoding for aminoglycoside 3′-phosphotransferase was purchased from Pharmacia Biotech. The base plasmid for site-specific mutagenesis, pEK405, was constructed by directly cloning the kanamycin-resistance gene from plasmid pUC4K into pUC119 using the single restriction endonuclease *Eco*RI. This plasmid construct uses the natural promoter of β-lactamase, *P3* (Goussard & Courvalin, 1999).

2.3. Methods

2.3.1. Construction of the S70G β-lactamases by site-specific mutagenesis. The oligonucleotide-directed mutagenesis procedure of Zoller & Smith (1982) was used to introduce the cysteine, alanine or glycine at position 70 of TEM-1 β-lactamase with previously described modifications (Carter *et al.*, 1985; Ladjimi *et al.*, 1988). Primers were 20 or 21 nucleotides in length, with a homologous sequence flanking the mutagenic site. The changes induced from the AGC (serine) codon were GGC and GGG (glycine). These mutagenic primers containing single- or double-nucleotide mismatches were phosphorylated with T4 polynucleotide kinase and annealed to the single-stranded DNA template of pEK405. Plasmid pEK405 contains the M13 intergenic region for production of single-stranded DNA when host cells are infected with helper phage M13KO7. After second-strand synthesis and ligation with T7 DNA polymerase and T4 DNA ligase, respectively, the mutagenesis reactions were transformed into the repair-deficient *E. coli* strain HB2154. For each mutagenic construct, approximately 200 colonies were screened for both kanamycin resistance and ampicillin sensitivity. Three or four candidates for each mutation were selected for sequencing. The entire $bla_{\mathrm{TEM\text{-}1}}$ gene was sequenced for each candidate to verify the incorporation of the desired mutation and to ensure no other mutations were introduced into the gene.

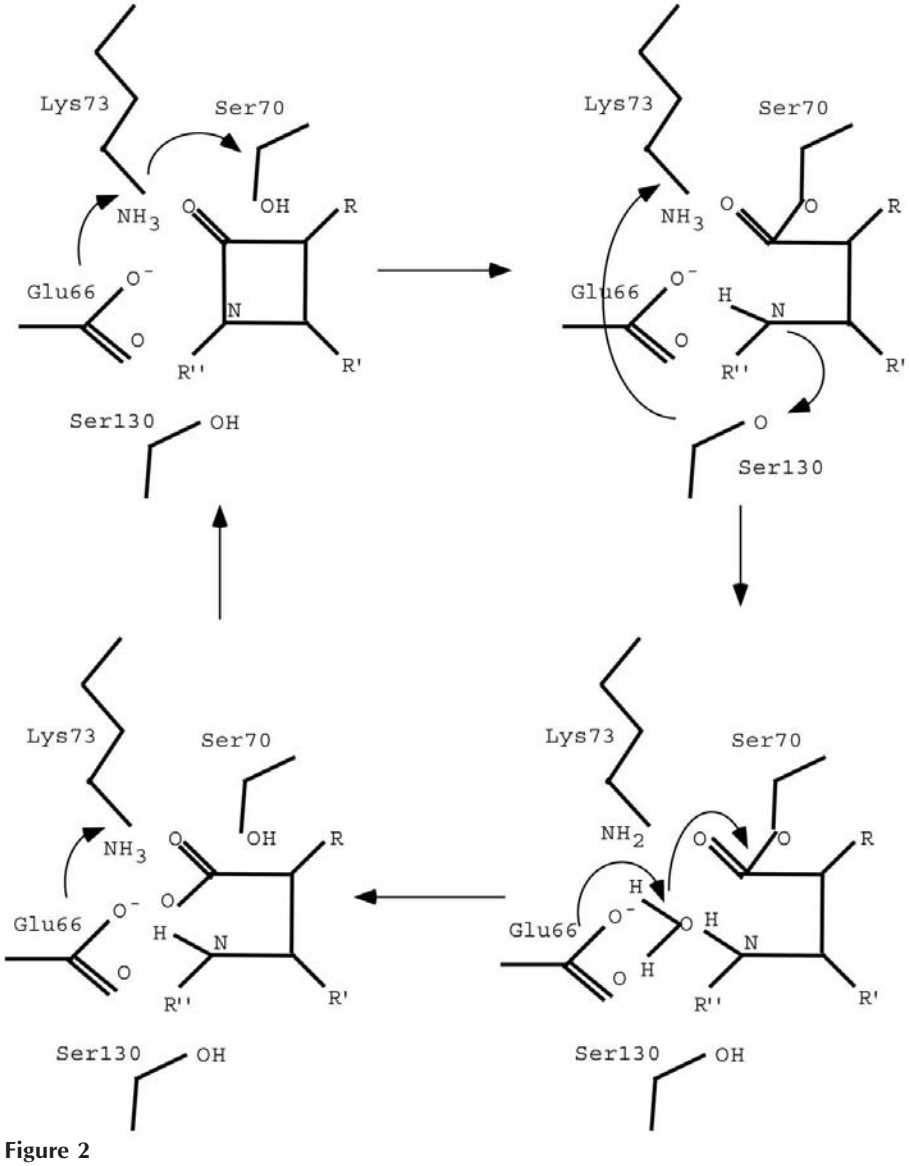

Figure 2
Schematic representation of the reaction catalyzed by TEM-1 β-lactamase.

2.3.2. Expression of the wild-type and mutant β-lactamases. The host strain MV1190 was used for expression of the wild-type and mutant β-lactamases. For the plasmid/strain combination pEK405/MV1190, a 1 l culture of YT media containing 150 µg ml^{-1} ampicillin was grown for ∼20 h to an A_{560} of 1.5–1.7 and then harvested. The cells were centrifuged and washed at least twice in 30 m*M* Tris buffer pH 7.2 prior to resuspension in a 20% sucrose solution containing 5 m*M* EDTA. After centrifugation, the cells were placed on ice and quickly resuspended in cold water to release the periplasmic space proteins. Expression of β-lactamase in the crude cell-free extracts was monitored by SDS electrophoresis. This procedure was followed for the mutant plasmid/strain combinations using 50 µg ml^{-1} kanamycin in the medium instead of ampicillin.

2.3.3. Purification. After isolating wild-type and mutant enzymes from the periplasmic space using the osmotic shock procedure described above, the resulting solution was centrifuged and the clarified supernatant was adjusted to 10 m*M* Tris pH 7 (using a 1 *M* stock solution). This solution was then further dialyzed against the same buffer (4 l at 277 K for 6 h) to remove sucrose. The dialyzed solution (∼400 ml) was centrifuged to remove precipitate, concentrated to ∼20–50 ml by ultrafiltration (Amicon) using a YM10 membrane and syringe-filtered (0.2 µm). The filtered solution was then loaded onto a Q

Table 1
Data-collection and refinement statistics for the wild-type and S70G β-lactamase structures.

Values in parentheses are for the last shell (1.55–1.6 Å for the wild type and 2.1–2.15 Å for the S70G mutant).

	Wild type†	S70G‡
Data collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
d_{min} (Å)	1.55	2.1
Completeness (%)	93.5 (72.9)	90.6 (85.3)
Average redundancy	2.9 (2.1)	2.3 (1.6)
$I/\sigma(I)$	14.1 (1.7)	13.7 (2.9)
Unit-cell parameters (Å)		
a	42.02	41.99
b	62.84	62.65
c	89.46	89.49
R_{merge}§ (%)	4.5 (27.1)	6.9 (21.5)
Refinement		
Final working R factor (%)		
4σ cutoff	16.0	15.9
All reflections	18.9	17.8
Final free R factor¶		
4σ cutoff	20.7	25.4
All reflections	24.1	27.6
No. of water molecules	194	157
R.m.s. deviations		
Bond lengths (Å)	0.016	0.018
Bond angles (°)	1.8	2.0
Dihedral angles (°)	22.8	23.6

† Phasing for this structure was accomplished using the coordinates of the TEM-1 β-lactamase from *E. coli* (PDB code 1btl). ‡ Phasing for this structure was accomplished using the coordinates of the TEM-1 β-lactamase structure refined in this study to 1.55 Å. § $R_{merge} = \sum_{hkl}\sum_i |I_{mean} - I_i|/\sum_{hkl}\sum_i I_i$. ¶ Reflections in the test set (5%) were not used in refinement.

Sepharose Fast Flow ion-exchange column (2 × 10 cm) and eluted with a linear gradient (0–0.2 *M* NaCl in 10 m*M* Tris pH 7) at a flow rate of 0.5 ml min^{-1}. In order to remove trace contaminants, additional purification steps were usually required. This was accomplished by Source 15Q anion-exchange chromatography, Phenyl Sepharose chromatography or gel filtration using SE100 on the BioLogic chromatography system from Bio-Rad. Enzyme purity was judged by SDS–PAGE as described by Laemmli (1970).

2.3.4. Determination of the protein concentration. The concentration of the wild-type enzyme was determined from absorbance measurements at 281 nm with an extinction coefficient of 29 400 M^{-1} cm^{-1} (Zafaralla *et al.*, 1992). The concentrations of the S70G enzyme were determined by the Bio-Rad version of the Bradford dye-binding assay (Bradford, 1976) using wild-type β-lactamase as the standard.

2.3.5. Determination of enzymatic activity. β-Lactamase activity was measured spectrophotometrically at 303 K. The decrease in absorbance upon hydrolysis of ampicillin was monitored at 235 nm. Assays were carried out in 50 m*M* Na_2HPO_4, 0.15 *M* KCl pH 7.0. The initial rates were measured for a range of substrate concentrations. The kinetic parameters K_m and V_{max} were determined from a non-linear least-squares fit of the data to the Michaelis–Menten equation.

2.3.6. Crystallization. Crystals of the wild-type and the S70G enzymes were obtained using the hanging-drop vapor-diffusion method. A concentrated solution of protein (~30 mg ml^{-1}) in 10 m*M* Tris buffer pH 7.0 buffer was combined with an equal volume of a PEG solution containing 30%(*w*/*v*) PEG 4000, 0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5. This protein drop (5–10 µl) was suspended over a reservoir containing 1 ml of the same PEG solution. After two weeks, the drops were seeded using a microcrystal attached to a whisker. Crystals usually formed within a week following this step.

2.3.7. X-ray data collection and processing. The diffraction data for the wild-type and S70G mutant β-lactamases were collected with Cu $K\alpha$ radiation on the Area Detector Systems MARK II system at the Crystallographic Facility in the Chemistry Department at Boston College. The data for the wild type and S70G mutant were collected to 1.55 and 2.1 Å resolution, respectively. Multiple measurements and symmetry-related reflections were scaled and merged using the software provided by Area Detector Systems. The final data sets for the wild type and S70G are over 90% complete, with R_{merge} values of 4.5 and 6.9%, respectively. A summary of the data collection is presented in Table 1.

2.3.8. Structure solution and refinement. The X-ray crystal structure of TEM-1 β-lactamase by Jelsch *et al.* (1993) was used as the initial model for the refinement of the wild-type enzyme at 1.55 Å resolution. This wild-type enzyme structure was then used as a starting model for refinement of the S70G enzyme. Both enzymes crystallized in space group $P2_12_12_1$, which is the same spatial arrangement as the crystal described by Jelsch *et al.* (1993), with similar unit-cell parameters.

Both X-ray crystal structures were refined using Silicon Graphics Indigo II workstations. Structure refinement was initiated by rigid-body minimization, continued by simulated annealing (*X-PLOR*) and completed by conjugate-gradient least squares (*SHELXL*; Sheldrick & Schneider, 1997). For both structures, a standard *X-PLOR* refinement macrocycle consisted of simulated annealing, positional refinement, temperature-factor refinement and water building. The difference Fourier maps and proper stereochemistry for hydrogen-bonding were used as criteria for the water-building procedure. The quality of the structures during all stages of the refinement was monitored by the free R factor and the standard R factor. The refinement was terminated when the R_{free} could not be reduced further.

3. Results

3.1. Kinetic characterization the wild-type and S70G enzymes

Kinetic parameters for the hydrolysis of ampicillin by the wild-type and mutant enzymes are provided in Table 2. The S70G enzymes created with two different glycine codons have almost identical kinetic properties. The turnover numbers represented by the k_{cat} value are essentially the same for the two different S70G mutant enzyme. These enzymes are both approximately 2000-fold less active than the wild type as measured by the k_{cat}/K_m ratio (Table 2).

Table 2
Kinetic parameters for the wild-type and the active-site mutant S70G β-lactamase.

Enzyme	k_{cat}† (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}\,M^{-1}$)
Wild type	1194 ± 88	133 ± 7	9.0×10^6
S70G‡	<0.024	>194	$\sim 1 \times 10^2$

† The k_{cat} values are calculated from V_{max} using a molecular weight of 29 000 Da. Assays were performed in 50 mM Na_2HPO_4, 0.15 M KCl pH 7 at 303 K using ampicillin as substrate. ‡ Data presented is for the S70G enzyme created by a single nucleotide change; the data from the S70G enzyme created by a double nucleotide change is almost identical (data not shown).

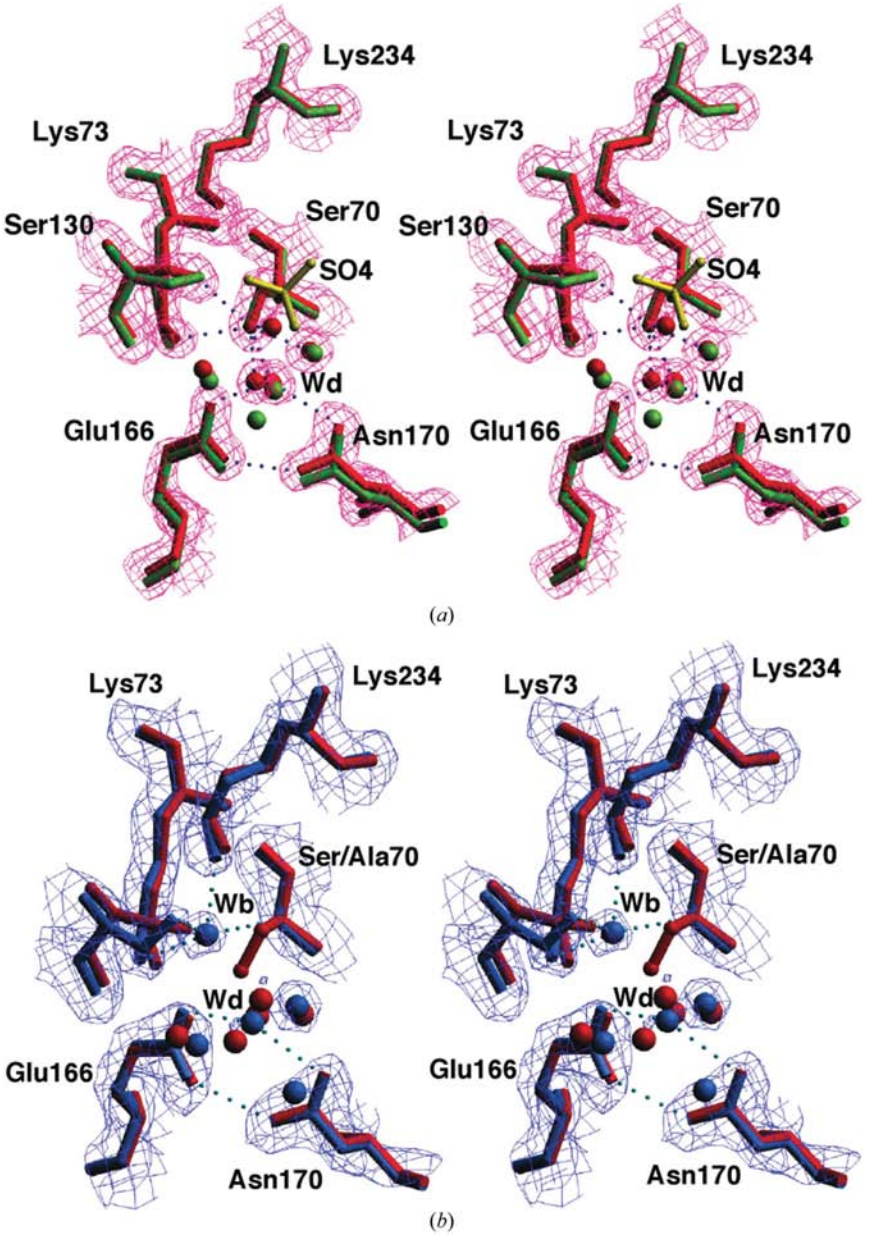

Figure 3
Active-site comparisons. (*a*) The wild-type β-lactamase at 1.55 Å (red) and the 1btl structure at 1.8 Å (green). The sulfate of the 1btl structure is shown in yellow. The deacylating water molecule (wat1046) is shown in both structures as Wd. (*b*) The comparison of the wild-type structure (red) and the S70G enzyme (blue). The postulated nucleophilic water molecule (wat440) unique to the S70G enzyme is marked as Wb. In both, the $2F_o - F_c$ electron density is contoured at 1.4σ (wild type in red and the mutant in blue).

3.2. Crystal structures of the wild-type and S70G enzymes

During attempts to find crystallization conditions for the S70G enzyme, crystals of the wild-type enzyme were also obtained that diffracted to 1.55 Å, significantly higher than previously reported for TEM-1 β-lactamase crystals (Jelsch *et al.*, 1993). As indicated above, both the wild-type and S70G enzymes crystallized in the same space group with unit-cell parameters that were similar to each other and to those reported in the literature. The structure of the wild-type enzyme was refined to a final working R factor of 18.9%, as determined using all reflections, and a final free R factor of 24.1% and contained 194 water molecules. The structure of the S70G enzyme was refined to a final working R factor of 17.8% and a final free R factor of 27.6% and contained 157 water molecules. A summary of the refinement process is provided in Table 1.

3.3. Active-site comparison of two wild-type β-lactamase structures

The molecule is folded into two domains, one predominantly α-helical and one $\alpha\beta$-domain. The active site is located at the interface of both domains close to the β-strands of the $\alpha\beta$-domain. The catalytically important Ser70 is located on the 3_{10}-helix initiating one of the helices of the α-helical domain. The active site appears to be highly charged with several conserved residues: Lys73, Lys234, Glu166 and two serine residues Ser70 and Ser130. The previously reported structure of TEM-1 β-lactamase (Jelsch *et al.*, 1993) was determined with a sulfate ion bound at the active site. Despite this difference, the conserved active-site motifs of both structures can be superimposed with very little divergence (Fig. 3*a*). The r.m.s. deviation between the original TEM-1 structure (Jelsch *et al.*, 1993) and the wild type reported here is 0.41 Å as calculated on 252 C^α positions and 0.7 Å if all atoms are included.

In the Jelsch *et al.* (1993) structure the presence of the sulfate stabilizes the protonated state of Ser70, judging from the distances to the O^γ ligands. In the wild-type structure presented here, Ser70 O^γ has six apparent ligands, including two water molecules with distances <2.5 Å (Table 3). The ligands are found in an approximate pentagonal bipyramidal arrangement, with five ligands in equatorial positions and one in an axial position (Fig. 3*a*). The other axial position is occupied by the covalent bond to Ser70 C^β. This axial water molecule

Table 3
Selected active-site distances in the wild-type and the mutant S70G β-lactamase (Å).

Interaction		Subunit	
		Wild type	Ser70Gly
Ser70 O^{γ}	Ser130 O^{γ}	3.15	
	Lys73 N^{ζ}	2.76	
	Watd1046	2.77	
	Wat1010	2.43	
	Wat1157	2.67	
	Wat1126	2.32	
Ser130 O^{γ}	Lys73 N^{ζ}	3.29	5.04
	Lys234 N^{ζ}	2.78	2.97
	Wat1157	3.25	
Glu166 $O^{\varepsilon 2}$	Watd1046	2.47	2.60
	Lys73 N^{ζ}	3.43	3.57
Glu166 $O^{\varepsilon 1}$	Asn170 $N^{\delta 2}$	2.94	3.02
Watd1046	Asn170 $O^{\delta 1}$	2.83	3.01
Watd319	Wat441		3.14
Watd319	Wat322		3.20
Watb440	Lys73 N^{ζ}		2.91
	Lys234 N^{ζ}		2.60
	Wat315		2.70
	Ser130 O^{γ}		3.10

is only 2.32 Å away from Ser70 O^{γ}. The environment of Ser70 in our structure suggests that it is deprotonated in this unliganded enzyme. Ser130 O^{γ}, which is one of the equatorial ligands, is relatively close to Ser70 O^{γ} (3.18 Å). This distance varies from that in the structure determined by Jelsch *et al.* (1993), in which Ser130 had a 3.54 Å contact distance with Ser70. It may be speculated that upon the loss of the Ser70 O^{γ}, Ser130 can take over the role of the primary nucleophile. However, the conformational change observed in the mutant structure precludes an easy approach to Ser130 O^{γ}, which makes this role unlikely. Additionally, the direct environment of Ser130 does not promote deprotonation.

3.4. Active-site comparison of the active sites in the X-ray crystal structures of the wild-type enzyme to 1.55 Å and S70G enzyme

Overall, the wild-type structure is very similar to the structure of the S70G enzyme. The least-squares superposition of the structures produced an r.m.s. deviation of 0.17 Å for C^{α} positions and 0.41 Å for all atoms. The major structural differences are seen in the active site, resulting from an altered conformation of the Ser130 side chain in the S70G enzyme. Small side-chain conformational differences and water-molecule positions are observed when the active sites of the wild-type structures are compared with the S70G enzyme structure (Fig. 3*b*). The water molecule hydrogen bonded to Glu166 (Wd) postulated to be involved in the deacylation step is observed in all three structures as hydrogen bonded (~2.5 Å) to the side chain of Glu166. In the S70G structure there is an additional water molecule (Wb) in close proximity to position 70 that is hydrogen bonded to the amino groups of Lys234 and Ser130 O^{γ} (3.14 Å). This water molecule is shifted approximately 2 Å away from the position occupied by Ser70 O^{γ} in the structure of the wild-type enzyme. What is important to note is that it is close to the position of Ser130 O^{γ} in the wild-type structure. In the wild-type enzyme the Ser130 O^{γ} is hydrogen bonded to the side chains of Lys234 and Ser70. This spatial arrangement apparently promotes the deprotonation of Ser70; therefore, it would also promote the deprotonation of water Wb.

3.5. Active-site comparison of the active sites in the X-ray crystal structures of the S70G enzyme and S70A mutant enzyme from *Staph. aureus*

Overall, the similarity between the *E. coli* and *Staph. aureus* at the primary sequence level is high. Therefore, the structures of both enzymes (Jelsch *et al.*, 1993; Chen *et al.*, 1996) show many similar features. The three-dimensional superposition carried out with the program *SEQUOIA* (Bruns, Scripps Institute) produced structures that show 1.5 Å r.m.s. divergence on 208 C^{α} atoms. The entire active site is conserved, including all the crucial residues. Less conservative changes occur ~10 Å away from the location of Ser70. Even the backbone atoms of the preceding residue, which is Met in *Staph. aureus* (Ala in *E. coli*) has a very small divergence (>0.3 Å). In particular, residues corresponding to Ser130 and Lys234 in the *E. coli* enzyme have very well preserved positions. There seems to be a native Ser130 conformation that is at variance with the Ser70G mutant of the *E. coli*.

The water molecule hydrogen bonded to Glu166 (Wd) postulated to be involved in the deacylation step is observed to be hydrogen bonded (~2.5 Å) to the carboxylate side chain of Glu166 in all three structures. The conserved water molecule and the oxyanion hole are also observed in all three structures. The additional water molecule observed in the S70G mutant enzyme (Wb) is not present in the S70A *Staph. aureus* enzyme. This suggests that an alternative explanation for the activities of these mutant enzymes is that Ser130 takes over the duties of the primary nucleophile in these enzymes. Verification of this hypothesis must await the mutational studies with a double mutation of Ser70 and Ser130 changed to Ala.

4. Discussion

4.1. The catalytic activity of the S70G enzyme

The S70G enzyme is significantly less active than the wild-type enzyme, but retains catalytic activity toward ampicillin despite the elimination of the serine nucleophile. The loss of activity on substituting the serine with a glycine reflects a change in the catalytic mechanism. Catalysis in the S70G mutants can no longer proceed through a covalent acyl-enzyme intermediate, given the lack of a side chain with nucleophilic properties in these two mutant enzymes.

The activity of the S70G β-lactamases is higher than expected, suggesting the possibility of wild-type contamination. It has been suggested that in the resulting S70G mutant with single nucleotide change from the original Ser codon (AGC) to the glycine codon (GGC) that mistranslation of the glycine codon back to AGC leads to wild-type contamination (Mazzella *et al.*, 1991). The same study has shown that the

glycine codon GGG having a double-nucleotide mismatch prevents wild-type contamination and reduces the activity of the S70G mutant β-lactamase 50-fold (Mazzella *et al.*, 1991). For this study, a second construct of the S70G mutation was made using the preferred glycine codon for *E. coli*, GGG, having a double nucleotide change. Despite the different codons, the resulting S70G mutant β-lactamases from both constructs have essentially the same kinetic properties toward ampicillin. Wild-type β-lactamase contamination can be essentially ruled out owing to the kinetic properties of the enzyme produced by the glycine GGG codon and also by care in preventing cross-contamination of the different β-lactamases during preparation. Therefore, the activity can be attributed to the nucleophilic water molecule in the S70G β-lactamase.

The kinetic parameters determined here show some discrepancies with previously reported literature values. According to Toth *et al.* (1988), the kinetic activities of the S70G enzyme produced with the GGA and GGC codons are roughly four and three orders of magnitude lower than the wild type, respectively. In this study, the kinetic properties of the S70G enzymes produced with both codons are indistinguishable and are at least four orders of magnitude lower than the wild-type enzyme. Furthermore, the kinetic activity of the S70A mutant β-lactamases from *Strep. albus* G and *Staph. aureus* are reduced by four to five orders of magnitude compared with the wild-type enzymes (Chen *et al.*, 1996; Jacob *et al.*, 1991). Even with correction for possible wild-type contamination, the S70G mutant enzymes display some catalytic activity.

4.2. Structure-based explanation for the catalytic activity of the S70G enzyme

The wild-type structure presented in this study provides a tentative base for understanding the catalytic details of the mutant enzymes. A brief inspection of our wild-type structure shows some crucial differences from the structure presented by Jelsch *et al.* (1993). In the Jelsch structure, the presence of the sulfate molecule at the active site appears to stabilize a protonated form of the Ser70 (Fig. 3*a*) as exemplified by relatively long hydrogen-bond distances to the ligands. Also, the distance of Ser130 O^{γ} to Ser70 O^{γ} (3.54 Å) is substantially different from the corresponding distance in our wild-type structure (3.18 Å). Furthermore, a short distance to two water molecules (2.32 and 2.34 Å) combined with a large number of ligands (six) suggests that the wild-type structure presented here represents an unprotonated form of the enzyme.

The structure of the S70G mutant provides a possible explanation for its catalytic activity. The overall structure of the molecule is very similar to the wild-type structure (Fig. 3*b*). Additionally, the conformations of the active-site residues are very similar in the two structures, with the obvious exception at position 70 (Fig. 3*b*). The main difference between the two structures is the organization of water molecules in the active site (Fig. 3*b*) and the conformational change of Ser130. The backbone atoms surrounding Ser130 are significantly shifted and Ser130 adopts a different conformer than in the wild-type enzyme. However, the crucial hydrogen-bond distance to other structural elements appears to be conserved. This underscores an important role of Ser130 in the catalytic mechanism and selectivity against a variety of different substrates (Vakulenko *et al.*, 1998).

The conserved water molecules involved in acylation and deacylation of the covalent acyl-enzyme intermediate present in the wild-type structure are also present in the S70G enzyme structure. The deacetylating water molecule participates in a strong hydrogen bond to Glu166 and Ser70 (Matagne & Frere, 1995; Matagne *et al.*, 1998) in all class A enzymes. In our wild-type β-lactamase structure, the deacylating water molecule (Wat46; Wd in Fig. 3*a*) is ~2.5 Å away from the carboxylate O atom of Glu166 and ~2.8 Å away from the hydroxyl of Ser102. In the wild-type structure presented by Jelsch *et al.* (1993), the corresponding distances are 2.7 and 2.9 Å, respectively.

In the S70G mutant enzyme structure, a new water molecule (Wat140; Wb in Fig. 3*b*) is observed in the vicinity of position 70 and is held in place by hydrogen bonds to Lys234 and Ser130 (Fig. 2*b*). If this water molecule were present in the wild-type structure it would be positioned too close (~2 Å) to the Ser102 side chain (O^{γ} as well C^{β}). We postulate that this water molecule functionally replaces the hydroxyl group of Ser70. The fact that there is similar coordination to Ser130 despite the different conformation of this residue strengthens this proposal.

The position of the conserved water molecules of the oxyanion hole is only slightly different in the structures of the wild-type and mutant enzymes. The changes in the water-molecule arrangements seem to correlate with the expected change in the catalytic mechanism for the S70G enzyme. Catalysis in the mutant enzyme must be achieved without formation of a covalent acyl-enzyme intermediate. Instead, catalysis must take place in one in-line displacement step, with an activated water molecule serving as a nucleophile. A water molecule (Wat140; Wb in Fig. 3*b*) is postulated to fulfill the role of nucleophile in the S70G enzyme. In order to participate in two hydrogen bonds with Lys234 and Ser130, this water molecule is shifted slightly back, approximately 2.0 Å away from the position occupied by Ser70 O^{γ} in the wild-type enzyme. While this displacement, in addition to the loss of covalent catalysis, drastically slows the hydrolysis of the β-lactam, some electrophilic assistance may still be provided by the intact oxyanion hole.

The ability of the S70G active site to adequately position an activated water molecule in close proximity to the β-lactam ring and offer stabilization of the negatively charged transition-state species may account for its accelerated rate over spontaneous solution hydrolysis of β-lactams. However, the absence of nucleophilic water in the latter enzyme from *Staph. aureus* is somewhat puzzling. It will be interesting to compare the structures of the S70A mutant from *Staph. aureus* to the same mutant from *E. coli*. Structural differences between the two class A β-lactamases may account for the higher catalytic activity of the *E. coli* S70A enzyme. A parallel can be drawn between the alkaline phosphatase mutants

(S102A, S102G, S102C; Stec *et al.*, 1998) studied by us earlier that eliminated the serine nucleophile from the structure and the present study. In those mutants the catalytic activity was significantly diminished but not completely eliminated. The explanation we offered for residual activity of the AP mutant enzymes was that the water molecule could provide the functionality of the primary nucleophile, changing the character of the catalysis from covalent to non-covalent.

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