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Altering enzymatic activity: Recruitment of carboxypeptidase activity into an RTEM β-lactamase/penicillin-binding protein 5 chimera

(mutagenesis/protein structure–function relationships/enzymatic catalysis)

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ABSTRACT **The D-Ala-D-Ala carboxypeptidases/transpeptidases (penicillin-binding proteins, PBPs) share considerable structural homology with class A β-lactamases (EC 3.5.2.6), although these β-lactamases have no observable D-Ala-D-Ala carboxypeptidase activity. With the objective of recruiting such activity into a β-lactamase background, we have prepared a chimeric protein by inserting a 28-amino acid segment of PBP-5 of *Escherichia coli* in place of the corresponding region of the RTEM-1 β-lactamase. The segment thus inserted encompasses two residues conserved in both families: Ser-70, which forms the acyl-enzyme intermediate during β-lactam hydrolysis, and Lys-73, whose presence has been shown to be necessary for catalysis. This chimera involves changes of 18 residues and gives a protein that differs at 7% of the residues from the parent. Whereas RTEM β-lactamase has no D-Ala-D-Ala carboxypeptidase activity, that of the chimera is significant and is, in fact, about 1% the activity of PBP-5 on diacetyl-L-Lys-D-Ala-D-Ala; in terms of free energy of activation, the chimera stabilizes the transition state for the reaction to within about 2.7 kcal/mol of the stabilization achieved by PBP-5. Furthermore, the chimera catalyzes hydrolysis exclusively at the carboxyl-terminal amide bond which is the site of cleavage by D-Ala-D-Ala carboxypeptidase. Though containing all those residues that are conserved throughout class A β-lactamases and are thought to be essential for β-lactamase activity, the chimera has considerably reduced activity ($\approx 10^{-5}$) on penams such as penicillins and ampicillins as substrates. As a catalyst, the chimera shows an induction period of ≈30 min, reflecting a slow conformational rearrangement from an inactive precursor to the active enzyme.**

The many examples of class A β-lactamases (EC 3.5.2.6) show striking similarities to a large group of enzymes, collectively known as penicillin-binding proteins (PBPs), that are involved in the cross-linking of the peptidoglycan in the final stage of the synthesis of the bacterial cell wall (1–3). In catalyzing the cleavage of a D-Ala-D-Ala peptide bond at the carboxyl terminus of one polypeptide chain and transferring the amino-terminal remainder to the amino terminus of another polypeptide in a transpeptidase reaction, these enzymes play an essential role in cross-linking the bacterial cell wall, thereby stabilizing the cells against rupture in hypotonic environments (4). The enzymes can also act as D-Ala-D-Ala carboxypeptidases (D,D-carboxypeptidases) by simple hydrolysis of the carboxyl-terminal D-Ala-D-Ala peptide bond.

The β-lactam substrates of the β-lactamases are close structural homologues of the D-Ala-D-Ala dipeptide. This led to the proposal of an evolutionary relationship between the β-lactamases and the PBPs (5). This relationship is further strengthened by similarities in several stretches of amino acid sequence, particularly among amino acid residues that are now known to lie within the active site region (6). Moreover, the general foldings of the polypeptide chains in β-lactamases and one PBP (the R61 carboxypeptidase of *Streptomyces*) show considerable conservation of secondary structural features (3). However, β-lactamases do not catalyze the hydrolysis of peptide bonds in noncyclic PBP substrates (though they can catalyze the hydrolysis of open chain esters), and PBPs do not effectively catalyze the hydrolysis of β-lactam antibiotics though they can form relatively stable acyl enzyme intermediates with β-lactams; indeed, the formation of these intermediates is the likely pathway of irreversible inhibition by which β-lactams are thought to manifest their action as antibiotics (1, 2, 4).

Can one recruit carboxypeptidase activity into a β-lactamase by replacing structural elements of a β-lactamase with homologous elements of a PBP? To explore this possibility and, more generally, the ways in which one might change the scope of an enzyme's catalytic activity, we have constructed a chimera of the RTEM-1 β-lactamase and the *Escherichia coli* PBP-5 carboxypeptidase. We chose PBP-5 of *E. coli* because it and the RTEM β-lactamase have the greatest sequence similarity (27%) in the active site (6). Moreover, most carboxypeptidases slowly degrade the penicilloyl group into phenylacetylglycine, whereas PBP-5 catalyzes hydrolysis to form penicilloic acid at a significantly higher rate than most PBPs, suggesting a closer evolutionary relationship to β-lactamases (7). In targeting the residues for replacement we were guided by sequence homologies rather than three-dimensional structural considerations because, at the time, no appropriate high-resolution structures were available.

Site-directed mutagenesis has previously been used, for example, to generate mutants with altered sequence recognition for DNA binding and substrate specificity in enzymatic catalysis and to create interspecies hybrids. By substituting five amino acids in the "outside" surface of the DNA recognition α-helix of the 434 repressor, with the corresponding amino acids from the P22 repressor, a chimera was produced with P22 DNA recognition in a 434 background (8). Replacement of the nonpolar substrate binding domain of ornithine transcarbamoylase with the analogous nonpolar domain of aspartate transcarbamoylase generated an active chimera that has aspartate transcarbamoylase activity, though at a level considerably lower than that of the native enzyme (9). For phosphoglycerate kinase, two interspecies chimeras were constructed, each composed of one domain from the human enzyme and one domain from the yeast enzyme. Despite a 35% difference in the amino acid composition between human and yeast phosphoglycerate kinases,

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Abbreviations: PBP, penicillin-binding protein; D,D-carboxypeptidase, D-Ala-D-Ala carboxypeptidase; Lac, lactate.

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the catalytic properties of the chimeras were very similar to those of the parental enzymes (10).

MATERIALS AND METHODS

Materials. All enzymes were purchased from Boehringer Mannheim. Antibiotics, peptide substrates, and 2-mercaptoethanol were from Sigma. Radioactive materials were supplied by Amersham. Isopropyl β-D-thiogalactoside (IPTG) was purchased from IBI. Molecular biology grade reagents agarose, phenol, and chloroform were also from IBI. *o*-Phthaldialdehyde and sodium tetraborate were from Fluka.

E. coli was used in all experiments. Plasmid pBR322 DNA was harbored in strain HB101; the pJN (11) expression vector was propagated in strain D1210, which is *lacI*^{q}. Bacteriophage were propagated in strain JM101. The culture medium was L broth unless otherwise indicated. Cells were made competent for transformation of plasmid DNA by using a process adapted from Hanahan (12).

Oligonucleotides were synthesized by using phosphoramidite chemistry (13) on the Applied Biosystems automated DNA synthesizer, model 380A. They were then purified by preparative polyacrylamide gel electrophoresis.

Wild-type plasmid pBR322 and bacteriophage M13mp18 replicative form (RF) DNA were purchased from Bethesda Research Laboratories. Mutant plasmids and RF phage DNA were purified from *E. coli* by the alkaline lysis method (14). Large-scale preparations were further purified by ultracentrifugation in cesium chloride/ethidium bromide gradients.

Construction of the RTEM-PBP-5 Chimera. The *Eco*RI/*Pst* I [752 base pairs (bp)] fragment containing part of the β-lactamase gene of pBR322-DC9 (15), a mutant containing a silent mutation creating a *Nar* I site at nucleotide 3924, was subcloned in M13mp8 for the cassette mutagenesis with the synthetic cassette corresponding to the PBP-5 sequence (Fig. 1). The replicative form DNA of the resulting M13 derivative was digested with *Xho* I/*Ban* II, and the resulting 1-kbp fragment was isolated. A second digestion of the M13 derivative was performed with *Nar* I/*Ban* II and the resulting 7-kbp fragment was isolated. Synthetic oligonucleotides were annealed by heating 50 pmol of each to 95°C in 100 μl of 10 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/50 mM NaCl and allowing the solution to cool to room temperature over 2 hr. Annealed oligonucleotides (0.4 pmol) were combined with approximately 0.04 pmol of vector [*Xho* II/*Ban* I fragment (1 kbp); *Nar* I/*Ban* II (7 kbp)] to give a 10:1 molar ratio of insert to vector. This was incubated in 10 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/5 mM dithiothreitol/1 mM ATP containing 2 units of T4 DNA ligase in a total volume of 25 μl for 12–14 hr at 16°C; 10 μl of the reaction mixture was used to transform competent *E. coli* JM101; the resulting cells were plated on 2× YT medium (16 g of Bactotryptone, 10 g of yeast extract, and 5 g of NaCl per liter). The three-piece ligation yielded the desired chimera as verified by sequencing. The mutant was then subcloned back into pBR322.

Plasmid DNA was sequenced by using a modification of the Sanger dideoxy method (16) for denatured, double-stranded DNA (17). Plasmid DNA was suspended in TE (10 mM Tris·HCl, pH 8.0/1 mM EDTA) with 100 pmol of sequencing primer in a volume of 10 μl. The mixture was incubated at 95°C for 4 min, then snap frozen in a dry ice/ethanol bath. Chain extension reactions were performed with a Sequenase kit from United States Biochemical (18). Labeling was accomplished with deoxyadenosine 5′-[α-[^{35}S]-thio]triphosphate.

Protein Purification and Analysis. Western blots (Fig. 2) were prepared to examine stability *in vivo*. Colonies harboring mutants were grown to late logarithmic phase ($OD_{600} \approx 1.0$); a 1.5-ml sample of each was pelleted by centrifugation and resuspended in 100 μl of protein sample buffer [10% (vol/vol) glycerol/5% (vol/vol) 2-mercaptoethanol/3% (wt/vol) SDS/62.5 mM Tris·HCl, pH 6.8/1.0 mM EDTA/0.05% bromophenol blue] and heated to 95°C for 10 min to lyse the cells. Aliquots (20 μl) were loaded onto a 15-cm, 12% polyacrylamide stacking gel and run at a constant current of 5 mA for 12–16 hr. Protein was then transferred from the gel onto DEAE-nitrocellulose by using a Bio-Rad Transblot cell for 2 hr at 100 mA. β-Lactamase was visualized following binding of rabbit anti-β-lactamase (19) by using the highly sensitive Vectastain ABC immunoperoxidase system (20).

Mutant genes were subcloned in pJN (11), an expression vector utilizing the *tac* promoter (21), for the overproduction of β-lactamase. Cells were grown in XB medium (25 g of Bactotryptone, 7.5 g of yeast extract, and 50 ml of 1 M Tris·HCl, pH 7.5, per liter) at 30°C until they reached stationary phase. Isopropyl β-D-thiogalatoside (0.1 M) was added and the culture was cooled to 0°C. After 30 min the cells were harvested and the crude protein was collected by osmotic extrusion (22). The chimera was then purified by anion-exchange (Whatman DE-52 DEAE-cellulose) followed by gel filtration (Sephadex G-100). Purity was checked by electrophoresis in SDS/12% polyacrylamide gels stained with Coomassie blue. The protein concentration was estimated by A_{281} using the extinction coefficient 29,400 $M^{-1}\cdot cm^{-1}$ (22). During purification of the chimera ampicillin was included in all buffers at 40 mg/liter.

Kinetic analyses of β-lactamase activities were performed at 30°C in 0.1 M potassium phosphate, pH 7.0. The hydrolysis of the lactam bond was monitored at 232 nm to obtain initial velocities. $\Delta\varepsilon = 500\ M^{-1}\cdot cm^{-1}$ for benzylpenicillin (23). D,D-Carboxypeptidase activities were determined at 37°C in 0.1 M Tris·HCl, pH 7.5. A fluorescence assay (24, 25) was employed to measure the carboxypeptidase activity, with N^{α},N^{ε}-diacetyl (diAc)-L-Lys-D-Ala-D-Ala as a substrate. Reactions (in 0.25 ml) were initiated at several substrate concentrations. Aliquots (50 μl) were collected at intervals of 30 min and added to 2 ml of buffered reagent [1.5 ml of *o*-phthaldialdehyde solution (5 mg/ml of ethanol), 1.5 ml of 2-mercaptoethanol solution (5 μl/ml of ethanol), and 90 ml of aqueous 0.05 M sodium tetraborate, pH 9.5]. The samples were read within 15 min of mixing on an SLM 4800 fluorescence spectrophotometer (SLM AMINCO, Urbana, IL). Excitation and emission monochromators were set at 340 nm and 455 nm, respectively, with a 2-nm slit width. The fluorescence value was corrected against the time-zero value and the concentration of D-alanine was determined from a calibration curve. To obtain the activity vs. pH plot, reactions were performed at 0.02 M diAc-L-Lys-D-Ala-D-Ala while varying pH and buffer systems: 0.1 M KOAc, pH 5.0 and 6.0; 0.1 M Tris·HCl, pH 7.0, 7.5, 8.0, 8.5, and 9.0; 0.1 M KH_2PO_4, pH 7.0 and 8.0; and 0.1 M K_2CO_3, pH 8.5 and 9.0. To assay the hydrolysis of the depsipeptide diAc-L-Lys-D-Ala-D-Lac (Lac = lactate), parallel reactions were initiated at a single substrate concentration. Reactions were quenched at timed intervals by snap-freezing in dry ice/ethanol. The remaining quantity of depsipeptide starting material in the samples was determined by isolation on reverse-phase HPLC using a linear gradient of acetonitrile/water (0.1% trifluoroacetic acid) and the protein content was quantified by A_{214}. A blank run (starting material only) was performed for calibration. Radiolabeled (^{3}H) substrate was used to detect activity for the lower-activity chimera. The products were separated by paper chromatography (26). To assay the chimeras for transpeptidase activity, depsipeptide digestions (in 0.1 M Tris·HCl, pH 7.5, 37°C) were initiated with glycine present in the reaction mixture. The reaction was quenched at 3 hr and the mixture was analyzed for the formation of the tripeptide diAc-L-Lys-D-Ala-Gly by HPLC.

RESULTS AND DISCUSSION

To construct the chimeric enzyme, we replaced a 28-amino acid sequence encompassing the active site region in RTEM-1 β-lactamase [residues 50–77 in the Ambler convention (27)] with a corresponding sequence from PBP-5 (residues 24–51 in the PBP convention, which does not include residues for any leader sequence) (Fig. 1). The gene encoding the chimeric protein was assembled in a three-piece ligation utilizing RTEM-1 β-lactamase encoded by plasmid pBR322 and a synthetic duplex encoding the PBP-5 insert. The resulting structural gene was subcloned in pJN (11), an expression vector that utilizes the *tac* promoter (21) and allows the overexpression even of inactive mutants of β-lactamase by encoding a gene for kanamycin resistance as a selectable marker.

E. coli strain D1210 cells containing the plasmid encoding the chimera do not show a resistant phenotype to penam antibiotics such as penicillins and ampicillins above the level conferred by a chromosomally encoded β-lactamase (6–10 μg/ml). Nevertheless, the chimeric protein can be isolated from the periplasm of such cells (grown in the presence of kanamycin at 50 μg/ml) by osmotic extrusion (22). The crude protein was then purified by anion-exchange followed by gel-filtration chromatography. Purity was confirmed by analysis on SDS/12% polyacrylamide gels. Protein concentration for kinetic analyses was estimated by A_{281} using the β-lactamase extinction coefficient 29,400 $M^{-1} \cdot cm^{-1}$ (22).

Preliminary assessment of the activity of the purified chimera toward benzylpenicillin (30°C, pH 7) revealed an induction period of about 30 min. After a particular reaction was allowed to proceed to complete conversion, newly added substrate was hydrolyzed without such an induction period. Also, whereas incubation of the chimeric protein at 37°C for 4 hr resulted in complete loss of activity, the chimera, when similarly incubated *in the presence of ampicillin*, retained full activity. These observations suggested that the initially inactive chimeric protein undergoes a slow conformational change to an active enzyme upon binding substrate. Moreover, they suggested that the presence of a β-lactam may stabilize a properly folded conformation of the chimera and thereby discourage its proteolytic degradation in the periplasm. To examine the latter possibility, cells were grown in the presence of sublethal concentrations (6 μg/ml) of ampicillin (and the normal concentrations of kanamycin); cells grown under these conditions contain about twice as much chimeric protein as those grown in the absence of β-lactam antibiotic (Fig. 2). Subsequent purifications of the chimera were performed with excess ampicillin (40 μg/ml) in all buffers.

Table 1 collects representative kinetic parameters for the chimeric enzyme acting both as a β-lactamase and as a D-Ala-D-Ala carboxypeptidase. These parameters for both β-lactamase and carboxypeptidase activities were determined for chimera that had been equilibrated with ampicillin to assist folding of the protein into the active conformation. (In the absence of either penam antibiotics or diAc-L-Lys-D-Ala-D-Ala substrate for periods in excess of about 30 min, the enzymatic activities as a β-lactamase and as a carboxypeptidase decay.) With benzylpenicillin as substrate, the turnover rate is sharply reduced relative to that for the RTEM-1-β-lactamase ($\approx 10^{-5}$) (19) and somewhat increased relative to that for PBP-5 ($\approx 10^{2}$) (28). Activity of the chimera as a D,D-carboxypeptidase was assessed by using as substrate diAc-L-Lys-D-Ala-D-Ala (26) and the depsipeptide analogue diAc-L-Lys-D-Ala-D-Lac (31). Whereas RTEM β-lactamase does not measurably catalyze the hydrolysis of either substrate, the chimeric enzyme has considerable such activity and shows about 1% the activity of PBP-5 (k_{cat}/K_m) toward the acylated tripeptide and 0.4% toward the acylated depsipeptide. In terms of free energies of activation, this places the stabilization by the chimera of the transition state for hydrolysis of D-Ala-D-Ala dipeptides within about 2.7 kcal/mol, and for hydrolysis of the depsipeptide within 3.3 kcal/mol, of that achieved by PBP-5 itself (1 kcal = 4.18 kJ). In both cases, hydrolysis occurs exclusively at the carboxyl-terminal peptide bond between the two D-Ala residues and at the terminal ester bond at which PBP-5 itself cleaves. The rate of nonenzymatic hydrolysis of a peptide bond in aqueous solution at neutral pH is on the order of 3×10^{-9} sec^{-1} (32), thus the rate of the reaction catalyzed by the chimera is about 10^{9} that for uncatalyzed amide hydrolysis at pH 7.

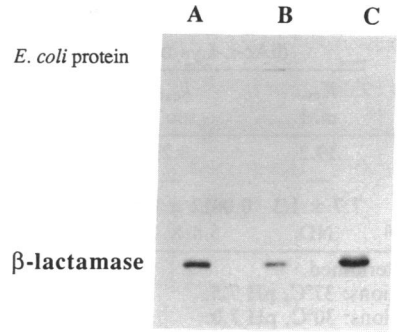

FIG. 2. Western blots of wild-type and chimeric enzymes. Lane A shows the amount of chimera present when cells were grown in the presence of sublethal levels of ampicillin; lane B shows the amount of chimera present at 37°C; and lane C shows the expression of wild-type β-lactamase at 37°C. Note that the increase in the intensity of the chimera band in the presence of ampicillin reflects the stabilizing effect of the antibiotic on the chimera.

In the course of this work, we also recovered a second chimera (E^+) that contains an extra glutamic residue between residues 59 and 60 [β-lactamase numbering (27)]. This chimera exhibited a k_{cat} for D,D-carboxypeptidase activity two orders of magnitude less and a β-lactamase activity toward benzylpenicillin about an order of magnitude greater than that of the chimera without this additional residue (Table 1). Also, in the chimera with the glutamic residue insertion (E^+) we observe an induction period during the hydrolysis of β-lactam antibiotics. We conclude that the additional residue must be accommodated by the flexibility of the random coil segment and, while it does hinder the carboxypeptidase activity, the augmented chimera (E^+) favors β-lactam hydrolysis compared to the chimera without the insertion.

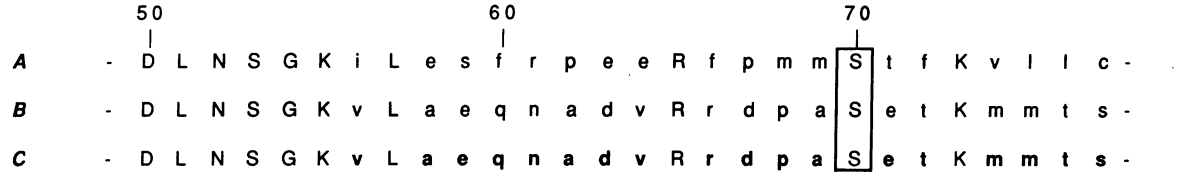

FIG. 1. Line A, the amino acid sequence of residues 50–77 of RTEM-1 β-lactamase (22); line B, the corresponding amino acid sequence of PBP-5 (6); and line C, the sequence of the resulting chimera. Standard one-letter symbols are used and the active serine is outlined. Conserved residues are capitalized and exchanged residues in the chimera are boldface.

Table 1. Kinetic parameters for wild-type PBP-5 from *E. coli*, wild-type RTEM-1 β-lactamase, and the RTEM/PBP-5 chimeric enzyme

Enzyme	diAc-L-Lys-D-Ala-D-Ala*			diAc-L-Lys-D-Ala-D-Lac*			Benzylpenicillin†		
	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$
PBP-5‡	19.2	0.29	15	2.4	1.1	260	ND	10^{-4}–10^{-3}	ND
RTEM-1§	—	—	—	—	—	—	0.02	2×10^3	1×10^8
Chimera¶	7.7 ± 1.3	0.0012 ± 0.00017	0.16	3.3 ± 1	0.0037 ± 0.0015	1.1	0.26 ± 0.03	0.051 ± 0.005	196
Chimera (E^+)‖	ND	5.8×10^{-5}	ND	ND	ND	ND	ND	1.3	ND

ND, not determined.
*Assay conditions: 37°C, pH 7.5.
†Assay conditions: 30°C, pH 7.0.
‡Kinetic parameters for PBP-5 were taken from ref. 28. Activity with benzylpenicillin is an estimate based on comparisons between PBPs involving the rates of deacylation (29, 30).
§The lack of observable activity is designated as —.
¶Kinetic parameters were calculated from initial velocities; ± indicates SD.
‖Specific activities determined from single reaction curves; E^+, extra glutamic residue.

Both chimeras exhibit a pH profile for carboxypeptidase activity similar to that we observe for the parent RTEM β-lactamase (33) on penam substrates and, as shown in Fig. 3, very different from that of the PBP-5 wild-type enzyme. Notable also is the fact that both chimeras show no buffer dependency, whereas PBP-5 itself shows a marked decrease in activity in carbonate and phosphate buffers.

We have also prepared a number of mutants with more moderate PBP-5 substitutions (i.e., Thr-71→Leu; Thr-71→Leu; Phe-72→Thr; Thr-71→Ile; Thr-71→Ile, Phe-72→Thr; and Phe-72→Thr) to examine the effect of generating the conserved triad Ser-Xaa-Thr-Lys present in all membrane-bound D,D-carboxypeptidases (6). These mutants were generally unstable and none showed any D,D-carboxypeptidase activity; indeed at 37°C the Thr-71→Leu mutant conferred only a modest resistance to β-lactam antibiotics (35) because of the susceptibility of this mutant to proteases in the periplasm. The other mutants with PBP-5-like sequences exhibited low to moderate β-lactamase activities (Table 2). These mutations likely interfere with the normal folding of the α-2 helix of β-lactamase (35, 36), as the side chain of Thr-71 points into the hydrophobic core of the enzyme. A similar effect has been observed in the case of substitutions of Thr-157 in phage T4 lysozyme (37).

In addition to having carboxypeptidase activity, PBPs generally function as transpeptidases, in which role they can transfer the carboxyl group of the cleaved amide to an amine such as glycine (4, 34). Our chimeras do not display any such activity; no transpeptidase transfer to glycine was observed even at 100 mM glycine. This lack of transpeptidase activity may reflect the absence in the β-lactamase/PBP-5 chimera (29 kDa) of helix G (3), which is likely to be present in PBP-5 (42 kDa); this additional helix, seen at low resolution in the R61 carboxypeptidase and therefore inferred in the closely homologous PBP-5, covers the face of the binding site for β-lactam substrate and may contain a structural component, such as an amino acid or peptide binding site, necessary for transpeptidase activity. This helix may also help to exclude water from the active site, thereby encouraging transpeptidase as distinct from carboxypeptidase activity.

The evidence that the D,D-carboxypeptidase activities reported in this paper truly reside in the chimeras and do not derive from contamination with PBP-5 includes the following observations. (*i*) Proteins purified by the procedures for isolating β-lactamase and its mutants from *E. coli* containing derivatives of pBR322 have observable D,D-carboxypeptidase activity only when the two specific chimeras that form the subject of this paper are encoded in the plasmids. (*ii*) The two chimeras have markedly different levels of activities from each other, and both have pH dependencies very different from those reported for PBP-5. (*iii*) Neither chimera catalyzes a transpeptidase reaction with glycine, whereas PBP-5 does.

CONCLUSIONS

We do not know precisely the particular residues in the chimera responsible for the altered catalysis and whether catalysis results from changes in a few particular interactions between enzyme and potential substrate or from larger, more general conformational alterations. We are trying both to narrow the changes necessary to achieve carboxypeptidase activity (the chimera has substitutions at 7% of the residues of the parent enzyme) and to create variants of this chimera with increased activity as a carboxypeptidase and eventually as a transpeptidase. Accordingly, the results of this work to

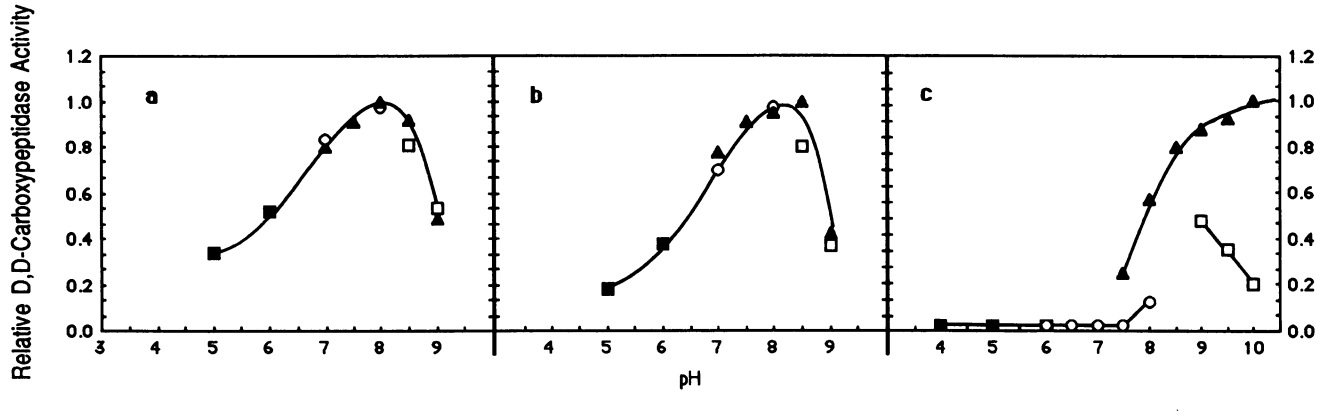

FIG. 3. pH dependency of the D,D-carboxypeptidase activity of RTEM/PBP-5 chimeras and of *E. coli* PBP-5. (*a*) RTEM/PBP-5 chimera. (*b*) E^+ chimera, which contains an additional glutamic residue inserted between residues 59 and 60 in β-lactamase numbering (see Fig. 1). (*c*) Wild-type PBP-5 (plot taken from ref. 34). ■, KOAc; ○, KH_2PO_4; ▲, Tris·HCl; □, K_2CO_3.

Table 2. Catalytic parameters for Thr-71 and Phe-72 mutants of RTEM-1 β-lactamase

Enzyme	Ampicillin			
	K_m, μM	k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$	Relative k_{cat}/K_m
Thr-71, Phe-72 (wild-type)	50	2000	4.0×10^7	1
Thr-71→Ile	50	145	2.9×10^6	7.2×10^{-2}
Thr-71→Ile, Phe-72→Thr	80	3.6	4.5×10^4	1.1×10^{-3}
Phe-72→Thr	16	338	2.1×10^7	5.2×10^{-1}

Kinetic parameters were determined from initial velocities at 30°C and pH 7.0.

date can be regarded as a significant first step toward the goal of designing proteins with altered and potentially novel catalytic activities.

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