Role of Asparagine 152 in Catalysis of β-Lactam Hydrolysis by *Escherichia coli* AmpC β-Lactamase Studied by Site-Directed Mutagenesis

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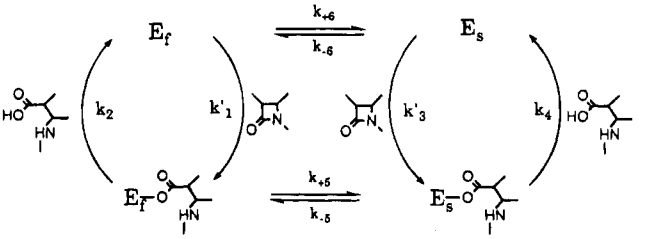
ABSTRACT: The role of asparagine 152 in the catalytic mechanism of *Escherichia coli* AmpC β-lactamase has been investigated by site-directed mutagenesis. The residue has been replaced by aspartic acid, glutamic acid, histidine, and leucine. All the substitutions had similar effects on the activity toward substrates and inhibitors. The rate of substrate hydrolysis decreased by factors of 500−5000. The rates of both acylation (2−50-fold decrease) and deacylation (50−500-fold decrease) were affected, indicating a role for Asn152 in both processes. The wild-type AmpC β-lactamase appears to exist as an equilibrium mixture of two forms, identified by their different kinetic properties. The Asn152 mutations affected the activity of the slow-reacting form much more than that of the fast-reacting form, but they did not appear to affect the interconversion of these two kinetic forms. Comparison of these observations with results obtained with mutation of the equivalent residues in other classes of penicillin-sensitive enzyme indicates that there are quite profound differences between the catalytic mechanisms of these enzymes despite a high degree of conservation of amino acids in the active center, and of the overall three-dimensional structure.

The principal mechanism of bacterial resistance to the commonly employed β-lactam antibiotics is the production of β-lactamases. The *Escherichia coli* AmpC β-lactamase is a representative of class C β-lactamases that readily inactivate cephalosporins (Ambler, 1980; Bush, 1989). These enzymes have become important in diseases caused by Gram-negative bacteria such as *E. coli* and *Citrobacter freundii*. Thus, particular emphasis has been placed on understanding the mechanism of action of these enzymes in order either to overcome or to circumvent their activity. The solution of the crystal structure of the class C β-lactamase from *C. freundii* 1203 (Oefner et al., 1990) suggested specific roles for residues in the active site, and these have been investigated by site-directed mutagenesis. In the related enzyme from *Enterobacter cloacae* 908R, serine 64, the residue that undergoes acylation, has been changed to cysteine, greatly decreasing the rate of hydrolysis (Dubus et al., 1993). The presence of a lysine group in position 67, juxtaposed to serine 64, has been shown to be essential for activity in the enzyme from *C. freundii* GN346 (Tsukamoto et al., 1990a). In the same enzyme (Tsukamoto et al., 1990b), and more recently in the *E. cloacae* 908R enzyme (Monnaie et al., 1994), lysine 315 was also shown to be important for catalytic activity. Mutation of tyrosine 150 (which lies between these two lysine residues and is within hydrogen-bonding distance of serine 64) gave very complicated results that depended on both the substitution made and the substrate being investigated (Dubus et al., 1994). In part, this complication arose from the propensity of many β-lactamases to undergo substrate-induced inactivation (Kiener et al., 1980; Carrey et al., 1984; Faraci & Pratt, 1985; Persaud et al., 1986; Fink et al., 1987; Charnas & Then, 1988; Waley, 1991). For class C β-lactamases, this behavior is most readily explained by the presence of two slowly interconverting conformations of the enzyme (Scheme 1). Upon addition of substrate, the enzyme forms will undergo parallel catalytic cycles with different kinetic parameters (Page, 1993; Dubus et al., 1994). It appeared that tyrosine 150 was involved in the isomerization of these two forms (Dubus et al., 1994).

Scheme 1: Interconversion of the Enzyme between Two Conformational States[a]



[a] The two conformations are denoted E_f (for fast reacting) and E_s (for slow reacting). The rate constant k'_1 takes the form $k_{cat}[S]/(K_M + [S])$, where S is the β-lactam substrate, and k_2 describes the breakdown of the acyl-enzyme complex. The rate constants k'_3 and k_4 describe the corresponding reactions of the E_s form.

In the crystal structure of the *C. freundii* 1203 β-lactamase, asparagine 152 was seen to lie at one end of a hydrogen-bonded network of residues connected *via* Lys67 to Ser64 and Tyr150. In addition, in the acyl-enzyme complex obtained with the very slowly hydrolyzed substrate aztre-

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[1] Abbreviations: CENTA, 7-(thienyl-2-acetamido)-3-[[(3-carboxy-4-nitrophenyl)thio]methyl]-3-cephem-4-carboxylic acid; FAP, 6-β-(furylacryloylamido)penicillanic acid; PADAC, 7-(thienyl-2-acetamido)-3-[2-[[4-(*N*,*N*-dimethylamino)phenyl]azo]pyridinium methyl]-3-cephem-4-carboxylic acid; DANS/phenylboronate, 3-[[5-(*N*,*N*-dimethylamino)-naphthalene-1-sulfonamido]-phenyl]boronate.

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onam, it was apparent that Asn152 could also form a hydrogen bond to the carbonyl group of the acyl side chain of the ligand (Oefner et al., 1990). Thus, three roles for this residue were possible: through the carbonyl oxygen of the amide function, it could be involved either in activation of Ser64 for attack (through its proximity to Lys67) or in conformational changes, and through the nitrogen of the side-chain amide, it could be involved in recognition of the substrate. We have used site-directed mutagenesis to evaluate the relative importance of these potential roles. The replacements made included those intended (i) to perturb both interactions by shifting the position of the hydrogen-bonding groups (Asn152Glu), by replacing Asn152 with an apolar residue (Asn152Leu), or by altering the ionization state (Asn152Asp) and (ii) to disrupt one of the interactions while preserving the potential for the other (Asn152Asp maintains the carbonyl oxygen; Asn152His maintains a nitrogen atom).

EXPERIMENTAL PROCEDURES

The methods used were those described in previous reports (Page, 1993; Dubus et al., 1994). Mutagenesis was performed with *Escherichia coli* SNO3 (*ampA1, ampC8, pyrB, recA, rpsL*) (Normark & Burman, 1977) using plasmid pAD7 (Dubus et al., 1994). The mutagenesis was an adaptation of the method described by Taylor et al. (1985), whereby T7 DNA polymerase (Sequenase) and T4 DNA polymerase were used for the first and second extensions, respectively. Oligonucleotides for mutagenesis were synthesized as before to allow replacement of Asn 152 by histidine, aspartate, glutamate, or leucine and were obtained from the Protein Chemistry Laboratory at the Department of Biochemistry and Molecular Biophysics, Washington University.

The wild-type and mutant proteins were purified by ion-exchange chromatography on Pharmacia S-Sepharose followed by affinity chromatography on immobilized (aminophenyl)boronate.

Kinetic measurements were performed in 0.1 M sodium phosphate at pH 7.0 and 37 °C. Steady-state kinetic parameters were determined with at least three replicates for each concentration of substrate and with two or three separate batches of enzyme. The parameters were obtained by a direct weighted fit to the Michaelis−Menten equation using the program GraFit (Leatherborrow, 1989). The pH dependence of activity was investigated using either 0.1 M sodium phosphate (pH 7.0−8.0) or 50 mM sodium acetate/50 mM Tris-HCl (pH 7.0−10.5).

RESULTS

(1) Protein Purification. The mutant proteins behaved rather differently from the wild type during purification. The first step in purification of a crude extract was chromatography over a column of Pharmacia S-Sepharose Fast Flow. The binding of the mutant proteins, except the Asn152His mutant, was markedly weaker than that of the wild type (data not shown). The second step of purification used for the wild-type protein was normally affinity chromatography over a column of Bio-Rad Affigel 15 coupled with (3-aminophenyl)boronate. The mutant proteins did not bind to this column very tightly, and the activity could be displaced by NaCl. Thus, it would appear that mutation of Asn152 caused a change in the isoelectric point of the protein (except when the replacement residue was histidine) and also decreased the affinity for boronic acids. There was no effect of the

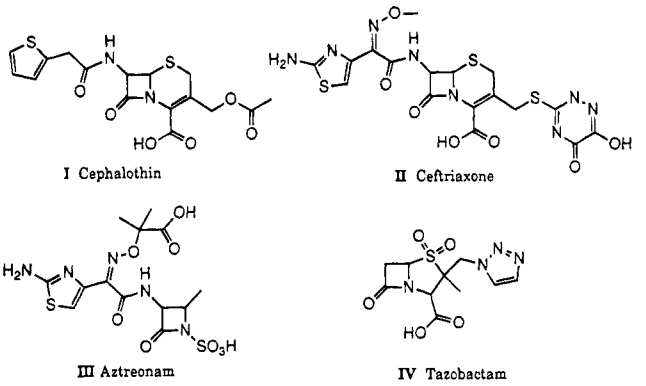

FIGURE 1: Substrates and inhibitors of AmpC β-lactamase.

mutations on the stability of the enzyme either during purification or upon storage at 4 °C.

(2) CD Spectroscopy. The wild-type and mutant proteins had identical CD spectra in the far-UV region. The contributions from aromatic side chains dominate the spectrum in the near-UV region. Here, too, the mutant proteins had very similar spectra (data not shown), indicating that the environments of the aromatic residues are rather similar. Thus there are no large-scale changes in protein structure caused by the replacements. Preliminary data from X-ray crystallographic studies suggest that there are no significant changes in the stucture of the Asn152Asp mutant protein (J. J. Daly, A. D'Arcy, C. Oefner, and F. Winkler, unpublished observations).

(3) Effects of the Mutations on the Reactions with β-Lactams. The catalytic efficiency (k_{cat}/K_M) of hydrolysis of cephalosporins with simple 7-thiopheneacetamido side chains (for example, cephalothin, Figure 1) was decreased more than 1000-fold in the Asn152 mutants, irrespective of the nature of the amino acid substitution. The major alteration in the mutants was a decrease in k_{cat}, and very little change in K_M was observed (Table 1). Similar decreases in the relative rate of hydrolysis were observed with several rapidly hydrolyzed cephalosporins and penicillins (supplementary material).

The pH dependence of cephalothin hydrolysis by the wild-type protein in the range pH 7−11 principally reflected two ionizations (Figure 2). One (pK 8.9−9.3) changed k_{cat} from a high value at neutral pH to a lower one at alkaline pH, and the other (pK 7.8−8.1) caused a change in K_M from lower affinity at neutral pH to higher affinity at alkaline pH (Figure 2). The latter effect was particularly marked in phosphate buffer (Dubus et al., 1994). These effects can be explained by a model with three protonation states [Scheme 2; see Dubus et al. (1994) for further discussion].

The pH dependence of the activity of the Asn152 mutants was much less marked than that of the wild-type protein, but it showed a trend toward decreased k_{cat} at alkaline pH (Figure 2). The pH profile was similar for all four mutants, despite the presence of different ionizable groups. The pK for this decrease is similar to that observed in the pH dependence of the activity of the wild type, although the difference in k_{cat} values between the EH_2 and EH states is much less marked in the mutants (Figure 2). None of the mutants showed evidence of the ionization affecting K_M, even in phosphate buffer. Thus, it would appear that Asn152 controls the transition between EH_2 and EH states (Scheme 2) and is involved in the high catalytic activity of the enzyme in these two states.

Table 1: Steady-State Parameters for Hydrolysis of 7-Thiophenacetamidyl Cephalosporins

substrate	protein	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1}/nM)
cephalothin	wild type	430 ± 12	48 ± 2.3	8950 ± 610
	Asn152Asp	0.12 ± 0.03	24 ± 1.9	5 ± 1.2
	Asn152Glu	0.022 ± 0.006	37 ± 3.3	0.59 ± 0.03
	Asn152His	0.013 ± 0.005	41 ± 12	0.31 ± 0.01
	Asn152Leu	0.34 ± 0.05	54 ± 13	6.2 ± 1.8
nitrocefin	wild type[a]	420 ± 60	380 ± 42	1780 ± 83
		25 ± 0.9	6.5 ± 0.6	15700 ± 3500
	Asn152Asp	0.12 ± 0.04	240 ± 53	0.50 ± 0.20
	Asn152Glu	0.11 ± 0.03	130 ± 32	0.85 ± 0.19
	Asn152His	0.042 ± 0.005	110 ± 33	0.38 ± 0.09
	Asn152Leu	0.20 ± 0.06	410 ± 87	0.49 ± 0.15
cephaloridine	wild type	180 ± 8	210 ± 9	860 ± 40
	Asn152Asp	0.27 ± 0.02	160 ± 8	1.7 ± 0.2
	Asn152Glu	0.11 ± 0.02	90 ± 10	0.50 ± 0.1
	Asn152His	0.041 ± 0.01	70 ± 8	0.59 ± 0.11
	Asn152Leu	0.34 ± 0.05	280 ± 22	1.2 ± 0.3
PADAC	wild type	835 ± 64	310 ± 24	2690 ± 430
	Asn152Asp	1.6 ± 0.2	250 ± 35	6.3 ± 0.5
	Asn152Glu	0.30 ± 0.09	120 ± 31	2.5 ± 0.3
	Asn152His	0.13 ± 0.2	80 ± 11	1.6 ± 0.4
	Asn152Leu	1.75 ± 0.43	470 ± 57	3.7 ± 0.23
CENTA	wild type[a]	1312 ± 25	833 ± 53	1.55 ± 0.027
		293 ± 15	49 ± 5	5.98 ± 0.1
	Asn152Asp	0.37 ± 0.23	560 ± 49	0.66 ± 0.21
	Asn152Glu	0.067 ± 0.019	130 ± 30	0.51 ± 0.15
	Asn152His	0.039 ± 0.007	60 ± 13	0.67 ± 0.18
	Asn152Leu	1.02 ± 0.09	890 ± 87	1.1 ± 0.2

[a]Biphasic Eadie–Hofstee plots. The two sets of kinetic parameters correspond to intercept and slope of asymptotes to the curve at high and low substrate concentrations, respectively.

The slowly reacting β-lactams with 7-β-aminothiazoyl-alkoxyimino-acetamido side chains (such as ceftriaxone and aztreonam, Figure 1) offer the opportunity to dissect the catalytic cycle. As with the fast-reacting cephalosporins, the turnover was greatly decreased (Table 2). However, the situation was more complex, as it appeared that only one form of the enzyme could react with these β-lactams. For example, approximately half of the nitrocefin hydrolysis activity could be inhibited by competition with ceftriaxone (Figure 3). Consistent with these observations was the finding that, in acylation experiments, only part of the enzyme was trapped as an acyl-enzyme complex and approximately half of the activity was not inhibited, except at very high concentrations of ceftriaxone (Figure 3). The rate of decay of the acyl-enzyme complex formed with the Asn152 mutants was considerably slower than that observed with the wild-type protein (Table 3).

Penam sulfones undergo a rather complex sequence of reactions with β-lactamases (Kemal & Knowles, 1981; Bush et al., 1993). Nevertheless, they are an interesting class of compound, as there is no acylamino side chain and, thus, they can be used to examine only the interactions taking place immediately around the catalytic center. In the reaction of the wild type with tazobactam, the accumulation of the acyl-enzyme was rather slow compared to rates obtained with cephalosporins (Table 3). The decrease in activity was monophasic (Figure 4), suggesting that tazobactam reacts at about the same rate with both forms of the enzyme or that the rate of isomerization of the protein is faster than the rate of reaction with the reagent (Rakitzis, 1984). The Asn152 mutants had a lower initial rate of reaction, and the reaction was clearly biphasic (Figure 4), such that half of the enzyme

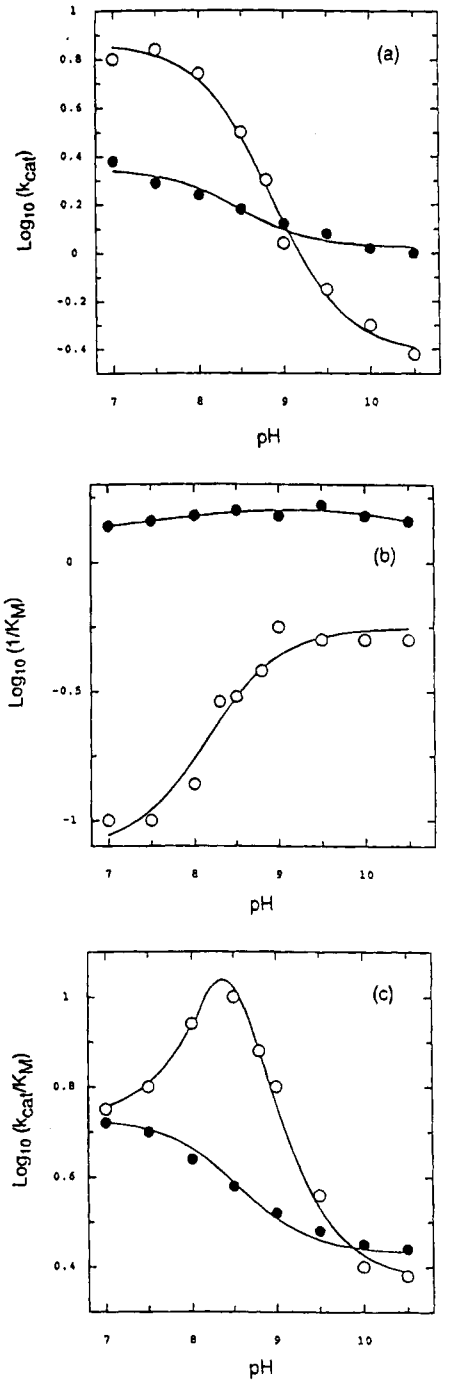

FIGURE 2: pH dependence of the kinetic parameters for cephalothin hydrolysis. (a) pH dependence of k_{cat}. Open circles represent log k_{cat} for wild-type enzyme, and closed circles represent 2 + log k_{cat} for the Asn152Leu mutant. (b) pH dependence of K_M. Open and closed circles represent log ($1/K_M$) for wild-type and Asn152Leu mutant, respectively. (c) pH dependence of k_{cat}/K_M. Open circles represent log k_{cat}/K_M for wild-type enzyme, and closed circles represent 2 + log k_{cat}/K_M for the Asn152Leu mutant. In (a) the curves were calculated using a two-state, single-pK model with the following parameters. Wild type: $k_{cat}(EH_2/EH)$ = 73.8 ± 5.1 s^{-1}; pK_E = 8.87 ± 0.18; $k_{cat}(E)$ = 3.8 ± 0.8 s^{-1}. Mutant: $k_{cat}(EH_2/EH)$ = 0.22 ± s^{-1}; pK_E = 8.46 ± 0.19; $k_{cat}(E)$ = 0.011 ± 0.004 s^{-1}. In (b) the curve for the wild-type protein was calculated using a two-state, single-pK model with the following parameters: $K_M(EH_2)$ = 12.9 ± 2.1 μM; pK_{EH} = 8.16 ± 0.14; $K_M(EH)$ = 1.78 ± 0.53 μM. In (c) the curves were calculated for the wild-type using a three-state, dual-pK model with the following parameters: $k_{cat}/K_M(EH_2)$ = 53 ± 4 μM^{-1} s^{-1}; pK_{EH} = 7.79 ± 0.12; $k_{cat}/K_M(EH)$ = 61.7 ± 6.0 μM^{-1} s^{-1}; pK_E = 8.94 ± 0.09; $k_{cat}/K_M(E)$ = 5.6 ± 1.2 μM^{-1} s^{-1}. The curve for the mutant was calculated using a two-state model with the following parameters: $k_{cat}/K_M(EH_2/EH)$ = 0.54 ± 0.11 μM^{-1} s^{-1}; pK_E = 8.55 ± 0.07; $k_{cat}/K_M(E)$ = 0.26 ± 0.07 μM^{-1} s^{-1}.

Scheme 2: pH Dependence of Enzyme Activity

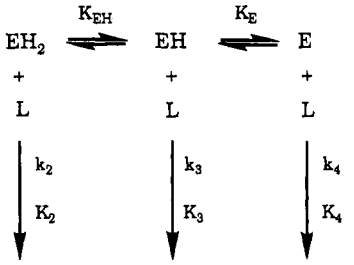

Table 2: Steady-State Kinetic Parameters for Slowly Hydrolyzed β-Lactams

		burst phase[a]			steady-state phase[a]		
substrate	enzyme	k_{cat} (s^{-1})	K_M (μM)	K_I[b] (μM)	k_{cat} (s^{-1})	K_M (μM)	K_I[b] (μM)
cefbuperazone	wild type	0.3	0.10	0.098	0.16	200	>500
	Asn152Asp	0.000 12	0.05	0.02	ND[c]	ND	>1000
	Asn152Glu			0.05			>1000
	Asn152His			0.06			>1000
	Asn152Leu	0.000 47	0.11	0.089	ND	ND	>1000
ceftriaxone	wild type	0.35	0.1	0.296	ND	ND	ND
	Asn152Asp	0.0002	0.05	0.04	ND	ND	>1000
	Asn152Glu			0.12			>1000
	Asn152His			0.06			>1000
	Asn152Leu	0.000 15	0.14	0.24	ND	ND	>1000
ceftazidime	wild type	0.05	10	35	ND	ND	ND
	Asn152Asp	0.000 01	22	10	ND	ND	>1000
	Asn152Glu	0.000 03	34	45	ND	ND	>1000
	Asn152His			34			>1000
	Asn152Leu	0.000 09	12	23	ND	ND	>1000
cefmenoxime	wild type	0.07	70	1.7	ND	ND	ND
	Asn152Asp	0.000 01	2	0.9	ND	ND	>1000
	Asn152Glu			0.5			>1000
	Asn152His			0.8			>1000
	Asn152Leu			1.9	ND	ND	>1000
aztreonam	wild type			0.011			ND
	Asn152Asp			3.0			>1000
	Asn152Glu			4.7			>1000
	Asn152His			2.1			>1000
	Asn152Leu			2.9			>1000

[a] Some slowly hydrolyzed substrates exhibit a nonstoichiometric burst of hydrolysis, followed by a steady-state phase (Page, 1993). The kinetic parameters are for the initial reaction (burst) and the slower second phase (steady-state). [b] Competition with nitrocefin. [c] ND = not detected.

reacted considerably more slowly (Table 3). The acyl intermediate formed with the wild-type enzyme was not very stable, and a saturating level of acylation is only obtained with a more than 100-fold excess of reagent. In contrast, the acyl complex obtained with the Asn152 mutants is quite stable, saturation being obtained with a 2-fold excess of reagent (Figure 4). Direct comparison of deacylation rates is complicated by the rearrangements of inhibitor and protein that occur during multiple turnovers of the wild-type protein. In the experiments shown in Figure 4, where only a slight excess of reagent was used, the rate of deacylation of the wild-type enzyme must be about 500 times the rate of deacylation from the Asn152Asp mutant. The spectral changes occurring during the reaction with the wild-type protein indicated the rapid formation of species with absorption maxima at 235 and 280 nm and a slower accumulation of a species with an absorption maximum at 295 nm (Figure 5). The changes at 235 nm have been associated with deacylation and formation of decomposition products in solution, while the changes at longer wavelength have been associated with opening of the thiazolidine ring and formation

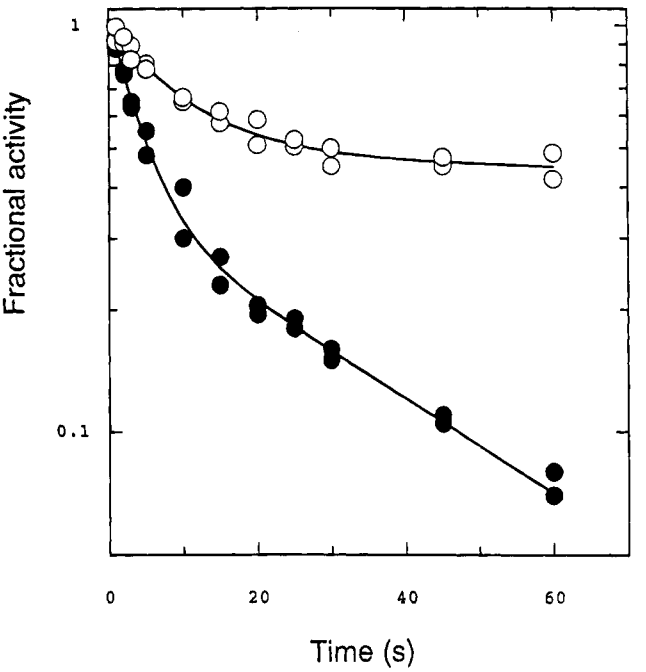

FIGURE 3: Acylation of wild-type and Asn152Asp mutant β-lactamases by ceftriaxone. The extents of acylation of (●) wild-type and (○) Asn152Asp enzymes were monitored as loss of activity toward nitrocefin, as described in the text. The ceftriaxone concentration was 2.5 μM. The data were fitted to a double-exponential decay of activity: $A_t = A_1(e^{-k1t}) + A_2(e^{-k2t})$. The observed values of the parameters were as follows. Wild type: $A_1 = 0.69 \pm 0.04$; $A_2 = 0.35 \pm 0.05$; $k_1 = 0.24 \pm 0.03$ s^{-1}; $k_2 = 0.027 \pm 0.005$ s^{-1}. Asn152Asp mutant: $A_1 = 0.53 \pm 0.06$; $A_2 = 0.48 \pm 0.07$; $k_1 = 0.10 \pm 0.02$ s^{-1}; $k_2 = 0.0011 \pm 0.0028$ s^{-1}.

of an aminoacryloyl intermediate both in solution and in the enzyme catalytic center (Kemal & Knowles, 1981; Brenner & Knowles, 1981; Bush et al., 1993). During the reaction with the Asn152 mutants there was no evidence for the formation of species with absorption maxima at 235 and 280 nm, and only a very slow increase at 292 nm (Figure 5). This suggests that the changes in A_{235} and A_{280} were due to reactions after deacylation and that the change in A_{292} is attributable to the acyl-enzyme. Thus, it seems that the acyl intermediate was formed more slowly with the mutant proteins and that it was longer-lived than that formed with the wild-type enzyme.

Overall, from the reaction with slow-reacting substrates and β-lactam inhibitors, it appears that Asn152 is not involved in the isomerization between the fast and slow forms of the enzyme (Scheme 1) but that it has a role in substrate recognition by the slow form of the enzyme. This residue is involved in formation of the acyl intermediate and in its destabilization toward attack by water in both forms of the protein.

(4) Effects of the Mutations on Reactions with Inhibitors. Boronic acid derivatives are reversible inhibitors of enzymes with activated serine residues in the catalytic center (Koehler & Lienhard, 1971; Lindquist & Terry, 1974). In the crystal structure of subtilisin complexed with phenylboronic acid, the boronate exists as a tetrahedral adduct with the active serine, analogous to the transition state thought to be formed during peptide bond formation (Matthews et al., 1975). The reactions of boronic acids with both serine proteases and β-lactamases shows the kinetic properties of slow binding inhibition (Kettner & Shenvi, 1984; Kiener & Waley, 1978). The reaction with boronates is the basis for affinity chro-

Table 3: Kinetic Parameters Describing the Formation of the Acyl-Enzyme Complex

		acylation (fast phase)			acylation (slow phase)			
substrate	enzyme	k_{on} ($\mu M^{-1} s^{-1}$)	K_S (μM)	amplitude	k_{on} ($\mu M^{-1} s^{-1}$)	K_S (μM)	amplitude	deacylation k_{off} (s^{-1})
ceftriaxone	wild type	6.6 ± 0.9	14.7 ± 15	0.51 ± 0.05	1.8 ± 0.03	28 ± 6	0.49 ± 0.06	$(5.0 \pm 0.2) \times 10^{-3}$
	Asn152Asp	1.3 ± 0.3	25.2 ± 5.3	0.46 ± 0.06	>0.01	>1000	0.54 ± 0.06	$(6.1 \pm 0.5) \times 10^{-6}$
	Asn152His	0.6 ± 0.1	11.3 ± 2.1	0.48 ± 0.04	>0.01	>1000	0.52 ± 0.05	$(1.2 \pm 0.4) \times 10^{-5}$
	Asn152Leu	0.8 ± 0.2	13.9 ± 3.1	0.47 ± 0.05	>0.01	>1000	0.53 ± 0.04	$(8.3 \pm 0.7) \times 10^{-6}$
aztreonam	wild type	2.8 ± 0.5	21 ± 4	0.45 ± 0.08	0.58 ± 0.05	105 ± 23	0.54 ± 0.1	$(5.5 \pm 0.8) \times 10^{-5}$
	Asn152Asp	0.8 ± 0.3	320 ± 56	0.49 ± 0.08	>0.01	>1000	0.51 ± 0.05	$(8.3 \pm 0.9) \times 10^{-7}$
	Asn152His	0.7 ± 0.2	280 ± 74	0.48 ± 0.04	>0.01	>1000	0.52 ± 0.05	$(6.7 \pm 0.5) \times 10^{-7}$
	Asn152Leu	0.3 ± 0.1	65 ± 15	0.45 ± 0.03	>0.01	>1000	0.55 ± 0.04	$(1.1 \pm 0.2) \times 10^{-6}$
tazobactam	wild type	0.080 ± 0.01	325 ± 35	1.0	ND	ND	0	<0.2[a] [$(9.6 \pm 1.1) \times 10^{-5}$][b]
	Asn152Asp	0.0019 ± 0.0005	115 ± 25	0.50 ± 0.04	$(4.5 \pm 0.3) \times 10^{-4}$	567 ± 29	0.50	$(3.9 \pm 0.8) \times 10^{-4}$[a]
	Asn152Leu	0.0012 ± 0.0003	85 ± 11	0.56 ± 0.05	$(6.1 \pm 0.8) \times 10^{-4}$	493 ± 54	0.44	$(2.5 \pm 0.6) \times 10^{-4}$[a]

[a] After a single turnover. [b] After >10 turnovers.

matography widely used to isolate β-lactamases, including the wild-type *E. coli* β-lactamase (Cartwright & Waley, 1984). DANSphenylboronate inhibited the wild-type enzyme in a slow, progressive manner, resulting in complete inhibition (Figure 6). The reaction with the mutant proteins showed simple, time-independent competition, but only partial inhibition was obtained. The fluorescence of the DANS moiety was enhanced when it was bound to the wild-type protein (supplementary material), presumably because it had entered the more hydrophobic environment provided by the substrate recognition site of the β-lactamase (Oefner et al., 1990). The fluorescence of the ligand bound to Asn152Asp or Asn152His was similar, but the emission was about 60% of that of the ligand bound to the same concentration of wild-type protein. Taken together, the results suggest that, while non-covalent binding to one form of the enzyme was not greatly affected by the mutations, the rearrangement leading to the more stable covalent modification of the protein occurred less rapidly in the mutant proteins and that a population of the enzyme did not bind the boronates readily.

Isatoic anhydride has been reported to be an inhibitor of active serine enzymes (Moorman & Abeles, 1982) including class C β-lactamases (Page, 1993). The reaction, which proceeds through an acyl intermediate, is very sensitive to the distribution of the enzyme between the different reactive states (Page, 1993). The reaction of the wild-type enzyme followed a biphasic exponential approach to a very slow steady-state rate of hydrolysis (Figure 7). In contrast, the exponential reaction with Asn152 mutants was monophasic, had a smaller amplitude (approximately 0.5 mol per mole of protein instead of 1.0 mol per mole of protein), and was somewhat slower than the fast phase of reaction of the wild-type protein. There was no significant subsequent reaction, suggesting that only one conformation of the mutant enzyme had appreciable reactivity toward this reagent and that it formed a more stable acyl-enzyme complex than the wild type did.

DISCUSSION

Inspection of the crystal structure of the complex between *C. freundii* AmpC β-lactamase and the slowly hydrolyzed substrate aztreonam (Oefner et al., 1990) suggested several possible roles for Asn152. These include substrate recognition and, through interaction with Lys67, activation of Ser64 for attack on the β-lactam ring.

The lack of effect of the substitutions on apparent affinity might be taken as an indication that the hydrogen bond between the amino group of Asn152 and aztreonam does not contribute largely to substrate recognition. This simple interpretation is somewhat complicated by the observation that, with slow-reacting substrates and the two non-β-lactam inhibitors, only one form of the enzyme is very reactive. The very much decreased reactivity of the second form is due to changes in both the rate of reaction and the apparent affinity, and thus it could be that Asn152 plays a role in binding to this form of the protein. This would imply that the crystal structure had been obtained from the slow-reacting form of the enzyme.

There was a marked effect on the reactivity of the active serine in both conformations of the enzyme, suggesting a role for Asn152 in its activation. The most obvious possibilities are an electrostatic interaction, inducing a shift in the pK_a of Lys67, or a structural function, maintaining the proper alignment of Lys67. It was somewhat surprising that the magnitude of the effect was not very dependent on the nature of the substitution made: for example, the change from asparagine to aspartate, which should maintain (or even strengthen) an electrostatic interaction, had as great an effect on the activity as did the change from asparagine to leucine, which would not support the same interaction. There was an even greater effect of the mutation on the stability of the acyl intermediate, which was much enhanced by all of the mutations. Again the effect was largely independent of the nature of the substituent replacing Asn152. The reasons for this are not yet fully understood, and this point is still under investigation.

Although Asn152 was clearly important in different ways to the activity of the two kinetic forms of the protein, its mutation did not appear to affect the interconversion between the two forms. For example, no indication of either a change in rate of interconversion between the two forms or the position of the equilibrium between the two forms could be detected. This is in contrast to observations made after mutagenesis of Tyr150, which clearly affected the isomerization reaction (Dubus et al., 1994). Thus it would appear that Asn152 is not directly involved in the isomerization reaction.

All of the residues that form the hydrogen-bonded network surrounding serine 64 (Oefner et al., 1990) have now been mutated in at least one of the class C β-lactamase (Tsukamoto et al., 1990a,b; Dubus et al., 1993, 1994; Monnaie et al., 1994). These studies have probably produced more questions

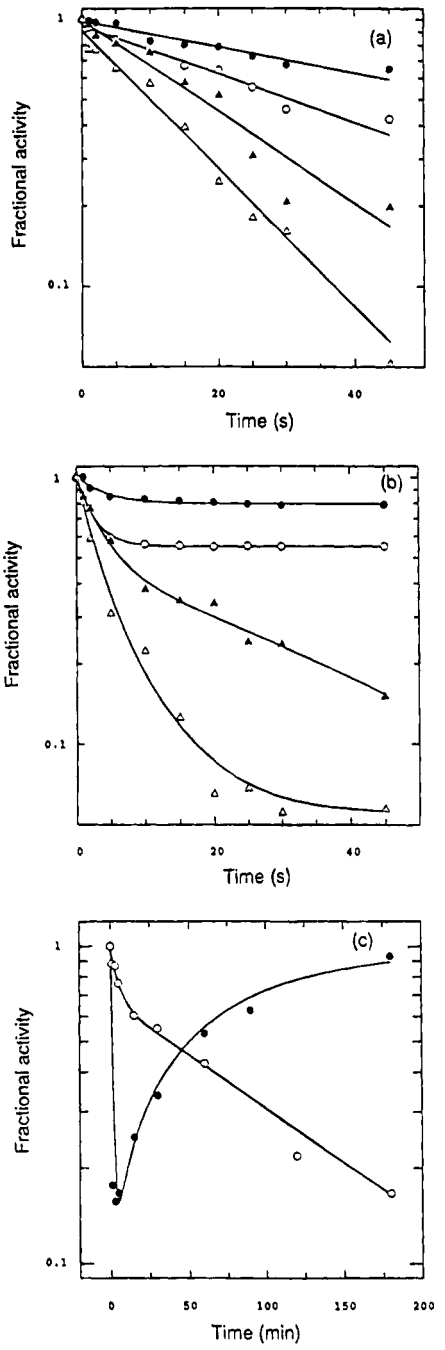

FIGURE 4: Time course of the formation of the inhibited species of the enzyme during the reaction with tazobactam. (a) Wild-type protein (1 μM) reacting with the following concentrations of tazobactam: (●) 2.5, (○) 5, (▲) 10, and (△) 20mM. (b) Asn152Asp mutant (2 mM) reacting with the following concentrations of tazobactam: (●) 0.5, (○) 1, (▲) 1.5, and (△) 2 mM. (c) Recovery of activity after reaction with tazobactam. The reaction conditions were adjusted to give approximately 80% inhibition of activity. Thus, the protein concentrations were 1 and 200 μM, and the tazobactam concentrations were 25 and 250 μM, for the wild-type enzyme and the Asn152Asp mutant protein, respectively.

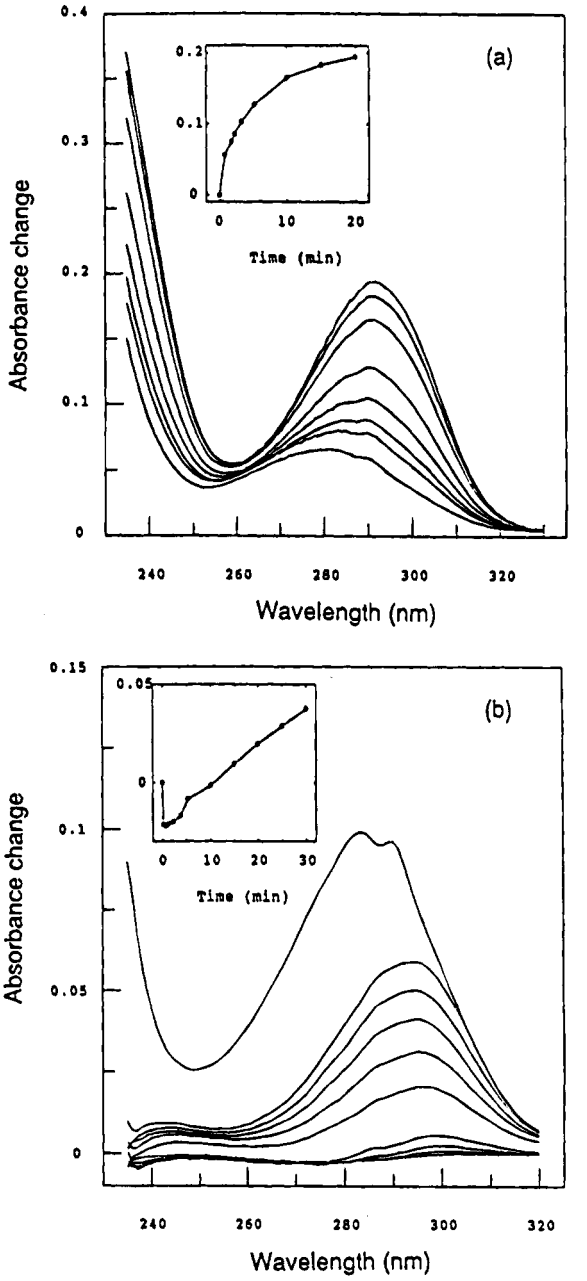

FIGURE 5: Spectral changes occurring during the reaction with tazobactam. (a) Wild-type protein (1 μM) reacting with 100 μM tazobactam in 0.1 M sodium phosphate buffer, pH 7.0. Inset: Plot of the absorbance change at 294 nm occurring during the reaction. (b) Reaction of the Asn152Asp mutant protein (10 μM) under the same conditions.

about the mechanism of β-lactamases than they have furnished answers, and a convincing, detailed mechanism for the β-lactamases remains to be formulated. The possibility of a single residue functioning as a general base [for example, Tyr 150 (Oefner et al., 1990) or Lys 67 (Strynadka et al., 1992)] now seems unlikely for class C β-lactamases (Dubus et al., 1994; Monnaie et al., 1994). Elucidation of the roles the individual members of the active site network will require more information about the effect of the mutations on invidual rate constants, about the structures of intermediates of the reaction, and about the pK_a values of the ionizable residues in the active site of the native enzyme.

X-ray crystallography of several class A β-lactamases (Dideberg et al., 1987; Moews et al., 1990; Herzberg, 1991; Strynadka et al., 1992; Jelsch et al., 1993), *Streptomyces* R61 D-peptidase (Kelly et al., 1989), and two class C β-lactamases (Oefner et al., 1990; Lobkovsky et al., 1993) indicates that an asparagine residue, highly conserved in the sequences of this family of enzymes (Joris et al., 1991), is also positioned in very similar positions in each type of enzyme. However, the role that it plays in each class appears to be rather different. With the R61 peptidase, mutation of the corresponding asparagine residue (Asn161) produced results that were dependent on the nature of the substitution (Wilkin et al., 1993). In general, acylation rates were not greatly

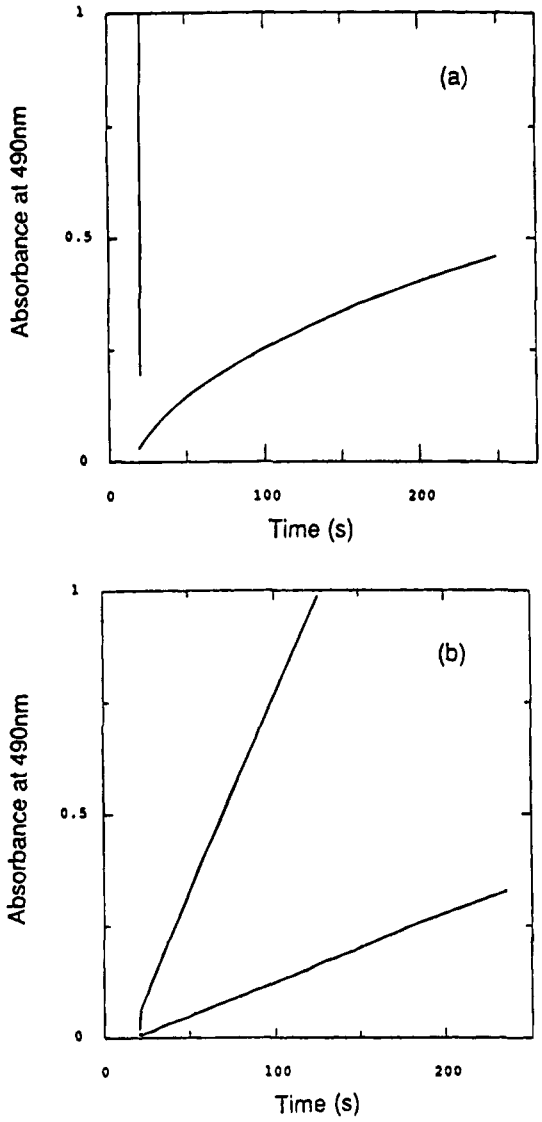

FIGURE 6: Competition between DANSphenylboronate and nitrocefin. (a) The upper trace represents the absorbance change at 490 nm occurring when the wild-type protein (0.05 μM) is mixed with 200 μM nitrocefin in 0.1 M sodium phosphate buffer, pH 7.0. The lower trace is the same reaction except that the enzyme was added to a mixture of 200 μM nitrocefin and 50 μM DANSphenylboronate. (b) The two traces represent the same experiment performed with the Asn152Asp mutant (1.75 μM).

affected, while deacylation rates decreased with one mutant and not another. The pH activity profile revealed a large effect on k_{cat} but not on K_M. The largest effects were a 200–400-fold decrease in activity, compared to the up to 2000-fold decreases in hydrolytic activity of the class C β-lactamase mutants. No indication of the presence of an unreactive conformation equivalent to that reported here for the class C β-lactamase was observed. Mutation of the equivalent asparagine residue of the *Streptomyces albus* G class A β-lactamase (Asn132) seemed to cause a change in substrate specificity. The mutants apparently lost the ability to hydrolyze penicillins while they retained the ability to hydrolyze cephalosporins (Jacob et al., 1990a,b). This is unlike any effect seen with the mutants of the class C β-lactamase. Thus, Asn152 has a very crucial role in the activity of class C β-lactamases that is not the same as the role of the homologous residues in related classes of penicillin-sensitive enzymes.

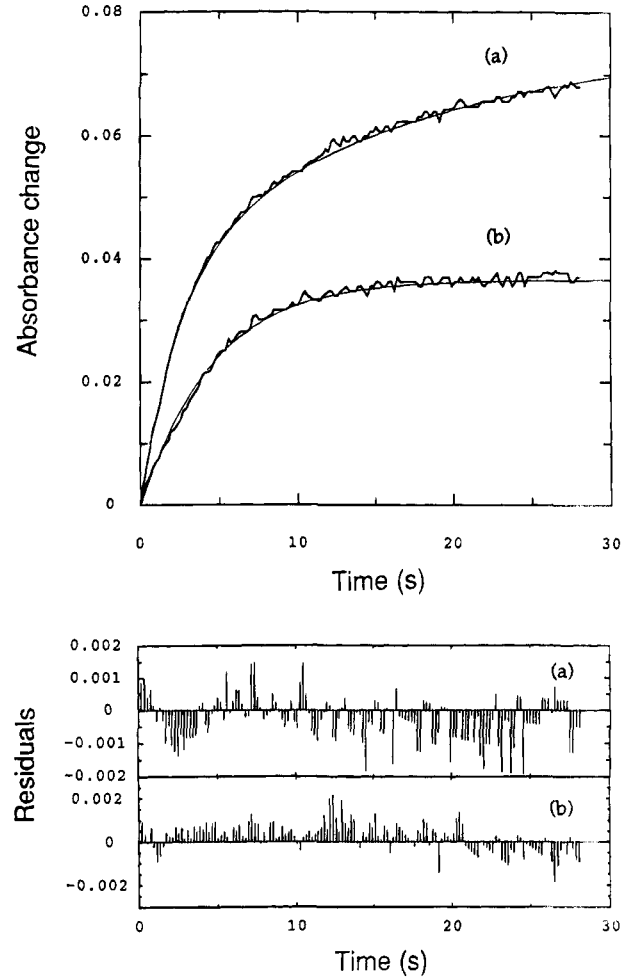

FIGURE 7: The change in A_{340} and residuals occurring during the reaction of (a) wild-type and (b) Asn152Asp mutant proteins with isatoic anhydride. The reactions were carried out with 0.5 μM protein and 100 μM isatoic anhydride in $NaHCO_3$ at pH 8.0. The smooth curves were calculated using parameters determined by fitting the data to a single-exponential increase in A_{340} for the Asn152 mutant [$\Delta A = A_0(1 - e^{-\alpha t})$, where $A_0 = 0.037 \pm 0.0001$ and $\alpha = 0.210 \pm 0.002$ s^{-1}] and, for the wild type, to a double-exponential approach to a steady-state rate of absorbance change [$\Delta A = A_0 t + A_1(1 - e^{-\beta t}) + A_2(1 - e^{-\gamma t})$, where $A_0 = (1.3 \pm 0.34) \times 10^{-5}$, $A_1 = 0.0383 \pm 0.0016$, $A_2 = 0.0381 \pm 0.0048$, $\beta = 0.39 \pm 0.014$ s^{-1}, and $\gamma = 0.056 \pm 0.011$ s^{-1}.

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SUPPLEMENTARY MATERIAL AVAILABLE

One table of relative rates of hydrolysis observed with wild-type and mutant proteins, plots of competition between nitrocefin and ceftriaxone, and a spectrum showing fluorescence changes associated with binding of DNSphenylboronate to wild-type and mutant proteins (5 pages). Ordering information is given on any current masthead page.

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