Inactivation of the *Enterobacter cloacae* P99 β-Lactamase by a Fluorescent Phosphonate: Direct Detection of Ligand Binding at the Second Site†

Marek Dryjanski and R. F. Pratt*

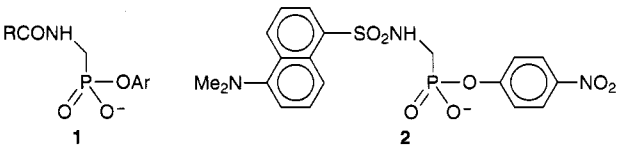
Department of Chemistry, Wesleyan University, Middletown, Connecticut 06459

Received May 31, 1994; Revised Manuscript Received December 7, 1994®

ABSTRACT: The synthesis of a fluorescent β-lactamase inhibitor, *p*-nitrophenyl [(dansylamido)methyl]-phosphonate is described. The compound inactivated the class C β-lactamase of *Enterobacter cloacae* P99 with stoichiometric release of *p*-nitrophenol, presumably, as with other phosphonate inhibitors, by phosphonylation of the active site serine. The inhibited enzyme exhibited typical dansyl fluorescence emission at 533 nm with excitation maxima at 345 and 283 nm; the latter excitation peak probably arises from radiationless energy transfer to the dansyl group from aromatic chromophores on the protein—inspection of the crystal structure shows that the closest are tyrosines. The fluorescence of the *p*-nitrophenyl phosphonate and the inhibited enzyme varied with pH in a very similar fashion, reflecting dissociation of the dimethylammonium ion in the ground state at low pH and of the sulfonamide in the excited state above pH 6. No perturbation of the fluorescence of the inhibited enzyme due to active site functional groups was observed. This may reflect the distance between the dansyl fluorophore and the phosphonyl group and/or the high pK_a's of the protonated active site functional groups in the presence of the phosphonate. The addition of certain small molecular weight *N*-acyl amino acids, of preferred structure D-RCONHCHR′CO_2^-, to the inhibited enzyme led to an enhancement of dansyl fluorescence intensity and a blue shift in the emission maximum. This suggested that these molecules bind to the β-lactamase at a site other than the active site and supports previous kinetic data to this effect [Dryjanski, M., & Pratt, R. F., (1995) *Biochemistry 34*, preceding paper in this issue]. Fluorescence titrations allowed the determination of dissociation constants for these ligands from this second site. The existence of the second site may well relate to the evolutionary relationship of β-lactamases to bacterial DD-peptidases, the targets of β-lactam antibiotics.

Class C β-lactamases are a significant factor in the resistance of Gram-negative bacteria to β-lactam antibiotics (Moellering, 1993; Ehrhardt & Sanders, 1993). They differ from typical class A β-lactamases in having higher turnover numbers for cephalosporins (Galleni & Frére, 1988; Galleni et al., 1988) and for acyclic substrates (Govardhan & Pratt, 1987; Murphy & Pratt, 1991). A detailed investigation of the kinetics of turnover of acyclic substrates by a typical class C β-lactamase, that of *Enterobacter cloacae* P99 (Pazhanisamy et al., 1989; Pazhanisamy & Pratt, 1989b), revealed the likely existence of a binding site or of binding sites on the enzyme surface for small peptide-like molecules other than the site involved in substrate turnover. It was suggested that this additional binding might relate to the presumed extended binding site of the evolutionarily ancestral DD-peptidases whose substrates are cell wall polymers. The kinetic method was subsequently employed to examine the binding of β-lactams and penicilloates at the alternative binding site(s) of the P99 β-lactamase (Dryjanski & Pratt, 1995). These studies were complicated however by the binding of substrates at the alternative site and of the test ligand at the active site. Further, the coupling between the active site and the other site(s) was small (Pazhanisamy et al., 1989b) such that it was possible to envisage effective binding at the latter site with little effect on catalysis. The present article describes our attempts to establish a simpler and faster method of determining whether a test ligand binds at an alternative site and of obtaining the dissociation constants of any such complexes.

Phosphonate monoester monoanions of general structure **1** have been established as specific inhibitors of serine



1 **2**

β-lactamases and in particular of the class C β-lactamase of *E. cloacae* P99 (Pratt, 1989; Rahil & Pratt, 1992). The inhibition is covalent and effectively irreversible and involves phosphonylation of the active site serine hydroxyl group (Rahil & Pratt, 1992; Chen et al., 1993; Lobkovsky et al., 1994). In the inert complex, the amido side chain is bound at the active site in the same way as thought to occur with the side chains of substrates during turnover. Thus, the amidophosphonyl group of **1** effectively blocks the active site (site 1) to other ligands. The binding of such ligands to another site (site 2), however, could perhaps be detected by an appropriate probe on the side chain (R) of **1**. To this end, we describe here the synthesis of the fluorescent dansylphosphonate **2**, its inhibition of the *E. cloacae* P99 β-lactamase, and its ability to detect the binding of other molecules to the enzyme through their perturbation of its fluorescence emission. The structural specificity of such binding has been explored. The pH dependence of the

† This research was supported by the National Institutes of Health Grant AI-17986 to R.F.P.

® Abstract published in *Advance ACS Abstracts,* February 15, 1995.

0006-2960/95/0434-3569$09.00/0

fluorescence of the probe has interesting features and is also described.

EXPERIMENTAL PROCEDURES

Materials. *N*-(Phenylacetyl)glycyl-D-phenylalanine, *N*-(phenylacetyl)glycyl-D-alanine, *N*-(phenylacetyl)glycine, *N*-(benzyloxycarbonyl)aminomethylphosphonic acid, and *m*-carboxyphenyl phenaceturate were available from previous work in this laboratory. Disodium penicilloate and monosodium methyl penicilloate were obtained as described in the preceding paper (Dryjanski & Pratt, 1995). *N*-Phenylacetyl derivatives of D-phenylalanine, D-norleucine, D-α-aminoadipic acid, and D-lysine were prepared from the amino acid and phenylacetyl chloride (Govardhan & Pratt, 1987). *N*-(Phenylacetyl)glycyl-D-*p*-iodophenylalanine was prepared as described for the deiodo analog (Govardhan & Pratt, 1987). *N,O*-Diacetyl-D-phenylalaninol was prepared from acetylation of D-phenylalaninol (Sigma) in pyridine by acetic anhydride. *N*-Benzyloxycarbonyl-D-glutamic acid and *N,N'*-diacetyl-L-lysyl-D-alanyl-D-alanine were purchased from Bachem Bioscience Inc., and *N*-acetyl-DL-phenylalanylglycine and D-phenyllactic acid were from Research Plus Inc. and Fluka Chemical Corp., respectively. The remaining ligands were commercial products of Sigma or Aldrich Chemical Companies.

Synthesis of p-Nitrophenyl [(Dansylamido)methyl]phosphonate (2). [(Dansylamido)methyl]phosphonic acid was prepared from reaction of aminomethylphosphonic acid (Lancaster Synthesis Inc.) with dansyl chloride (Sigma Chemical Co.) in aqueous sodium bicarbonate. The condensation of the phosphonic acid with *p*-nitrophenol was mediated by thionyl chloride. Thus, a solution of the phosphonic acid (50 mg), dimethylformamide (1 equiv), and thionyl chloride (0.5 mL) was stirred at room temperature for 1 h. The volatiles were removed by rotary evaporation, chased with methylene chloride, and then put through 3 h of high vacuum pumping. The residue, the phosphonyl dichloride, was dissolved in the minimum volume of dimethylformamide and stirred in an ice bath to 0 °C. A solution of *p*-nitrophenol (1 equiv) in dimethylformamide (1 mL) containing triethylamine (1.1 equiv) was then added dropwise, and the mixture was stirred to room temperature and then at 40 °C for 5 h. At that stage, 1 M sodium hydroxide solution (0.43 mL, 3 equiv) was added with stirring. The mixture was evaporated to dryness by rotary evaporation followed by lyophilization from water. The product (**2**) was purified (final yield of the sodium salt, 7%) by Sephadex QAE and Sephadex G-10 chromatography as previously described (Rahil & Pratt, 1991, 1992) and characterized spectroscopically [^{1}H NMR (^2H_2O, Na^2HCO_3) δ 2.77 (s, 6, Me_2N), 3.27 (d, J = 12 Hz, 2, CH_2P), 6.68 (d, J = 10 Hz, 2, *p*-NO_2Ph), 7.69 (d, J = 10 Hz, 2, *p*-NO_2Ph), 7.3−8.4 (m, 6, Np); ^{31}P NMR (^2H_2O) δ 9.79 (vs 85% H_3PO_4); λ_{max} (pH 7) 300 nm (ϵ = 21 100 M^{-1} cm^{-1}); λ_f (pH 7) 530 nm, λ_{ex} 337 nm (wavelengths uncorrected)].

The product salt was hygroscopic and not suitable for elemental analysis. The concentrations of **2** in stock solutions were determined by the addition of a suitable aliquot (10 μL) to 1 M sodium hydroxide solution (1 mL). Alkaline hydrolysis in a sealed ampule at 80 °C was complete after 48 h. The original concentration of **2** could be obtained by measurement of *p*-nitrophenoxide absorption at 400 nm (ϵ = 18 700 M^{-1} cm^{-1}).

Preparation of Labeled Enzyme. The *E. cloacae* P99 β-lactamase was purchased from the Centre for Applied Microbiology & Research (Porton Down, Wiltshire, U.K.) and used as received. A solution of the enzyme (ca. 1 mg) in 0.5 mL of MOPS buffer was treated with **2** (2−3 equiv). After 5 min, the β-lactamase activity determined against benzylpenicillin had fallen to zero. The inhibited enzyme was separated from small molecules by Bio-Gel P-6DG chromatography at 5 °C; no return of enzyme activity was observed.

Kinetics of Enzyme Inhibition by 2. The reaction between the P99 β-lactamase (4 μM) and **2** (25 μM) in 20 mM MOPS buffer pH 7.5, 25 °C, was monitored spectrophotometrically (410 nm; release of *p*-nitrophenoxide) in a Durrum D110 stopped-flow spectrophotometer. The absorbance vs time data were fitted to an integrated second-order rate equation to give the rate constant for the reaction.

Titrations of the Inactivated Enzyme with Ligands. These were carried out by monitoring the perturbation of dansyl fluorescence on ligand binding by means of a Perkin-Elmer 44A spectrofluorimeter. Typically, 1-μL aliquots of a ligand stock solution were added to 0.5 mL of the inactivated enzyme (0.5 μM) in 20 mM MOPS buffer at pH 7.5, 25 °C. Excitation and emission wavelengths of 340 and 540 nm, respectively, were generally used. After corrections were made for dilution effects, the ligand dissociation constants were determined from the measured fluorescence intensities F by means of eq 1 and a nonlinear least-squares computer

$$F = F_0 + \frac{(F_\infty - F_0)[L]}{K + [L]} \quad (1)$$

program. In this equation F_0 represents the initial fluorescence intensity ([L] = 0), F_∞ is the fluorescence intensity at saturating ligand concentrations, and K is the dissociation constant of the ligand.

In some instances, the titrations were complicated by apparent emission from the ligands above 400 nm; such emission is termed apparent because most of the ligands involved would not be expected to be fluorescent. Such artifactual fluorescence probably arose from trace impurities readily conceivable as a problem in commercial products when used at high concentrations (0−30 mM). In such cases, a competitive titration was employed where the enzyme sample in the presence of a fixed concentration of the test ligand was titrated with a ligand of known affinity; *N*-(phenylacetyl)glycyl-D-*p*-iodophenylalanine **3** was generally used. With the assumption of simple competitive binding (Scheme 1), the dissociation constant, K_2, of the test ligand could be calculated from the apparent dissociation constant of **3**, K_{app}, obtained from the experiment by eq 2. In Scheme

Scheme 1

$$E\text{-}P + L_1 \underset{K_1}{\rightleftharpoons} E\text{-}PL_1$$

$$E\text{-}P + L_2 \underset{K_2}{\rightleftharpoons} E\text{-}PL_2$$

$$K_2 = K_1\{[L_2]/(K_{app} - K_1)\} \quad (2)$$

1, L_1 represents the titrant ligand of known dissociation constant, K_1, and L_2 is the test ligand; E-P is the phosphonyl enzyme. The competitive method was mainly used to show

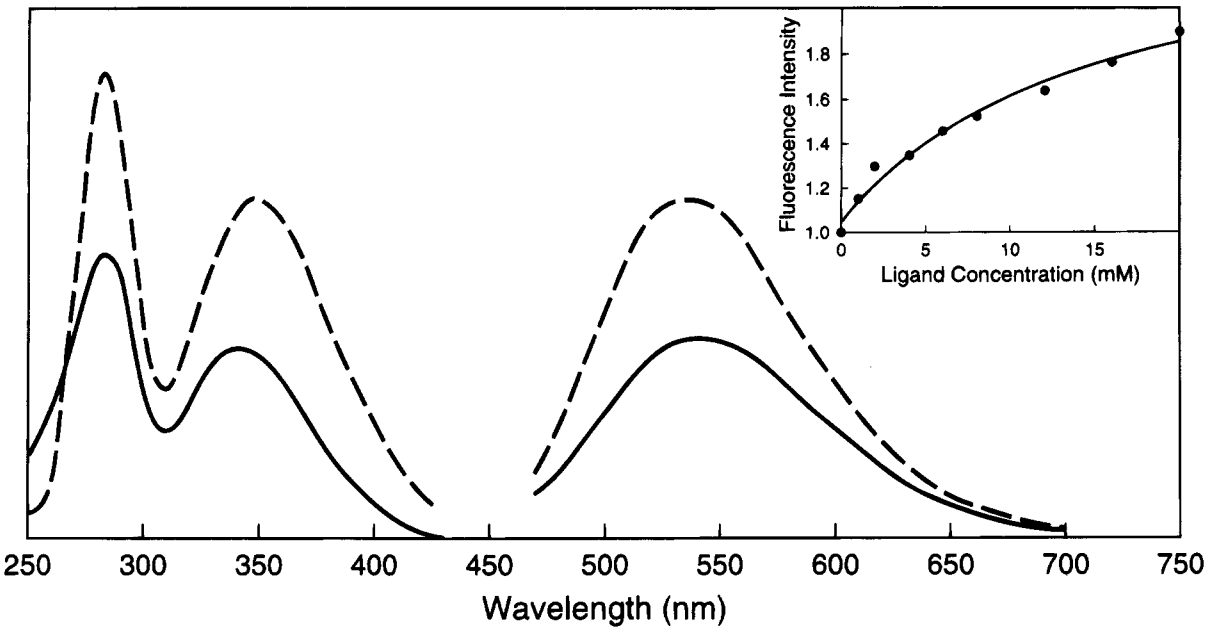


FIGURE 1: Excitation (250−450 nm) and emission (450−700 nm) spectra of the dansylamidomethylphosphonyl-β-lactamase (0.5 μM) in the absence (—) and presence (– –) of *N*-(phenylacetyl)-D-phenylalanine (20 mM). (Inset) Titration of the labeled enzyme (0.5 μM) with *N*-(phenylacetyl)-D-phenylalanine, as monitored by fluorescence emission at 540 nm on excitation at 340 nm.

that there was no evidence of binding of certain ligands even at high concentrations.

pH Titrations. The effect of pH on the absorption and fluorescence spectra of **2** and the enzyme inactivated by **2** was determined at 25 °C in a mixed buffer system containing 20 mM each of sodium acetate, MES, MOPS, HEPES, AMPSO, and 1 M sodium chloride, the latter to dampen ionic strength changes during the titration. Titrations were performed by the addition of small aliquots of 1 M sodium hydroxide solution to 0.75-mL samples of **2** or the enzyme (concentrations of 25 and 0.5 μM were employed for absorption and fluorescence measurements, respectively), generally beginning at a pH between 3 and 4. Absorption and fluorescence emission spectra were recorded as a function of pH, and the data were fitted where possible to proton dissociation equilibrium equations by a nonlinear least-squares procedure.

RESULTS AND DISCUSSION

Properties of the Inhibited Enzyme. The phosphonate **2**, like a series of similar compounds (Rahil & Pratt, 1992), was a strong inhibitor of the *E. cloacae* P99 β-lactamase under essentially stoichiometric conditions. The inhibition was accompanied by release of *p*-nitrophenol, and presumably proceeds by the same mechanism as occurs with the other phosphonates, viz. phosphonylation of the active site serine hydroxyl group. The second-order rate constant of the inhibition reaction, 2.0×10^4 s^{-1} M^{-1} at pH 7.5, 25 °C, is very similar to that previously determined for *p*-nitrophenyl [(phenylacetylamido)methyl]phosphonate under the same conditions, viz. 5.62×10^4 s^{-1} M^{-1} (Rahil & Pratt, 1992). This result indicates that, at least in the phosphonylation transition state, the dansyl group of **2** does not interact unfavorably with the enzyme.

The absorption spectrum of **2** at pH 7.5 exhibited a broad maximum at 300 nm, presumably involving absorption by both the *p*-nitrophenyl and dansyl chromophores. The excitation spectrum for fluorescence showed a single peak above 250 nm with a maximum at 337 nm. The fluorescence emission maximum was at 530 nm. Both of these features are typical of dansylamides (Lagunoff & Ottolenghi, 1965; Chen, 1967) and suggest a densyl chromophore in **2** not significantly perturbed by the *p*-nitrophenyl chromophore.

The inhibited enzyme also showed typical dansyl fluorescence, confirming the presence of covalent modification by **2**. The emission and excitation spectra are shown in Figure 1, with maximal emission at 533 nm. The excitation spectrum, also shown in Figure 1, differs from that of **2** in that it has as well as a normal dansyl maximum at 345 nm a more intense peak at 283 nm. This suggests light absorption by an aromatic protein chromophore (or chromophores), probably tyrosine(s), nonradiative energy transfer to the dansyl chromophore, and finally dansyl emission. This phenomenon is well-known in the literature with dansyl moieties either covalently (Steinberg, 1971; Stryer, 1978) or noncovalently (Latt et al., 1970) bound to proteins. Inspection of the crystal structure of the P99 β-lactamase after phosphonylation by *m*-carboxyphenyl [(*p*-iodophenylacetamido)methyl]phosphonate (Lobkovsky et al., 1994) showed that the closest aromatic residues to the naphthalene ring of the phosphonate to be Tyr 221 (whose closest aromatic carbon atom is about 4 Å from the phosphonate side chain methylene group) and Tyr 150 (whose closest ring carbon is 7.6 Å from the same methylene group). One of these residues may well be involved in the energy transfer process.

The fluorescence emission maximum of the dansyl chromophore is also well-known to be sensitive to the polarity (dielectric constant) of the adjacent medium (Chen, 1967; Li et al., 1975), moving to lower wavelength in solvents of lower polarity. The wavelength of this emission from dansyl groups positioned at the active sites of enzymes has been used to estimate a general average polarity of the active sites concerned (Himel et al., 1970). In the present instance, the close similarity between the emission maxima of **2** and of the phosphonylated enzyme might be interpreted to mean

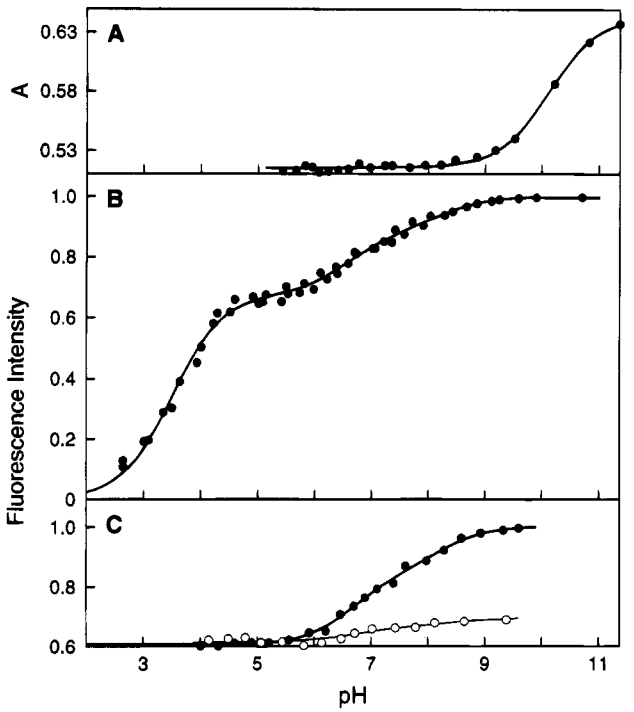


FIGURE 2: Variation with pH of the absorbance at 300 nm (A) and the fluorescence emission at 550 nm (B) of **2**; concentrations of **2** in the two experiments were 24 and 0.5 μM, respectively. Panel C shows the relative variation with pH of the fluorescence emission at 550 nm of the dansylamidomethylphosphonyl-β-lactamase (0.5 μM) in the absence (●) and presence (○) of 25 mM *N*-(phenylacetyl)-D-α-aminoadipate.

that the area of the enzyme surface that the dansyl group finds itself in the complex is very polar and likely extensively hydrated. An alternative interpretation would be that the similarity is coincidental. The emission maxima of dansylamide itself and dansylglycine were 556 and 564 nm, respectively, under the same aqueous conditions as the phosphonates. The much lower maximum for **2** suggests a less polar environment that might be provided in solution by intramolecular hydrophobic contact between the dansyl nucleus and the *p*-nitrophenyl group. The loss of this interaction (and the *p*-nitrophenyl group) on phosphonylation of the enzyme could be compensated by hydrophobic interaction with the enzyme. The side-chain binding pocket of the P99 β-lactamase active site, although open to hydration from one side, does contain a hydrophobic surface and favors aromatic side chains in substrates (Xu et al., 1994).

Effect of pH on the Fluorescence of **2** *and the Labeled Enzyme.* Figure 2B shows the variation with pH of the fluorescence intensity at 550 nm on irradiation of **2** at 340 nm. The pH was controlled by the mixed buffer system as described in Experimental Procedures. The data could be fitted to Scheme 2, with four species and three dissociation constants. The best fit to the data (solid line of Figure 2B) was achieved with pK_a's of DH_3, DH_2, and DH_1 of 3.50 ± 0.05, 6.5 ± 0.3, and 8.2 ± 0.6, respectively. Relative

Scheme 2

$$DH_3 \overset{K_{a1}}{\rightleftharpoons} DH_2 + H^+ \overset{K_{a2}}{\rightleftharpoons} DH + H^+ \overset{K_{a3}}{\rightleftharpoons} D + H^+$$

fluorescence intensities of DH_3, DH_2, DH, and D were 0, 0.67, 0.88, and 1.0, respectively. A much poorer fit to the data (not shown) was achieved if only two dissociations (i.e., only one above pH 5) were included. The low pH dissociation (pK_a 3.5) from a species weakly fluorescent at long wavelengths is typical of dansylamides (Lagunoff & Ottolenghi, 1965) and corresponds to dissociation of the 6-dimethylammonium group in the ground state.

The increase in the fluorescence intensity of **2** between pH 5 and pH 10 however is unusual. Normally, one observes dissociation of the sulfonamide group accompanied by an absorption and fluorescence intensity increase only above pH 9.0. Such a phenomenon is observed, for example, with dansylsulfonamide itself and with dansylglycine. Although the excited-state pK_a of the sulfonamide is probably lower than the ground-state pK_a, the two usually appear to coincide, presumably because of an excited state short-lived in comparison with the rate of proton dissociation. The ground-state dissociation behavior of the dansylphosphonate **2** is in accord with these expectations with a pK_a of 10.1 ± 0.1, also shown in Figure 2 (panel A). Virtually no change in absorption and hence of the dominant ground-state molecular structure occurs between pH 5.5 and pH 8.5. The contrast between this behavior and the excited-state behavior is striking. It is likely that the fluorescence intensity increase between pH 5 and pH 9 (Figure 2B) represents proton dissociation from the excited sulfonamide at rates comparable to the fluorescence decay rate and which increase with pH because of base catalysis. There is considerable precedent for this behavior in naphthalenes with acidic substituents (Schulman, 1976). This interpretation would mean however that the apparent pK_a's of 6.5 and 8.2 extracted from the data of Figure 2B are spurious. The shape of the curve of Figure 2B would then be dictated by rates of proton dissociation from the excited state of **2**, which would vary in a complicated way with pH, especially if the buffer bases also act as general bases (Schulman, 1976). In accord with this hypothesis, a gradual blue shift in the emission maximum (from 540 nm at pH 5.5 to 515 nm at pH 11) is observed as the pH and the fluorescence intensity increase. A similar shift (from 556 and 520 nm) is observed in the emission spectrum of dansylamide between pH 9 and pH 13. No further change to the spectrum of **2** was observed above pH 10. In further support of this interpretation, the emission intensity of **2** at pH 6.5 increased linearly with MOPS concentration up to 1 M.

The pH dependence of dansyl fluorescence in the modified enzyme is shown in Figure 2C. It differs from that of **2** at low pH in that the dimethylammonium group pK_a must be lower at the enzyme surface. This may reflect the effect of the overall positive charge of the adjacent protein whose isoelectric pH is reported to be 7.9 (Ross, 1975). Above pH 5 however, the pH dependence of the enzyme-bound dansyl group is very similar to that of **2** (the apparent but probably spurious pK_a's derived from fitting the data to Scheme 2 and yielding the solid line of Figure 2C are 6.65 ± 0.05 and 8.10 ± 0.25 with intensities corresponding to DH_2, DH, and D of 0.61, 0.85, and 1.0). The simplest interpretation of this result would be that the dansyl group in the inhibited enzyme is in an environment closely resembling bulk solution. This is in accord with the dansyl emission spectrum (see above), but is somewhat surprising in one respect, in that the sulfonamide proton which must on the basis of the pH dependence of fluorescence dissociate in the excited state of the enzyme complex with equal facility to that of **2** in solution might be expected to be hydrogen-

bonded into the active site in the same way believed of carboxamido side chains (Herzberg & Moult, 1987; Moews et al., 1991; Strynadka et al., 1990; Oefner et al., 1990; Lobkovsky et al., 1994). It could be of course that the sulfonamide group is *not* firmly hydrogen-bonded in this way in the phosphonyl enzyme—although it may be so in the pentacoordinated intermediate of the inactivation reaction—and, indeed, there is evidence that sulfonyl derivatives may not bind well in β-lactamase active sites (Davern et al., 1994). It may also be possible that the structural changes in the molecule, resulting from excitation, lead to loss of direct contact between the side chain and the protein; if so, this process must be faster than fluorescence decay and result, like the observation of competing proton transfer, from a relatively long-lived excited state.

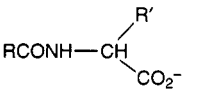
The evidence described above suggests overall that the dansyl reporter group of the phosphonyl enzyme is in a close-to-aqueous environment and is probably not strongly interacting with the enzyme. This would certainly be in accord with the generally broad specificity of β-lactamases toward the amido substituents of their substrates. In further accord with this scenario, no evidence of perturbation of the pH dependence of dansyl emission by the dissociation of enzymic functional groups was observed. In particular, no dissociation of the active site functional groups was detected. This however may be because of their strong interaction with the phosphonate moiety (Lobkovsky et al., 1994). A comparable situation occurs in phosphonate monoesters of serine proteinases (Liang & Abeles, 1987; Bachovchin et al., 1988).

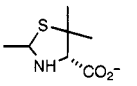
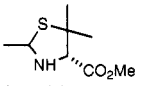
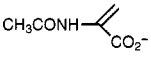
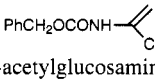
Figure 2C also shows the titration of the labeled enzyme in the presence of *N*-(phenylacetyl)-D-α-aminoadipic acid, a site 2 ligand, at a concentration (25 mM) sufficient to largely saturate the site at pH 7.5 (see below). The increase in fluorescence intensity between pH 4 and pH 10 is much smaller than in the absence of the ligand. A blue shift of dansyl emission was observed as the pH increased, as observed in the absence of ligand (see above), suggesting that the fluorescence enhancements produced by a ligand and pH are different in kind. As the pH is raised, the ligand probably dissociates, but the fluorescence intensity at any wavelength does not change greatly because of the pH effect.

Ligand Interactions with the Inhibited Enzyme. The addition of a variety of molecules to a solution of the fluorescent-labeled β-lactamase led to enhanced dansyl fluorescence emission. Figure 1, for example, shows the enhancement achieved on the addition of *N*-(phenylacetyl)-D-phenylalanine (20 mM) to the labeled enzyme (0.5 μM). This observation suggests that these molecules bind to the enzyme−**2** complex in such a way that either through direct contact with the enzyme-bound dansyl group or, perhaps more likely, through indirect means, modulated by protein conformational changes, the return of the dansyl group to the ground state is influenced such that emission is enhanced. The effect could result either from direct promotion of radiative decay or by suppression of a competing nonradiative pathway. The dansyl emission maximum moves to slightly lower wavelengths in these complexes, possibly signifying the creation of a slightly less polar environment. The excitation spectrum after addition of these ligands indicates that emission from both direct dansyl excitation and from the nonradiative energy transfer pathway, arising through excitation of aromatic protein chromophores, is comparably enhanced by ligand binding. This presumably means that the position of the relevant protein chromophore with respect to the dansyl group is not significantly affected by ligand binding. The extent of fluorescence enhancement varied from ligand to ligand, from a factor of ca. 1.5 to one of ca. 3. Apparently, the different ligands do not affect the dansyl photophysics identically, i.e., the structural perturbation leading to the enhancement is not identical for all ligands.

Titration of the β-lactamase−**2** complex with these ligands led to hyperbolic plots of fluorescence intensity vs concentration such as shown in Figure 1 (inset) for *N*-(phenylacetyl)-D-phenylalanine. From these curves, dissociation constants for the various complexes could be calculated as described in the Experimental Section. Table 1 presents the dissociation constants for a variety of ligands. These data are discussed below.

Table 1: Dissociation Constants of Complexes of Ligands with the Inactivated P99 β-Lactamase

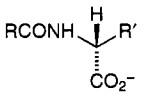


entry	R	R′	*K*(mM)
1	$PhCH_2CONHCH_2$-	D-CH_2Ph	11.7 ± 2.6
2	$PhCH_2CONHCH_2$-	D-CH_2Ar-*p*-I	7.5 ± 2.2
3	$PhCH_2CONHCH_2$-	D-CH_3	26 ± 12
4	$PhCH_2$-	D-CH_2Ph	14.1 ± 6.0
5	$PhCH_2O$-	D-CH_2Ph	18.0 ± 8.0
6	$PhCH_2$-	D-$(CH_2)_3CH_3$	15.0 ± 5.0
7	$PhCH_2$-	D-$(CH_2)_3CO_2^-$	13.0 ± 4.0
8	$PhCH_2O$-	D-$(CH_2)_2CO_2^-$	13.0 ± 4.0
9	$PhCH_2O$-	D-$(CH_2)_4NH_3^+$	35 ± 10
10	$PhCH_2O$-	L-$(CH_2)_4NH_3^+$	≥50
11	$PhCH_2$-	D-$(CH_2)_4$-NHCOCH_2Ph	28 ± 10
12	$PhCH_2$-	H	12.0 ± 2.0
13	$PhCH_2O$-	H	15.0 ± 5.0
14	$PhCH_2O$-	H (PO_3^{2-})	>20[a]
15	CH_3-	D-CH_2Ph	14.4 ± 4.3
16	CH_3-	L-CH_2Ph	25 ± 7
17	CH_3-	D-CH_2Indole	≥20[a]
18	$H_3N^+CH_2$-	D-CH_2Ph	10.2 ± 3.0
19	(DL)$H_3N^+CH(CH_3)$-	DL-CH_2Ph	>20
20	(L)$H_3N^+CH(CO_2^-)CH_2CH_2$-	L-CH_2Ph	>20[a]
21	(DL)$CH_3CONHCH(CH_2Ph)$-	H	≥20[a]
22	Ac_2-L-Lys-D-Ala-	D-CH_3	≥20[a]
23	$PhCH_2$-		7.7 ± 2.3
24	$PhCH_2$-		>20
25	6β-aminopenicillanic acid		>20[a]
26	adipic acid		>20
27	D-α-aminoadipic acid		>20
28	citric acid		>20
29	*m*-hydroxybenzoic acid		≥20[a]
30	D-phenylalanine		>50
31	D-phenyllactic acid		>20
32			≥20
33			≥20
34	*N*-acetylglucosamine		≥50
35	*N*-acetylmuramic acid		≥50
36	*N*,*O*-diacetyl-D-phenylalaninol		>20

[a] A competition experiment against **3** was also performed.

The first conclusion from Table 1 is that none of the ligands yet discovered binds very tightly—the strongest complexes appear to be those with benzylpenicilloate (entry 23) and *N*-(phenylacetyl)glycyl-D-*p*-iodophenylalanine (entry 2) with dissociation constants of around 7.5 mM. Two points should be made with respect to this. First, no peptides and peptide analogs, even substrates, are known to bind tightly in a noncovalent fashion to these enzymes, β-lactamases, or DD-peptidases. It would be therefore surprising to find strong binding. Secondly, as discussed in the preceding paper (Dryjanski & Pratt, 1995), the dissociation constants in Table 1 may be greater (or smaller) than for the corresponding complexes of the ligands with the free enzyme because of interactions between the sites and the fact that the complex with **2** probably closely resembles a transition-state structure (Rahil & Pratt, 1994).

The second point arising from the data of Table 1 is that site 2 binding has broad but certainly not unlimited structural specificity. Of the compounds examined, the data show a peference for structure **4**, of D-configuration adjacent (α) to



4

the amide nitrogen atom. The following specific conclusions with respect to structural specificity can be made:

(i) A D-configuration at the α-carbon is preferred but not strongly so (compare entries 9 vs 10 and 15 vs 16). It is likely that space is available in the binding site beyond the hydrogen at the α-carbon of **4**.

(ii) A planar α-carbon is not well-accommodated (entries 32 and 33).

(iii) The carboxylate seems important. Compare entry 15 with entry 36. Phosphonate is not an adequate replacement (entry 14 vs entry 13).

(iv) R′ may be hydrophobic (entries 1−6, 11, 15, and 18) or negatively charged (entries 7, 8, and 23—compare the latter with entry 24); positive charge seems less favorable (entry 9). The R′ subsite has limited size—it accommodates aliphatic chains and the aromatic side chains of D-phenylalanine and D-*p*-iodophenylalanine, but not so readily the side chain of D-tryptophan (entry 17). The absence of an extended R′ side chain (entry 12) is quite acceptable.

(v) The amido side chain appears quite critical. D-Phenylalanine itself (entry 30) and its anionic analog, D-phenyllactate entry 31 (where a neutral amine is mimicked by a hydroxyl group), do not bind measurably. Simple di- (entries 26 and 27) and tri- (entry 28) carboxylic acids do not bind detectably. The acylamide substituent R may have rather broad specificity (entries 1, 15, and 18). There is the suggestion that branching α to the amide carbonyl (entries 19, 21, and 22) may not be readily accommodated, although more data are needed on this point. The positive charge β to the carbonyl group (entry 18) seems permitted, but it may be the neutral amine that binds.

(vi) The bacterial cell wall monosaccharides, *N*-acetylglucosamine and *N*-acetylmuramic acid, showed no affinity, nor did the penicillin nucleus as exemplified by 6-β-aminopenicillanic acid (entry 25).

These results suggest therefore that site 2 has rather broad specificity directed toward a peptide α-D-carboxylate. The absence of D-phenylalanine (D-phenyllactate) binding suggests that site 2 (as defined by specificity for the moiety **4**) may *not* be the acyl acceptor binding site. The possibility that it might be was raised earlier by Pazhanisamy and Pratt (1989b). The converse conclusion is still not completely tight however since the binding of D-phenylalanine is known to be weak—dissociation constants of complexes from the free enzyme and from an enzyme−**4** complex were shown to be greater than 15 mM (Pazhanisamy & Pratt, 1989a,b). It should be noted however that the absence of a fluorescence change on the addition of D-phenylalanine to the labeled enzyme was not accepted as definitive of the absence of binding. In a further experiment, the addition of D-phenylalanine, up to 50 mM, did not affect the fluorescence of a complex of *N*-(phenylacetyl)glycyl-D-phenylalanine and the labeled enzyme, i.e., there was no evidence of competition between these two ligands for site 2. Kinetically, however, D-phenylalanine and ligands of site 2 appear to interact competitively (Pazhanisamy & Pratt, 1989b).

The results of the experiments with substrates and their analogs are also interesting when considered together with the results of the kinetics studies (Dryjanski & Pratt, 1995). Both studies indicate that the penicilloates bind weakly to site 2. In contrast perhaps, the same comparison indicates considerably tighter binding of substrates to site 2 in the presence of *m*-(dansylamido)phenylboronic acid {for example, *m*-carboxyphenyl phenaceturate, 1.7 mM [K_1 of Scheme 2, Dryjanski and Pratt (1995)]; 6-β-aminopenicillanate 0.8 mM} than in the presence of the phosphonate (both >20 mM). This may only reflect differences in the nature of interaction between the sites depending on the specific occupants of the sites or, given the differences in structure between *m*-carboxyphenyl phenaceturate and 6-β-aminopenicillanate on one hand and **4** on the other, indicate the existence of yet another binding mode. The competitive interaction of D-phenylalanine in the aminolysis pathway with *m*-carboxyphenyl phenaceturate in its second binding site (cf. D-phenylalanine and **4** above) (Pazhanisamy & Pratt, 1989b) might also be consistent with this interpretation. On the other hand, however, the dissociation of *N*-(phenylacetyl)glycyl-D-phenylalanine from the phosphonate−enzyme complex (11.7 ± 2.6 mM) is also markedly more favorable than from the boronate−enzyme complex (1.0 mM) (Dryjanski & Pratt, 1995). The conservative conclusion, viz. only two sites with ligand-specific linkage between them, should presumably be tentatively accepted at present.

A final matter that might be considered is the function, if any, of site 2. Previous thought on this as mentioned in the introduction and elsewhere (Dryjanski & Pratt, 1995; Pazhanisamy & Pratt, 1989b) has centered on the idea of an evolutionary relic of a peptide substrate binding site. The present result, of the peptide α-carboxylate specificity of site 2, is certainly in accord with this idea. A C-terminal D-Ala-D-Ala specificity would not be expected, since this, if found anywhere, should correspond to site 1, and the apparently weak binding of *N,N′*-diacetyl-L-lysyl-D-alanyl-D-alanine to site 2 (Table 1) supports this idea. A more likely candidate from the perspective of cell wall structure would be the L-Ala-γ-D-Glu moiety found in peptidoglycan cross-linking peptides of many bacteria (Rogers et al., 1980). A specific binding site for such a moiety, as part of a bacterial disaccharide tetrapeptide, has been recently described in the T4 phage lysozyme (Kuroki et al., 1993). Of course, it is

certainly very possible that neutral mutations (little reason for selective pressure is evident) have changed the specificity of the present site from that in an ancestral DD-peptidase; a general broadening of specificity may result from a series of enzyme-wide mutations.

Direct attempts to determine the location of site 2 have not yet been successful. No discrete electron density corresponding to the position of a ligand was obtained on soaking crystals of the P99 β-lactamase with *N*-(phenylacetyl)glycyl-D-*p*-iodophenylalanine (J. R. Knox, personal communication). The position of the Tyr 150 side chain in the enzyme was however affected, which might indicate that site 2 is close to the active site.

The method of investigation of additional or extended binding sites employed here should be of general applicability to β-lactam-recognizing enzymes. We intend to use it with some other typical examples. The recruitment of these sites to future design of bivalent inhibitors (Szewczyk et al., 1993; Fitzpatrick et al., 1993) is conceivable.

ACKNOWLEDGMENT

The synthesis of dansylamidomethylphosphonic acid was performed by Ms. Sandhya Subramanian, a NECUSE Summer Fellow. We are grateful to Dr. James R. Knox of the University of Connecticut for access to details of the crystal structure of the P99 β-lactamase.

REFERENCES

Bachovchin, W. W., Wong, W. Y. L., Farr-Jones, S., Shenvi, A. B., & Kettner, C. A. (1988) *Biochemistry 27*, 7689−7697.
Chen, C. H. C., Rahil, J., Pratt, R. F., & Herzberg, O. (1993) *J. Mol. Biol. 234*, 165−178.
Chen, R. F. (1967) *Arch. Biochem. Biophys. 120*, 609−620.
Davern, P., Sheehy, J., & Smyth, T. (1994) *J. Chem. Soc. Perkin Trans 2*, 381−387.
Dryjanski, M., & Pratt, R. F. (1995) *Biochemistry 34*, 3561−3568.
Ehrhardt, A., & Sanders, C. C. (1993) *J. Antimicrob. Chemother. 32* (Suppl. B), 1−11.
Fitzpatrick, P. A., Steinmetz, A. C. U., Ringe, D., & Klibanov, A. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8653−8657.
Galleni, M., & Frére, J.-M. (1988) *Biochem. J. 255*, 119−122.
Galleni, M., Amicosante, G., & Frére, J.-M. (1988) *Biochem. J. 255*, 123−129.
Govardhan, C. P., & Pratt, R. F. (1987) *Biochemistry 26*, 3385−3395.
Herzberg, O., & Moult, J. (1987) *Science 236*, 694−701.
Himel, C. M., Mayer, R. T., & Cook, L. L. (1970) *J. Polymer Sci. 8*, 2219−2230.
Kuroki, R., Weaver, L. H., & Matthews, B. W. (1993) *Science 262*, 2030−2033.
Lagunoff, D., & Ottolenghi, P. (1965) *C. R. Trav. Lab. Carlsberg 35*, 63−83.
Latt, S. A., Auld, D. S., & Vallee, B. L. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1383−1389.
Li, Y.-H., Chan, L.-M., Tyer, L., Moody, R. T., Himel, C. M., & Hercules, D. M. (1975) *J. Am. Chem. Soc. 97*, 3118−3126.
Liang, T.-C., & Abeles, R. H. (1987) *Biochemistry 26*, 7603−7608.
Lobkovsky, E., Billings, E. M., Moews, P. C., Rahil, J., Pratt, R. F., & Knox, J. R. (1994) *Biochemistry, 33*, 6762−6772.
Moellering, R. C., Jr. (1993) *J. Antimicrob. Chemother. 31* (Suppl. A), 1−8.
Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P., & Frére, J.-M. (1990) *Proteins: Struct., Funct., Genet. 7*, 156−171.
Murphy, B. P., & Pratt, R. F. (1991) *Biochemistry 30*, 3640−3649.
Oefner, C., D'Arcy, A., Daly, J. J., Gubernator, K., Charnas, R. L., Heinze, I., Hubschwerlen, C., & Winkler, F. K. (1990) *Nature 343*, 700−705.
Pazhanisamy, S., & Pratt, R. F. (1989a) *Biochemistry 28*, 6870−6875.
Pazhanisamy, S. & Pratt, R. F. (1989b) *Biochemistry 28*, 6875−6882.
Pazhanisamy, S., Govardhan, C. P., & Pratt, R. F. (1989) *Biochemistry 28*, 6863−6870.
Pratt, R. F. (1989) *Science 246*, 917−919.
Rahil, J., & Pratt, R. F. (1991) *J. Chem. Soc. Perkin Trans. 2*, 947−950.
Rahil, J., & Pratt, R. F. (1992) *Biochemistry 31*, 5869−5878.
Rahil, J., & Pratt, R. F. (1994) *Biochemistry 33*, 116−125.
Rogers, H. J., Perkins, H. R., & Ward, J. B. (1980) *Microbial Cell Walls and Membranes*, Chapman & Hall, London.
Ross, G. W. (1975) *Methods Enzymol. 43*, 678−687.
Schulman, S. G. (1976) in *Modern Fluorescence Spectroscopy* (Wehry, E. L., Ed.) Vol. 2, Chapter 6, Plenum Press, New York.
Steinberg, I. Z. (1971) *Annu. Rev. Biochem. 40*, 83−114.
Stryer, L. (1968) *Science 162*, 526−533.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819−846.
Strynadka, N. C. J., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C., Sutoh, K., & James, M. N. G. (1992) *Nature 359*, 700−705.
Szewczyk, Z., Gibbs, B. F., Yue, S. Y., Purisma, E., Zdanov, A., Cygler, M., & Konishi, Y. (1993) *Biochemistry 32*, 3396−3404.
Xu, Y., Soto, G., Adachi, H., van der Linden, M. P. G., Keck, W., & Pratt, R. F. (1994) *Biochem. J. 302*, 851−856.

BI9411833