Mechanism of Inhibition of the β-Lactamase of *Enterobacter cloacae* P99 by 1:1 Complexes of Vanadate with Hydroxamic Acids†

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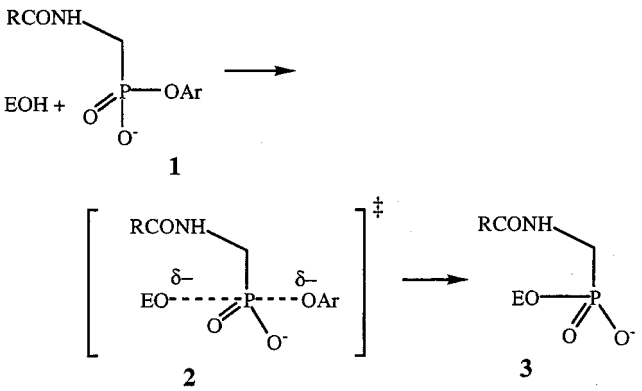
ABSTRACT: The class C β-lactamase of *Enterobacter cloacae* P99 is competitively inhibited by low concentrations of 1:1 complexes of vanadate and hydroxamic acids. Structure−activity studies indicated that the hydroxamic acid functional group was essential to this inhibition. Both aryl and alkyl hydroxamic acids form inhibitory ternary complexes with vanadate and the enzyme, although, in certain cases of the latter, the inhibition may not be seen because of the low formation constants of the vanadate−hydroxamic acid complex. After all of the vanadate species present in solution had been taken into account, "real" K_i values for the vanadate complexes could be determined. The K_i value of the best of the inhibitors that were investigated, the 1:1 complex of vanadate with 4-nitrobenzohydroxamic acid, was 0.48 μM. Kinetics studies showed that the association and dissociation rate constants of this complex with the enzyme were 1.48×10^6 s^{-1} M^{-1} and 0.73 s^{-1}, respectively; the magnitude of the latter indicates covalent interaction of the complex with the enzyme. ^{51}V NMR and UV−vis spectra suggest that the structure of the vanadate complex bound to the enzyme may be very similar to that in solution. A ^{13}C NMR spectrum of the enzyme complex with 4-nitrobenzo[^{13}C]hydroxamic acid and vanadate yields a coordination-induced shift (CIS) of 7.74 ppm. This is significantly larger than that of the vanadate complex in free solution (3.62 ppm), suggesting either, somewhat contrary to the ^{51}V and UV−vis spectra, greater interaction between vanadium and the hydroxamate carbonyl oxygen in the enzyme complex than in free solution or, more likely, polarization of the hydroxamate by interaction, e.g., hydrogen bonding, with the enzyme. Molecular modeling indicates that a pentacoordinated vanadate complex may well be able to snugly occupy the enzyme active site; Asn 152 is suitably placed to hydrogen bond to the hydroxamic acid oxygen atom. The experimental results are in accord with a model whereby the vanadate−hydroxamate−enzyme complex is a moderately good analogue of the transition state of the reaction of the β-lactamase with phosphonate inhibitors.

Bacterial resistance to β-lactam antibiotics has been an ever-present threat to human health since β-lactams were introduced into clinical practice some 50 years ago. This resistance has a number of sources, but one of the most important is through bacterial production of β-lactamases that catalyze the hydrolysis of these antibiotics. Inhibitors of these enzymes have been actively sought over the same 50-year period (*1*, *2*). At present, the class C β-lactamases of Gram-negative bacteria have become a particular challenge (*3*), and new classes of inhibitors of these enzymes are very welcome. In this laboratory, we have been particularly interested in the potential of phosphonate inhibitors and analogues derived from them (*4*−*9*). The initially discovered phosphonates, of general structure **1**, inhibit serine β-lactamases (classes A and C) covalently by reaction with the active site hydroxyl group (Scheme 1). This reaction yields a tetrahedral covalent adduct **3** that appears to be a good transition state analogue for the normal acyl transfer reaction catalyzed by these enzymes (*10*−*12*). The transition state for the phosphonylation reaction presumably resembles **2**, containing pentacoordinated phosphorus.

Scheme 1



Rapid inhibition by phosphylating agents is also a property of serine proteases (*13*, *14*). Since the reactions of these compounds with both serine β-lactamases and proteases are much more rapid than with water, it is clear that the active sites of these enzymes must stabilize transition states such as **2** that contain pentacoordinated phosphorus (*9*, *15*−*17*). The structural basis of this stabilization is not clearly defined

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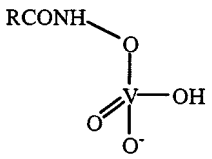
at present. Nonetheless, it follows from these observations that suitably constructed stable molecules that could react with these enzymes to generate a structure resembling **2** might represent a new class of inhibitor.

One approach to such molecules would be to begin with a tetrahedral species, but one which would, unlike phosphyl derivatives, generate stable pentacoordinated species on addition of the active site nucleophile. Compounds of vanadium meet this criterion well (*18*). Indeed, vanadate (V^V) complexes have been employed for many years as inhibitors of phosphoryl transfer enzymes (*19*−*21*), and structural studies show that the inhibitory complexes contain pentacoordinated vanadium (*22*−*25*). Although a direct vanadium analogue of **1** is not synthetically feasible, an isostere **4** can be easily generated, in principle, from a complex of vanadate and hydroxamic acids, formed in situ.



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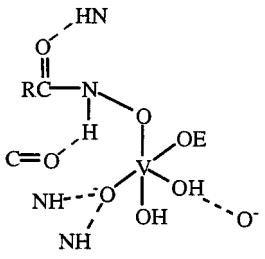
This simple complex does not have the aryl oxide leaving group of **1**; however, there is no evidence for strong interaction between the leaving group and the β-lactamase active site (*4*−*9*), and thus, as a first approximation at least (ternary complexes containing a leaving group analogue could be a second approximation), complexes of structure **4** could reasonably be assessed as inhibitors. According to the rationale described above, **4** would inhibit a β-lactamase by forming a complex with the enzyme such as **5**, where the usual stabilizing hydrogen bonds of the active site (*12*) are also shown. A complex containing tetracoordinated vanadium would also be possible.



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We have, in fact, provided preliminary evidence that mixtures of hydroxamic acids and vanadate do inhibit serine amidohydrolases, both β-lactamases and proteases (*26*). In this paper, we look in detail at the inhibition mechanism, focusing on quantitation of the inhibition, on structure−activity studies, and on the structure of the inhibitory species, both in solution and at the active site of a class C β-lactamase.

MATERIALS AND METHODS

Materials. The β-lactamase of *Enterobacter cloacae* P99 was purchased from the Centre for Applied Microbiology and Research (Porton Down, Wilts, U.K.) and used as received. The following potential vanadate ligands and inhibitors were purchased commercially and used as received: *O*-benzylhydroxylamine (Fluka), benzoic hydrazide (Aldrich), salicylic acid (Aldrich), *O*-phenylhydroxylamine (Fluka), benzaldehyde oxime (Fluka), *N*-hydroxyphthalimide (Aldrich), L-mandelic acid (Sigma), D-lactic acid (Aldrich), D-phenylactic acid (Aldrich), benzamidoxime (Maybridge), salicylamide (Aldrich), 2-hydroxypyridine *N*-oxide (Aldrich), *N*-(hydroxymethyl)benzamide (Fluka), L-lysine hydroxamic acid (Sigma), 1-hydroxy-3-phenylurea (Aldrich), *N*-hydroxyurea (Aldrich), *N*-hydroxybenzenesulfonamide (Fluka), benzohydroxamic acid (Aldrich), salicylhydroxamic acid (Aldrich), acetohydroxamic acid (Aldrich), and L-phenylalanine hydroxamic acid (Sigma). Phenylacetohydroxamic acid was prepared in this laboratory by M. Ernst following a literature procedure (*27*).

N-Methylbenzohydroxamic acid was synthesized as described by Exner et al. (*28*). The general protocol of Weber and Fox (*29*) was employed to prepare *N*-acetyl-L-phenylalanyl-, *N*-acetyl-L-prolyl-, and *N*,*N*′-diacetyl-L-lysylhydroxamic acid. These three compounds were first purified by Dowex 50W X4-400 chromatography. The first of the three was then recrystallized from an ether/light petroleum mixture, and the latter two were purified further by anion exchange chromatography (Dowex-2 2X8-800 and Sephadex QAE A25 for the proline and lysine compounds, respectively). *O*-Methylbenzohydroxamic acid and *O*-methylsalicylhydroxamic acid were synthesized by the procedure of Hosangadi et al. (*30*). The remaining hydroxamic acids were prepared by the general method of Stolberg et al. (*31*) and, in general, purified by recrystallization from methanol or ethanol. All of the hydroxamic acids were pure by ^{1}H NMR and tested strongly positive in the $FeCl_3$ test (*32*).

Kinetics and Spectroscopic Methods. β-Lactamase activity was routinely determined spectrophotometrically against 100 μM nitrocefin (Unipath), employing a Hewlett-Packard 8453A spectrophotometer. All kinetics experiments were carried out in 20 mM MOPS buffer at pH 7.5 and 25 °C. Stock solutions (10 mM) of vanadate were prepared by dissolution of sodium orthovanadate (99.99%, Aldrich) in MOPS buffer. These were diluted into buffer containing hydroxamic acids as described elsewhere (*33*). Equilibrium and kinetic data were routinely fitted to reaction schemes by the Dynafit program (*34*). Apparent K_i values were obtained by fitting enzyme activity values versus hydroxamic acid concentrations at a fixed vanadate concentration (Figure 1) to Scheme 2, where VH[1] represents the inhibitor and V represents, in this approximation, total free vanadate; E and EVH represent free and inhibited β-lactamase, respectively; and S and P represent the substrate, nitrocefin, and its hydrolysis product, respectively. Values of k_{cat} and K_m for nitrocefin were determined to be 200 s^{-1} and 50 μM, respectively, from initial velocity measurements. Better estimates of the inhibitory ability of VH, taking into account other species in solution, were determined using Scheme 5 as described in the Results and Discussion.

Stopped-flow experiments were performed on a Durrum D-110 stopped-flow spectrophotometer. A solution contain-

[1] Abbreviations: V, monomeric vanadate, $H_nVO_4^{n-3}$; VH, 1:1 complex of vanadate with hydroxamic acid; VH_2, 1:2 complex of vanadate with hydroxamic acid; EVH, 1:1 complex of VH with β-lactamase; CIS, coordination-induced (NMR) shift.

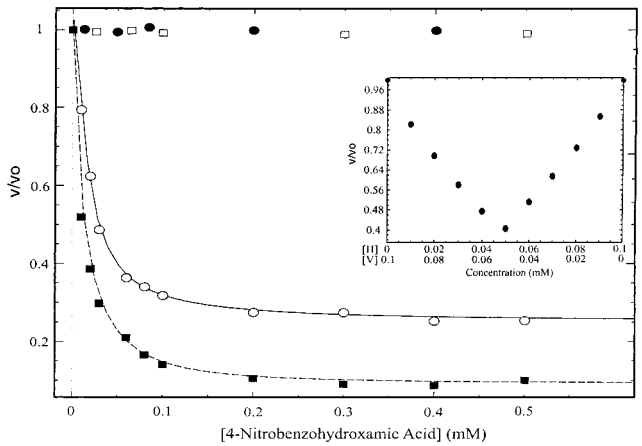


FIGURE 1: Inhibition of turnover (v/v_o, the ratio of observed initial velocity to initial velocity in the absence of inhibitor, is shown) of nitrocefin by the P99 β-lactamase in the presence of 0.03 (○) and 0.1 mM (●) vanadate as a function of 4-nitrobenzohydroxamic acid concentration. The points represent experimental data, and the solid line represents the fit to Scheme 5 calculated with Dynafit (*34*). The effect of vanadate (●) and 4-nitrobenzohydroxamic acid (□) separately on enzyme activity is also shown. The inset shows a Job plot of the effect of continuous variation of the vanadate concentration, [V], and the 4-nitrobenzohydroxamic acid concentration, [H], on the activity of the β-lactamase against nitrocefin.

Scheme 2

$$V + H \underset{}{\overset{K_I}{\rightleftharpoons}} VH$$

$$E + VH \underset{K_i}{\rightleftharpoons} EVH$$

$$E + S \underset{K_M}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P$$

ing 0.67 μM β-lactamase, 20 μM total vanadate, and 20 μM 4-nitrobenzohydroxamic acid was mixed with an equal volume of a solution containing 200 μM nitrocefin in the apparatus described above. The appearance of the nitrocefin hydrolysis product was monitored at 482 nm. The data were fitted with Dynafit (*34*) to Scheme 5, where the equilibrium constant K_i was replaced by the rate constants k_r and k_f; the latter parameters were determined from the calculated best fit to the data.

^{13}C and ^{51}V NMR spectra were obtained as described elsewhere (*33*). Preparation of 4-nitrobenzo[^{13}C]hydroxamic acid is also described in ref *33*.

Molecular Modeling. The computations were set up essentially as previously described (*35*) and run on an SGI Octane 2 computer with Insight II 2000 (MSI, San Diego, CA). The starting point for the simulations was the crystal structure of the P99 β-lactamase with a phosphonate inhibitor covalently bound to the active site serine residue [PDB entry 1bls (*12*)]. The pentacoordinated vanadate dianion was constructed using the V−O bond distances and angles calculated by Krauss and Basch (*36*), and the positions of the vanadium atom and the five oxygen ligands were fixed in the subsequent computations; this procedure was necessary since molecular mechanics parameters for this type of structure were not available. Molecular dynamics (20 ps) followed by molecular mechanics energy minimization on a typical snapshot was then performed.

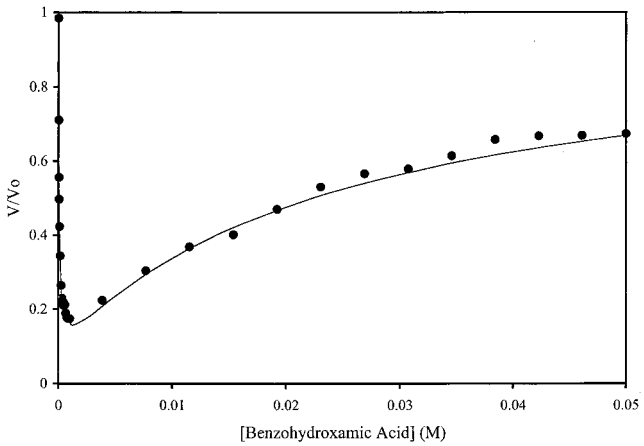


FIGURE 2: Effect of higher concentrations of benzohydroxamic acid on the inhibition of the P99 β-lactamase produced by 0.3 mM vanadate and the hydroxamate. The points represent experimental data, and the solid line represents the fit to Scheme 5 calculated with Dynafit (*34*).

RESULTS AND DISCUSSION

As communicated previously (*26*), the class C β-lactamase of *E. cloacae* P99 is inhibited by mixtures of vanadate ($H_2VO_4^-$) and benzohydroxamic acid at submillimolar concentrations. Other hydroxamic acids, described in more detail below, also produce this inhibition. For example, Figure 1 shows the inhibition produced by 4-nitrobenzohydroxamic acid when it is mixed with vanadate. Neither vanadate nor hydroxamic acid alone is an inhibitor at the concentrations that were employed. The inhibition is fast and reversible on a manual mixing time scale. A kinetics Job plot (β-lactamase activity vs a continuous variation of vanadate and benzohydroxamic acid concentrations; Figure 1, inset) showed that the inhibitory species derived from a 1:1 combination of vanadate with hydroxamic acid. We have shown (*33*) that vanadate forms both 1:1 and 1:2 complexes with benzohydroxamic acid in aqueous solution at pH 7.5 and that the former predominates at submillimolar concentrations. Thus, it is likely that the inhibitory species is a 1:1 complex of vanadate and the hydroxamic acid. Further evidence favoring this conclusion is seen in Figure 2 where enzyme activity is plotted against benzohydroxamic acid concentration where the latter is extended to 50 mM. The inhibition seen at low benzohydroxamic acid concentrations is lost at higher concentrations. This strongly suggests that an inhibitory 1:1 complex is supplanted by a noninhibitory 1:2 complex at higher benzohydroxamic acid concentrations. The solid line describes the fit of these data to such a model (see below).

At low concentrations of the 1:1 vanadate−hydroxamic acid complex (VH), calculated as described elsewhere (*33*), the pattern of inhibition appears to be competitive (Figure 3A), although at higher concentrations, a noncompetitive element appears (Figure 3B; note the intercept is different compared to Figure 3A). The latter, presumably arising from the binding of VH at a site other than that immediate to catalysis, is perhaps not surprising. Peptides and peptide-like molecules are known to bind to an extended substrate-binding region on this enzyme, as well as at the catalytic site (*37*−*39*).

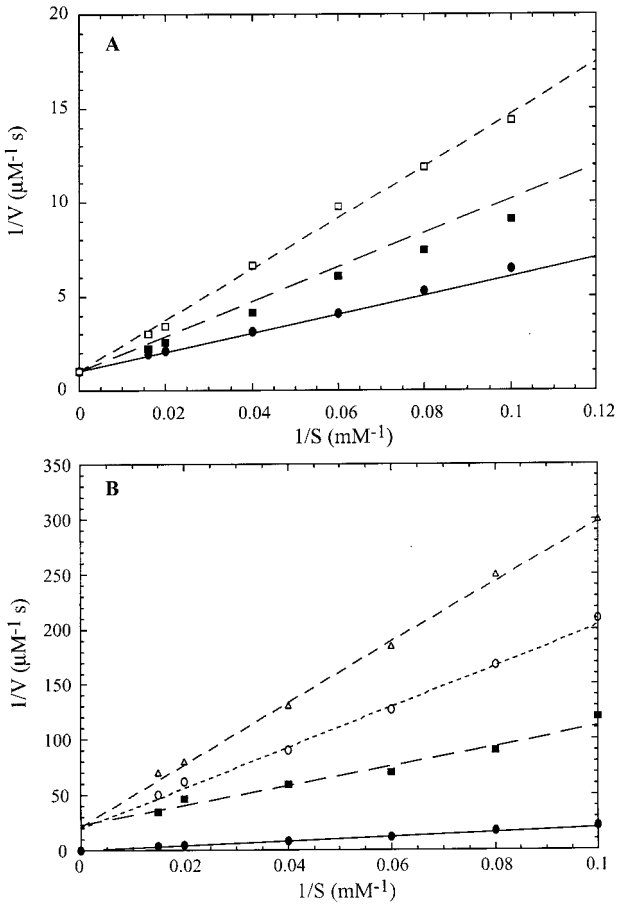
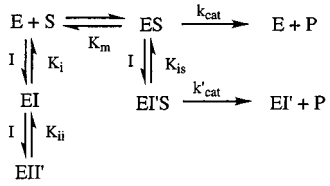


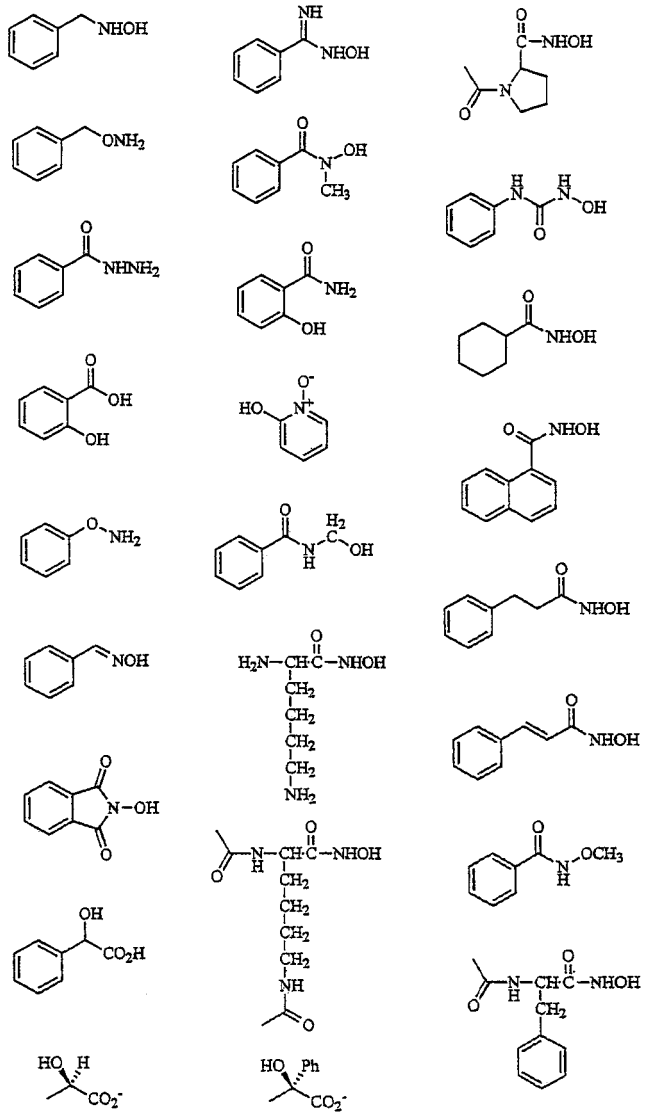
FIGURE 3: Double-reciprocal plots showing the inhibition patterns produced by vanadate (V)/benzohydroxamic acid (H) mixtures affecting the hydrolysis of nitrocefin by the P99 β-lactamase. (A) Low vanadate and hydroxamic acid concentrations: 0 mM V and H (●), 0.125 mM V and 0.038 mM H (■), and 0.125 mM V and 0.085 mM H (□). (B) High vanadate and hydroxamic acid concentrations: 0 mM V and H (●), 1 mM V and 0.3 mM H (△), 1 mM V and 0.7 mM H (○), and 1 mM V and 1 mM H (■). The lines were calculated from inhibition parameters (see the text) derived from treatment of the data by the method of Cleland (*51*).

Scheme 3



The data of Figure 3 are most simply interpreted in terms of Scheme 3. The important features of this scheme are that there are two binding sites for I, one of which is competitive with the substrate for the active site, and the other is separate from the active site. Binding in the latter site, however, does still permit substrate binding at the active site to form EI′S, and this ternary complex is productive (k_{cat}'), as shown by the intersection of the lines in Figure 3B at the $1/V$ axis. These data show that the binding of I at the second site is some 3000-fold weaker than the binding that is competitive with substrate (i.e., $K_i/K_{ii} = 0.003$) and the EI′S complex is 12 times less productive than ES (i.e., $k_{cat}/k_{cat}' = 12$). Attempts to directly detect additional binding of VH by (dansylamido)methylphosphonate fluorescence perturbation (*39*) were unsuccessful however; VH bound at its second site must not affect the fluorescence of the dansyl phosphonate covalently bound at the active site.

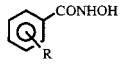
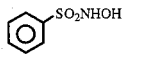
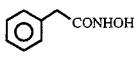
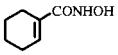
Scheme 4



A few experiments were performed to explore the variation of the inhibition with pH. The value of the "real" K_i (see below) of the vanadate−benzohydroxamic acid complex did not vary significantly within experimental uncertainty (±30%) between pH 6.5 and 8.5 but increased at higher pH, presumably due to the decrease in the concentration of the active enzyme form (*40*), the weakening of the vanadate−hydroxamic acid complex in solution (*33*), or both.

Structure−Activity Relationships of Noninhibitors. Scheme 4 shows the structures of compounds that, when mixed with vanadate (1 mM each) at pH 7.5, did not inhibit the P99 β-lactamase. There are two obvious reasons for the failure of any particular hydroxamic acid analogue to act as an inhibitor. The compound may not form a complex with vanadate, or the complex may not have the correct structure to be an inhibitor. The latter could occur because the required chemical functionality of the complex is not available to interact with the enzyme, or the complex, although containing the correct functional structure, is not of a size or shape to fit tightly enough into the active site. We did not attempt to distinguish between these possibilities for most of the

Table 1: Apparent and Real Inhibition Constants

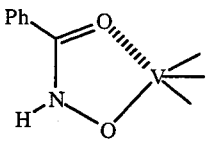
Hydroxamic Acid		K_i^{app} (μM)[a]	K_i (μM)[b]
	R = H	55±3	6.0±0.1
	R = 2-OH	35±11	c
	R = 4-NO_2	5.3±1.0	0.48±0.05
	R = 4-OMe	28±9	1.58±0.03
		378±5	c
		392±6	1.15±0.02
		265±10	c
$CH_3CONHOH$		$(1.0\pm0.2)\times10^3$	120±10
DL-PheNHOH		$(2.1\pm0.1)\times10^3$	c

[a] Determined from Scheme 1. [b] Determined from Scheme 5. [c] Not determined.

compounds of Scheme 4. Certainly, one would expect all of them to form complexes with vanadate, although perhaps only to a small extent at millimolar concentrations. α-Amino acid and small peptide hydroxamates, for example, do not appreciably complex with vanadate under these conditions (ref *41* and unpublished observations of J. H. Bell and R. F. Pratt); this fact alone could lead to our inability to observe inhibition by several such compounds.

Some general points, however, can be made with respect to Scheme 4. Benzoic hydrazide, the aza analogue of benzohydroxamic acid, had no inhibitory activity, nor did other potential nitrogen ligands. Simple nitrogen ligands generally bind more weakly to vanadate than do oxygen ligands (*18*). N-Substituted hydroxamic acids were not inhibitory. This category includes, particularly, *N*-methylbenzohydroxamic acid. Because of the close similarity between this compound and its inhibitory parent, and the relationship of this to β-lactamase substrate specificity (see below), we did look into the ability of this compound to form vanadate complexes.

Absorption spectra indicated the formation of two complexes with absorption maxima at 270 and 510 nm. Job analyses indicated that the former represented a 1:1 vanadate−hydroxamic acid complex and the latter a 1:2 complex. Spectrophotometric titrations, performed in the same fashion as those of the parent acid (*33*), gave K_1 and K_2 values of 2100 ± 40 and 420 ± 60 M^{-1}, respectively. These values are very similar to those obtained for benzohydroxamic acid (*33*), viz., 3000 and 420 M^{-1}, respectively. A ^{51}V NMR spectrum of a solution containing 0.75 mM vanadate and 1.0 mM *N*-methylbenzohydroxamic acid with V, VH, and VH_2 concentrations calculated from the above equilibrium constants of 0.29, 0.32, and 0.09 mM, respectively, displayed a single resonance, presumably due to VH, at −518 ppm. This too is similar to the resonance of the analogous benzohydroxamic acid resonance at −498 ppm (*33*). These results therefore indicate that *N*-methylbenzohydroxamic acid forms vanadate complexes with structures similar to those formed by benzohydroxamic acid itself and add further support to a hydroxamate **6** rather than hydroximate **7** structure for the latter complexes.



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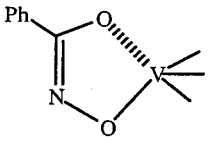

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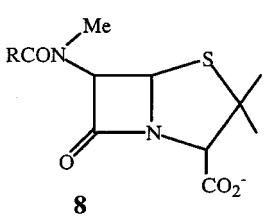
A hydroxamate structure for the latter is clearly indicated by $^1H-^{15}N$ HSQC spectroscopy (*33*).

It is now clear then that although *N*-methylbenzohydroxamic acid forms vanadate complexes very similar to those from benzohydroxamic acid, the former complexes do not inhibit the P99 β-lactamase whereas the latter do. The *N*-methyl substituent could certainly preclude effective binding at the active site in the mode depicted in **5**. It is known that the *N*-methylpenicillin **8** does not have antibiotic activity (*42*), i.e., does not bind to penicillin-binding proteins, and **9** is not a β-lactamase substrate (R. F. Pratt, unpublished).



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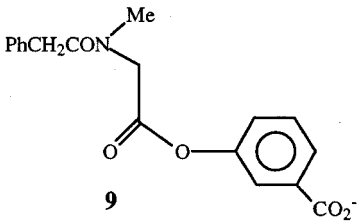

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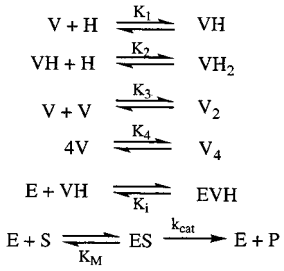
The impotence of the *N*-methylbenzohydroxamic acid−vanadate complex as an inhibitor thus supports the proposed mode of inhibition by means of complex **5**.

Structure−Activity Relationships of Inhibitors. Table 1 contains the structures of ligands that generated β-lactamase inhibitors in the presence of vanadate, along with their apparent K_i values (Scheme 2 in Materials and Methods). A combination of the qualitative results of Table 1 with those described above with reference to Scheme 4 indicates that the hydroxamic acid moiety (-CONHOH) is necessary, but perhaps not sufficient, to produce an inhibitor. Aliphatic hydroxamic acids appear to be less effective than aromatic (see below, however), but the presence of a planar sp^2 carbon α to the hydroxamic acid carbonyl appears to generate activity. This is present in the aromatic hydroxamic acids of course and also in cyclohexene-1-hydroxamic acid which has some activity, but not in cyclohexanehydroxamic acid which does not (Scheme 4). Spectrophotometric investigation of mixtures of vanadate and cyclohexanehydroxamic acid showed the presence of complexes with spectra and strength similar to the spectra and strength of those formed between vanadate and acetohydroxamic acid; the former complexes therefore cannot be inhibitory. Elaboration of the aromatic group of benzohydroxamic acid does uncover some apparently more effective inhibitors, in particular, 4-nitrobenzohydroxamic acid. The sulfonyl analogue of benzohydroxamic acid, *N*-hydroxybenzenesulfonamide, appears to be less effective than the carbonyl compound. This result mirrors the generally poorer performance of sulfonamido β-lactams as antibiotics and β-lactamase substrates (*43*).

Scheme 5



Real versus Apparent Inhibition Constants. The K_i^{app} values of Table 1 are apparent in the sense that their calculation from the data assumed that all free vanadate in solution was in the form of the monomer. This is not true of course (*33*). With the assumption taken from the evidence described above that the inhibitory species is indeed the 1:1 complex VH, Scheme 5 was used to obtain more accurate K_i values for this complex from inhibition data such as those shown in Figure 1. In Scheme 5, VH and VH_2 represent the 1:1 and 1:2 vanadate−hydroxamic acid complexes, respectively, V_2 and V_4 are divanadate and tetravanadate, respectively, EVH is the competitive inhibitory complex, and S is the substrate (nitrocefin) turned over by free enzyme E to product P. Values of K_3 and K_4 (310 M^{-1} and 3.0×10^8 M^{-3}, respectively) were from the literature (*30*); values of K_1 and K_2 were available for several hydroxamic acids (*33*), and thus, K_i values could be obtained in these cases by iterative least-squares curve fitting [Dynafit (*34*)] to inhibition data such as those shown in Figure 1. The real K_i values obtained are also presented in Table 1. Inspection of these values shows, first, not surprisingly, that all VH inhibitors are better than they appear. In fact, in one case, that of 4-nitrobenzohydroxamic acid, the best of those studied, the K_i value is submicromolar. A second interesting observation is that when the stability of VH is taken into account, the complex derived from the aliphatic phenylacetohydroxamic acid is as effective an inhibitor as those from the purely aromatic compounds. This result is certainly more in line with general β-lactamase specificity and thus **5**. It could be, therefore, that a number of the compounds in Scheme 4 would be observed to inhibit if their complexes with vanadate were stronger. Finally, no clear electronic effect is seen in the K_i values of benzohydroxamic acid and its 4-methoxy and 4-nitro derivatives; both of the latter are more effective inhibitors than the parent compound. The order of effectiveness here then probably reflects the specificity of interaction of these compounds with the enzyme.

Kinetics of Formation and Dissociation of EVH. A stopped-flow experiment was performed to determine the rate constants for formation and dissociation of the EVH complex. Figure 4 shows that there is a distinct lag in the formation of the product when a β-lactamase/vanadate/4-nitrobenzohydroxamic acid mixture is reacted with a good substrate of the enzyme (nitrocefin). The linear initial rate in the absence of the vanadate−hydroxamic acid complex is also shown for comparison. The lag in product formation is interpreted in terms of a slow dissociation of the inhibitor from the enzyme. When the curve shown in Figure 4 is fit to Scheme 5, rate constants of formation (k_f) and dissociation (k_d) of EVH with the free enzyme were found to be 1.48×10^6 s^{-1} M^{-1} and 0.73 s^{-1}, respectively. These rate constants give a

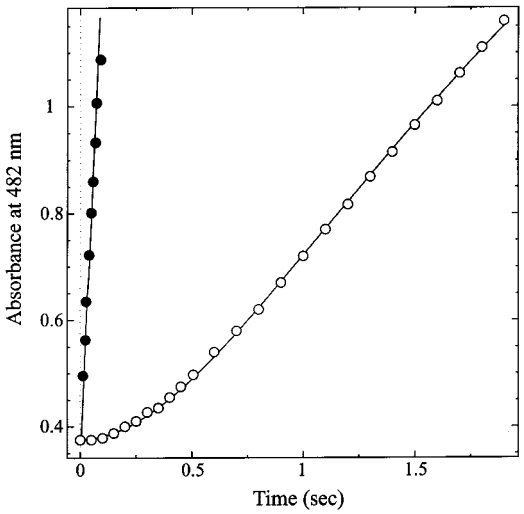


FIGURE 4: Absorption changes observed on mixing equal volumes of solutions containing 200 μM nitrocefin and 0.67 μM P99 β-lactamase, the latter in the absence (●) and presence (○) of 20 μM vanadate and 20 μM 4-nitrobenzohydroxamic acid. The points are experimental, and the lines were calculated with Dynafit (*34*) and Scheme 5.

value for K_i ($=k_d/k_f$) of 0.48 μM which is in excellent agreement with that obtained from steady state measurements (Table 1).

The mechanism of formation of EVH could involve direct reaction of E with VH. Also possible would be a stepwise process of reaction of the enzyme with vanadate followed by reaction of that complex with H. Although there is no direct evidence for either at present, the latter seems less likely since vanadate alone at concentrations up to 1 mM does not inhibit the enzyme. The slow dissociation of VH from the enzyme is certainly suggestive of a covalent interaction between E and VH, as would be found in **5**. It is informative to compare the above rate constants with those for exchange of ethanol into the coordination sphere of vanadate, for which the rate constants ($k_f = 115$ s^{-1} M^{-1} and $k_d = 1.2 \times 10^3$ s^{-1}) have been determined (*45*). The much greater rate constant for formation of EVH than of ethyl vanadate suggests that the enzyme catalyzes formation of this complex. This would favor a mechanism of direct reaction at the active site serine since that is where the activated nucleophile resides; the latter can certainly attack carbonyl and phosphyl centers with enhanced rates. The slow dissociation to re-form the free enzyme presumably reflects the specificity of binding for this complex available at the active site.

Structure of the EVH Complex. The ^{13}C NMR spectrum of 5 mM 4-nitrobenzo[^{13}C]hydroxamic acid in the presence of 1 mM vanadate and 0.25 mM P99 β-lactamase in 5 mM MOPS buffer at pH 7.5 is shown in Figure 5A. Three carbonyl resonances, at 173.24, 174.04, and 176.86 ppm, are observed. From a comparison with the spectrum in the absence of enzyme (*33*), the upfield and downfield resonances are assigned to free VH and free hydroxamic acid, respectively, with a coordination-induced shift (CIS) of 3.62 ppm therefore produced on formation of the complex. As discussed elsewhere (*33*), a CIS of this magnitude indicates only weak, if any, interaction between the hydroxamate carbonyl and vanadium. It is noticeable that both the free and complexed resonances have moved downfield by ca. 7

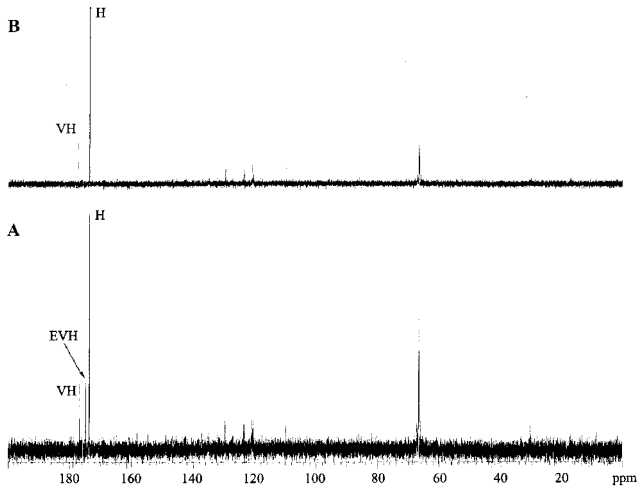


FIGURE 5: (A) ^{13}C NMR spectrum of a mixture of vanadate (V, 1 mM), 4-nitrobenzo[^{13}C]hydroxamic acid (H, 5 mM), and the P99 β-lactamase (E, 0.25 mM). Calculated concentrations of H, VH, and EVH at equilibrium (Scheme 5) were 3.9, 0.71, and 0.249 mM, respectively. (B) As in panel A after the addition of *m*-nitrophenyl phenylacetamidomethylphosphonate (1 mM) as described in the text.

ppm in the presence of the enzyme [the corresponding chemical shifts in the absence of enzyme were 166.30 and 170.08 ppm (*33*)]. This probably reflects the presence of fast reversible interaction between both free and complexed ligand and the enzyme. Such chemical shifts have been observed previously (*46*). Bovine serum albumin (1 mM) had a similar effect in the present case. The resonance of the 174.04 ppm chemical shift in Figure 5A is assigned to the EVH complex. In accord with this assignment, its line width (5.2 Hz) exceeds that of the upfield and downfield resonances (1.8 and 1.2 Hz, respectively), indicating some degree of immobilization of the carbonyl group in the ternary complex.

The assignment of the 174.04 ppm resonance to EVH was confirmed by an experiment where *m*-nitrophenyl phenylacetamidomethylphosphonate was added to the NMR tube (final concentration of 1 mM) containing the sample described above. This compound is an essentially irreversible covalent inhibitor of the P99 β-lactamase which rapidly phosphonylates the active site serine residue (*9*). A ^{13}C NMR spectrum of the reaction mixture immediately after addition of the phosphonate is shown in Figure 5B. Peaks assigned to free hydroxamic acid and VH remain, but the resonance at 174.04 ppm has disappeared. This observation confirms the assignment of this peak to EVH and supports the proposal of a competitive interaction between active site ligands and VH; i.e., the inhibition by VH is caused by its binding to the active site of the enzyme.

From the point of view of structure, the important parameter is the chemical shift of the bound inhibitor. With respect to the chemical shift value of the carbonyl carbon in the free hydroxamic acid in the absence of enzyme, a CIS value of 7.74 ppm is therefore observed upon binding the hydroxamic acid to both vanadium and the enzyme. This value suggests either a stronger chelation of the hydroxamic acid carbonyl to vanadium in the EVH complex than in VH in solution or, alternatively, polarization of the carbonyl group by another mechanism on binding of VH to the enzyme. These alternatives are further discussed below.

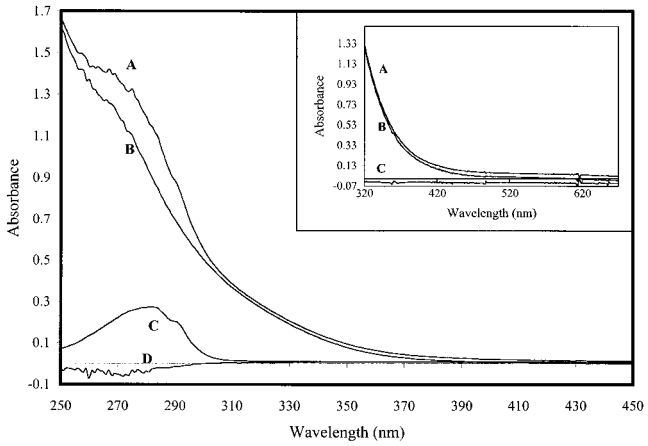


FIGURE 6: (A) Absorption spectrum of a mixture of 0.3 mM vanadate, 1 mM benzohydroxamic acid, and 0.2 mM P99 β-lactamase in 20 mM MOPS buffer (pH 7.5). The path length of the cuvette that was employed was 0.2 cm. (B) Absorption spectrum of a mixture of 0.3 mM vanadate and 1 mM benzohydroxamic acid under the same conditions as described for panel A. (C) Absorption spectrum of 0.2 mM P99 β-lactamase under the same conditions as described for panel A. (D) A difference spectrum obtained by subtraction of the spectra of B and C from that of A; it should represent the difference spectrum between an enzyme-bound and free vanadate−hydroxamic acid complex. The inset shows longer-wavelength spectra taken in a 1 cm path length cuvette. A and B contained the same mixtures as those above. C represents the difference spectrum between A and B.

The absorption spectrum of EVH also held structural information. Figure 6 shows the relevant spectra in the benzohydroxamic acid case. No absorption corresponding to EVH is seen beyond 400 nm (Figure 6, inset), and thus, the enzyme-bound species cannot be the 1:2 complex, a result in accord with all other evidence. The EVH complex does appear to absorb around 270 nm, however, as does vanadate itself and VH. Subtraction of spectra of E and (V + H) from that of (E + V + H) should yield, at the specified concentrations employed for Figure 6, a difference spectrum of the bound inhibitor versus free VH in solution. As seen in Figure 6, the difference spectrum contains essentially no absorption above 250 nm. The conclusion must be that the enzyme-bound inhibitor has essentially the same absorption spectrum as free VH, viz., a peak at ca. 270 nm (*33*). Therefore, the structure of the bound ligand may be very similar to that of the VH complex in solution.

Finally, the ^{51}V NMR spectrum of an EVH complex is shown in Figure 7. This represents the spectrum taken of an enzyme/4-nitrobenzohydroxamic acid/vanadate mixture which should contain, according to calculations based on Scheme 5, 0.015 mM V, 0.003 mM VH, and 0.282 mM EVH, i.e., essentially all EVH. The chemical shift of the enzyme-bound vanadium complex is thus −494 ppm. The chemical shift of the analogous complex of benzohydroxamic acid (not shown) was also approximately −490 ppm. In free solution, the chemical shifts of the VH complex of 4-nitrobenzohydroxamic acid and benzohydroxamic acid were −497 and −498 ppm, respectively (*33*). Apparently, the environment of vanadium is not greatly changed on binding of these complexes to the enzyme.

Concluding Discussion. The class C β-lactamase of *E. cloacae* P99 is rather effectively inhibited by mixtures of vanadate and certain, particularly aryl, hydroxamic acids. The

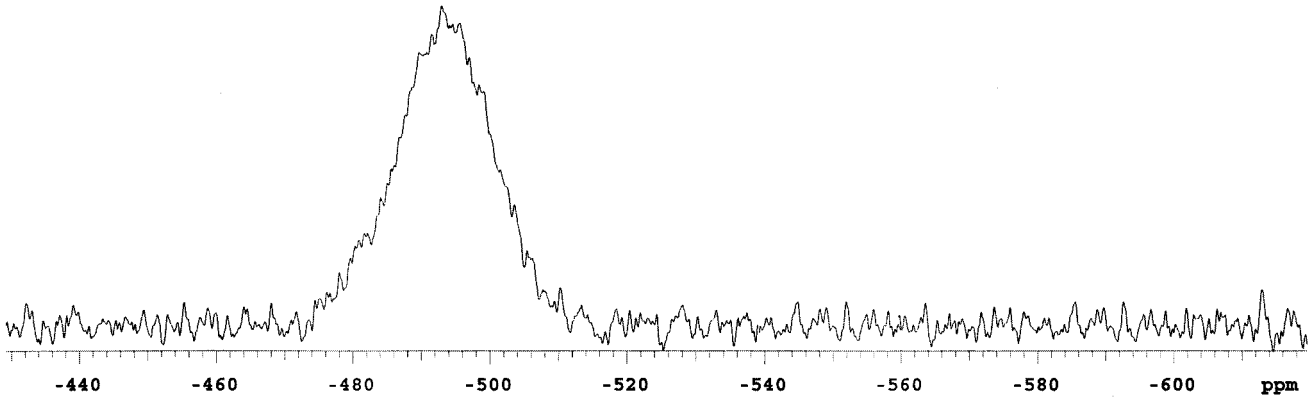


FIGURE 7: ^{51}V NMR spectrum of the enzyme-bound 1:1 vanadate−4-nitrobenzohydroxamic acid complex taken in 20 mM MOPS buffer in ^2H_2O at p^{2}H 7.5 and at 25 °C. The sample contained 0.3 mM vanadate, 0.3 mM 4-nitrobenzohydroxamic acid, and 0.328 mM P99 β-lactamase. Calculated concentrations of free vanadate monomer, free complex, and bound complex at equilibrium (Scheme 5) were 0.015, 0.003, and 0.282 mM, respectively.

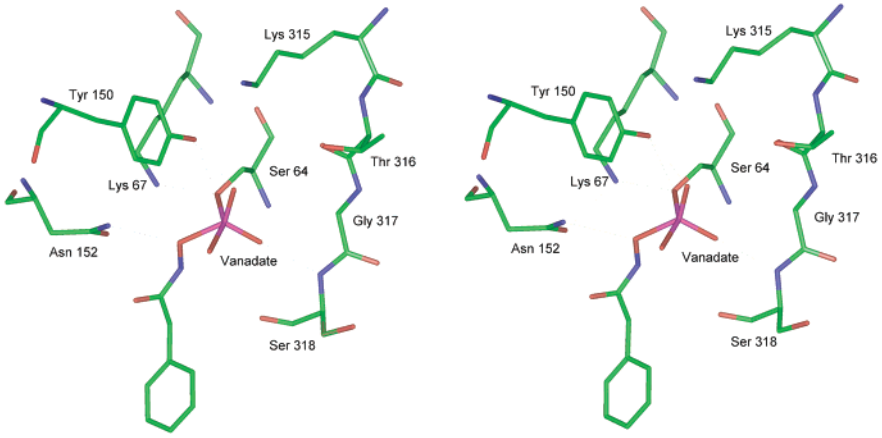
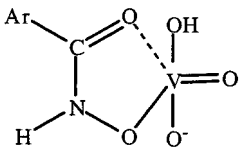


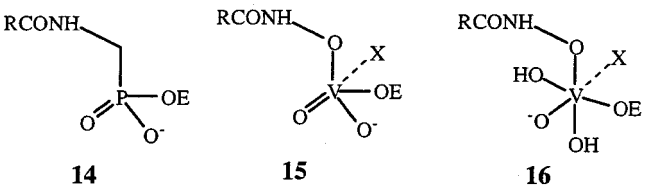
FIGURE 8: Stereoview of the energy-minimized ternary complex of vanadate, phenylacetohydroxamic acid, and the P99 β-lactamase. In this view, the (five-coordinated) vanadium is coordinated to the hydroxamic acid, the active site Ser 64 hydroxyl group, and three hydroxyl groups, two of which are dissociated and thus anionic; one of the latter occupies the oxyanion hole.

inhibition, which is fast and reversible on a manual mixing time scale (*26*), is caused by a 1:1 complex of the latter two species. Such complexes are found in significant amounts in even submillimolar concentrations of vanadate and aryl hydroxamic acids (*33*). Spectroscopic examination (*33*) suggested the structure of these complexes in solution probably involves weak interaction of the hydroxamic acid carbonyl with a pentacoordinated, **13**, or perhaps hexacoordinated vanadium.



13

The spectroscopic observations reported in this paper suggest that the enzyme-bound inhibitor species has a similar structure with respect to the electronic nature of the environment of the hydroxamate carbonyl and vanadium, but may differ in the nature of the functionality responsible for that environment. The competitive nature of the inhibition at low vanadate concentrations, observed in correlation studies with a substrate on one hand and an active site-directed inhibitor on the other, suggests that the vanadate complex most likely inhibits by interaction with the active site of the enzyme. Since structurally analogous phosphonates inhibit by direct covalent modification of the active serine to form structure **14**, it seems certainly plausible to suggest that the vanadate hydroxamates **13** inhibit by a similar reaction to form **15** or **16**.



14 **15** **16**

In **15** and **16**, X represents a weakly bound oxygen ligand, which may be, as in **13**, the hydroxamic acid carbonyl, another water molecule, or an enzyme functional group, e.g., the Tyr 150 hydroxyl group. If the hydroxamate carbonyl of **15** were occupied, as it is in the phosphonate crystal structure (*12*) and indicated in **5**, in formation of a hydrogen bond to the side chain of Asn 152, this interaction might then be responsible for the increased CIS value of the carbonyl in the enzyme complex. The specificity of hydroxamic acids as vanadate ligands for inhibition certainly suggests this may be true. In the extended conformation required in **5** to achieve this hydrogen bonding, interaction

between vanadium and the carbonyl group would probably not be possible.

If it is assumed that the inhibitory species in solution is **13**, then the best inhibitor achieved to date, the complex of vanadate with 4-nitrobenzohydroxamic acid, has a K_i value of ca. 0.5 μM. If the structure of the bound complex is not closely similar to **13**, particularly with respect to the issue of ligand chelation, then the real K_i of the inhibitory species, therefore a minor component in solution, would be smaller than 0.5 μM. Presumably better inhibitors could be achieved by optimization of the aryl group.

The specific and quite tight binding of **13** to the enzyme may well reflect the resemblance between **15** or **16** and either the tetrahedral carbon intermediate of substrate turnover or the pentacoordinated phosphorus intermediate of phosphonate inhibition. The dissociation constant of 0.5 μM, however (unless this is also, as mentioned above, an apparent value), does not suggest a very close transition state analogue. This could be, in part, because of the replacement of the α-methylene of a substrate or phosphonate inhibitor with a more polar oxygen.

Another factor in determining the value of K_i could be the differences in structure between POX_4 and VOX_4. Calculations suggest that VOX_4 may be significantly different in geometry and charge distribution than POX_4, and thus, pentacoordinated vanadate may not be a very close analogue of phosphoryl transfer transition states (*36*). Recent experiments appear to support this proposition (*47*−*49*). It appears that vanadates may be structurally very plastic in the active site environment of enzymes (*50*), and thus, mutual adaptation of vanadate and enzyme occurs on binding. The resultant complex therefore may or may not strongly resemble the transition state of phosphoryl transfer. Nonetheless, a modeled pentacoordinated vanadate adduct does seem to fit into the active site rather well (Figure 8).

The structure shown is a dianionic vanadium complex with Tyr 150 in the neutral form. There is a tight hydrogen bond between one VO^- and Tyr 150 in the structure shown. The alternative protonation state containing a monoanionic vanadate and anionic Tyr 150 fitted much less strongly into the active site due to the V−OH bond being longer than the V−O^- bond (*23*). The second VO^- is accommodated in the oxyanion hole, although a good hydrogen bond is made only with the backbone NH of Ser 318; a water molecule (not shown) is also strongly associated with this VO^-. The VOH moiety is directed out into solution, mimicking the position of the leaving group in a phosphorylation transition state. The other notable feature of the structure is that the amido side chain is not hydrogen bonded well into Asn 152 and Ser 318 as it is in the phosphonate crystal structure and as believed to occur with substrates. This again is likely a result of the longer V−O bonds. Because of this, perhaps, the NH_2 group of the Asn 152 side chain is well-placed to hydrogen bond to the hydroxamate oxygen. Asn 152 also maintains a hydrogen bond to Lys 67 which, in turn, is hydrogen bonded to Ser 64 O_γ, as in the phosphonate structure.

Thus, although the evidence to date is consistent with a structure such as **15** or **16**, an X-ray structure of the enzyme−inhibitor complex will probably be needed to completely resolve the issues discussed above. The structure of the complex could lead to new insight into how a polar molecule may occupy the active site and inhibit these enzymes.

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