

Inhibition of Serine Amidohydrolases by Complexes of Vanadate with Hydroxamic Acids

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Serine β -lactamases are inhibited by phosphonate monoester monoanions. These compounds phosphorylate the active site serine hydroxyl group to form inert, covalent complexes. Since spontaneous hydrolysis of these phosphonates is generally quite slow, the β -lactamase active site must have considerable affinity for the (presumably) pentacoordinated phosphonyl transfer transition state. Structural analogs of such a transition state might well therefore be effective and novel β -lactamase inhibitors. Complexes of vanadate with hydroxamic acids may be able to achieve such a structure. Indeed, mixtures of these two components, but neither one alone, were found to inhibit a typical class C β -lactamase. A Job plot of the inhibition by vanadate/benzohydroxamic acid mixtures indicated that the inhibitor was a 1:1 complex for which an inhibition constant of 4.2 μ M could be calculated. A bacterial DD-peptidase, structurally similar to the β -lactamase, was also inhibited ($K_i = 22 \mu$ M) by this complex. A similar rationale would suggest that other serine hydrolases might also be inhibited by these mixtures. In fact, chymotrypsin was inhibited by a complex of vanadate with benzohydroxamic acid ($K_i = 10 \mu$ M) and elastase by a complex with acetohydroxamic acid ($K_i = 90 \mu$ M). © 2000 Academic Press

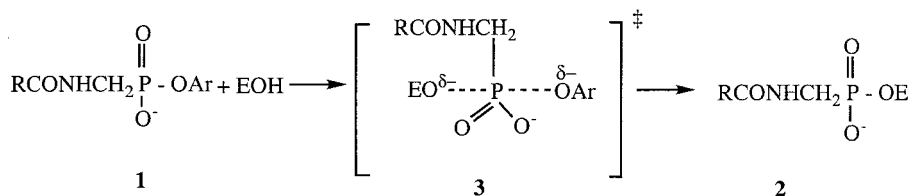
Key Words: enzyme inhibition; vanadate; hydroxamic acid; β -lactamase; DD-peptidase; chymotrypsin; elastase.

Serine β -lactamases are inhibited by phosphonate monoesters of structure 1 (1, 2). These compounds phosphorylate the active site serine hydroxyl group (Scheme 1) to form inert covalent complexes 2 (3, 4) that are believed to be structural analogs of the transition state of the normal acyl transfer reactions catalyzed by these enzymes (3–5). Phosphorylation of the β -lactamase active site by 1 is much faster than spontaneous phosphonate hydrolysis and therefore the β -lactamase active site must have significant affinity for the pentacoordinated phosphonylation transition state 3 (2). Commonly used analogs of phosphoryl transfer transition states are vanadate or vanadate complexes (6), but direct extension of this theme to phosphoryl transfer is not synthetically feasible. We hypothesized however that complexes of hydroxamic acids with vanadate (4) [although this is unlikely to be the dominant form of the complex in free solution (7, 8)] might yield effective analogs (5) of 3, and thus β -lactamase inhibitors, after reaction at the active site (Scheme 2). We report here that 1:1 complexes of hydroxamates and vanadate do indeed inhibit a class C β -lactamase and a structurally similar DD-peptidase. Two representative serine proteases were also inhibited suggesting that such inhibition may be general to serine amidohydrolases.

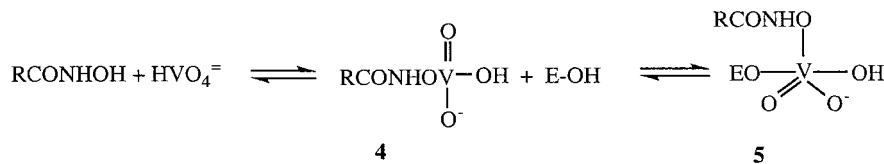
MATERIALS AND METHODS

The β -lactamase of *Enterobacter cloacae* P99 was purchased from the Centre for Applied Microbiology and Research (Porton Down, Wilts., UK). Enzyme activity was determined spectrophotometrically

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SCHEME 1



SCHEME 2

against nitrocefin (Unipath) in 20 mM MOPS, pH 7.5, 25°C. A K_m value for nitrocefin of 50 μM was used in the fitting of data to Scheme 3. Concentrations of enzyme and substrate were ca. 2 nM and 0.1 mM, respectively. A rate equation derived from Scheme 3 was fitted to the data of Fig. 1 by a non-linear least squares program.

Bovine α -chymotrypsin was purchased from Sigma Chemical Co. Enzyme activity was determined spectrophotometrically (9) against *N*-succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide (Sigma) in 0.2 M Tris buffer, pH 8.0. The K_m of the substrate was taken to be 43 μM (9).

Porcine pancreatic elastase was purchased from Sigma Chemical Co. Enzyme activity was determined spectrophotometrically (10) against *N*-succinyl-alanyl-alanyl-prolyl-leucyl-*p*-nitroanilide (Sigma) in 0.1 M Tris, 0.01 M CaCl_2 buffer, pH 7.8. The K_m of the substrate was taken to be 0.49 mM (10).

The ^{51}V NMR spectrum of the enzyme-inhibitor complex was taken in $^2\text{H}_2\text{O}$ at 25°C on a Varian Unityplus-400 spectrometer operating at 105.1 MHz. Data acquisition parameters were: 20 kHz spectral width, 4K data points, 0.05 s acquisition time, 7.8 μs (35.1°) pulse, 0.4 s delay time, 360 K transients accumulated. The sample, in 20 mM MOPS pH 7.5, contained 0.328 mM P99 β -lactamase, 0.8 mM vanadate, and 1.0 mM benzohydroxamic acid.

RESULTS AND DISCUSSION

Shown in Fig. 1 is the effect of varying concentrations of benzohydroxamic acid on the initial activity of the class C β -lactamase of *Enterobacter cloacae* P99 in the presence of fixed concentrations (0.1 and 0.03 mM) of vanadate. Neither vanadate or hydroxamic acid alone had any effect on the enzyme activity (also shown). A Job plot (Fig. 2) of β -lactamase activity vs hydroxamic acid (0–0.1 mM) and vanadate (0.1–0

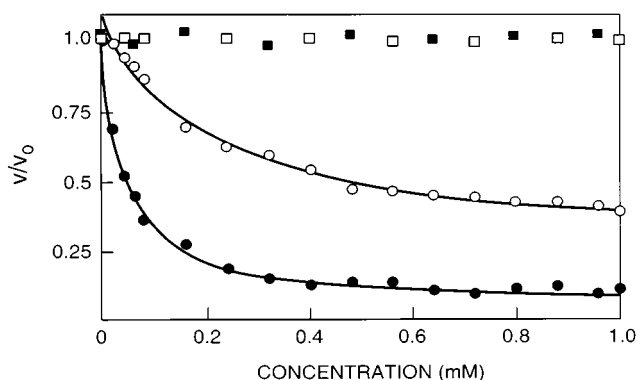


FIG. 1. Inhibition of turnover of nitrocefin by the P99 β -lactamase in the presence of 0.03 mM (○) and 0.1 mM (●) vanadate as a function of benzohydroxamic acid concentration. Also shown is the effect of vanadate alone (□) and benzohydroxamic acid alone (□) on the enzyme activity.

mM) concentrations suggested that the inhibitor is a 1:1 complex of the components. The loss of activity was not time dependent and could be reversed by dilution. The data of Fig. 1 was therefore fitted to Scheme 3, where V represents vanadate, H the hydroxamic acid, E the enzyme, and S the assay substrate.

Only a 1:1 complex, VH, was included in these calculations because this stoichiometry dominated at the concentrations employed ($[\text{benzohydroxamic acid}] \leq 1$ mM). At higher concentrations of vanadate and benzohydroxamic acid, a red color ($\lambda_{\text{max}} = 520$ nm) in solution was observed; a Job plot of this absorption data (not shown) indicated a 2:1 hydroxamate/vanadate ratio in these complexes. Absorption titration curves indicated a VH_2 dissociation constant of around 65 mM. Hydroxamic acids are well-known to form 2:1 complexes with vanadate, although more strongly at low pH (7, 8). Application of Scheme 3 to the data of Fig. 1 yielded K_1 and K_i values of 1240 M^{-1} and 4.2 μM respectively. Since Scheme 3 fitted the data adequately (Fig. 1) at several vanadate concentrations of 1 mM and less, polymerized vanadate species are not involved in the inhibition.

In Table 1, inhibitory data for a series of relevant ligands is presented. These results indicate the requirement for the hydroxamic acid–CONHOH moiety for inhibition, in accord with the original concept, 5. The apparently greater inhibitory activity of aromatic

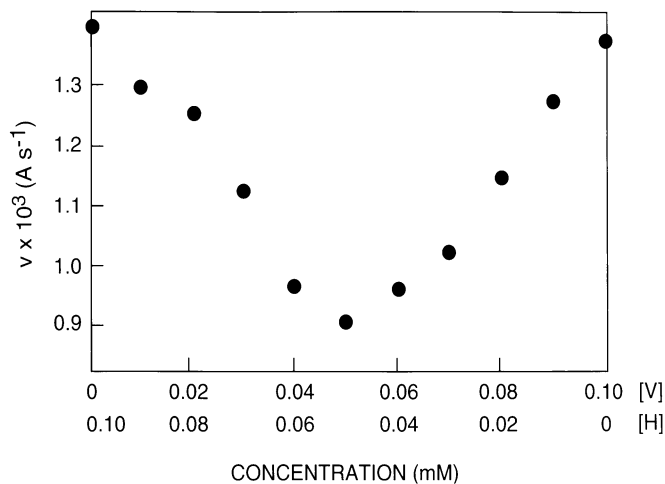
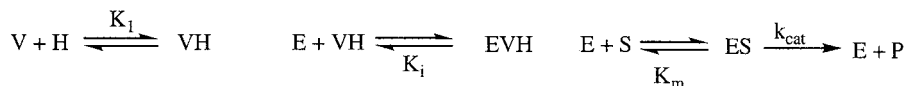


FIG. 2. A Job plot of the effect of continuous variation of the vanadate concentration, [V], and benzohydroxamic acid concentration, [H], on the activity of the P99 β -lactamase against nitrocefin.



SCHEME 3

hydroxamic acids is also in accord with the side chain specificity of the β -lactamase active site. The benzohydroxamic acid/vanadate mixture did not inhibit the class A TEM β -lactamase but did inhibit the DD-peptidase of *Streptomyces* R61 ($K_i = (22 \pm 5) \mu\text{M}$), an enzyme that is structurally similar to the P99 β -lactamase (10).

Mixtures of hydroxamic acids and vanadate also inhibited representative serine proteases in a similar fashion (Scheme 3). These enzymes of course are also inhibited by phosphonate esters, although much more strongly by diesters than monoesters (2). The K_i of the 1:1 benzohydroxamic acid complex of vanadate against α -chymotrypsin was $(10 \pm 1) \mu\text{M}$ at pH 8.0 and that of acetohydroxamic acid against porcine pancreatic elastase was $(91 \pm 9) \mu\text{M}$ at pH 7.8; benzohydroxamic acid and vanadate appeared to have no activity against the elastase. It will be of interest to see whether the subsite specificity of these enzymes is apparent in any inhibition by vanadium complexes of extended peptide hydroxamic acids.

Although the evidence mentioned above suggests that a 1:1 vanadate/hydroxamate complex is responsible for the β -lactamase inhibition, it is not clear that the inhibitory species is necessarily the dominant 1:1 species in solution; the inhibitory power of the active complex might therefore be greater than indicated by the data in Table 1. Since vanadate complexes with simple ligands are labile in solution (12), knowledge of the structure of the dominant species present in these solutions does not necessarily and directly reveal the structure of the enzyme-bound complex. Absorption spectra of the inhibited enzymes show that they are not

colored, i.e., do not have $\lambda_{\text{max}} > 300 \text{ nm}$. This suggests that the structure of the inert complex does not involve a hydroxamate or hydroximate chelate; the structurally well-characterized vanadate hydroxamates all possess this feature (7, 8, 13, 14). ^{51}V spectra of both the β -lactamase and α -chymotrypsin complexes contained a broad resonance with maximum intensity around -510 ppm (vs VOCl_3). This is consistent with, but does not unequivocally prove, the presence of a 5-coordinated dioxovanadium species (6, 15); it is, however, similar to the ^{51}V NMR resonance of most likely pentacoordinate (16) vanadium in the inosine/vanadate/ribonuclease T_1 complex (17). Tetrahedral vanadate at the active site is also possible. Detailed knowledge of the structure of the inhibitory complexes will probably require crystallographic studies.

A new class of inhibitors of serine amidohydrolases has thus been described. It is not clear at present whether the original rationale for their mode of action (Scheme 2) is correct, but studies of the structure of these inhibitors at representative active sites may suggest new directions in the design of inhibitors, phosphonate and otherwise, of these enzymes. The rationale employed here in the design of 4 may generally apply to enzymes where inert covalent complexes are formed by chemistry (and thus transition states) that differ from those employed during the catalysis of reactions of normal substrates.

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TABLE 1

Inhibition of the P99 β -Lactamase by Hydroxamic Acids with Vanadate^{a,b}

Ligand	$K_i/\mu\text{M}$
PhCONHOH	4.2 ± 0.3
CH ₃ CONHOH	60 ± 7
PhCH ₂ CONHOH	15 ± 5
PhCON(Me)OH	— ^c
PhCH ₂ NHOH	— ^c
PhCH ₂ ONH ₂	— ^c
PhCONHNH ₂	— ^c
PhCH=NOH	— ^c

^a 20 mM MOPS buffer, pH 7.5, 25°C.

^b Vanadate concentrations 1 mM or less.

^c No inhibition at ligand concentrations to 1 mM.

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