Thiols as Classical and Slow-Binding Inhibitors of IMP-1 and Other Binuclear Metallo-β-lactamases†

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ABSTRACT: The inhibitory effect of a variety of thiol compounds on the function of binuclear metallo-β-lactamases, with a particular focus on IMP-1 from *Pseudomonas aeruginosa*, has been investigated. Thiol inhibitors, depending on their structural features, fall into two categories, one in which inhibition at neutral pH was instantaneous and the other in which inhibition was time-dependent. While mercaptans with anionic substituents in the vicinity of their SH groups exhibited the former type of inhibition, neutral thiols appear to induce a slow, time-dependent isomerization of the initially formed EI complex to a tighter EI* complex. Kinetic parameters describing the latter process were obtained by fitting progress curves of substrate hydrolysis using standard and numerical procedures. The failure of charged thiols to exhibit slow binding is suggested to be due to a rapid isomerization of the initial EI complex. Slow binding in the case of neutral thiols was observed only below pH 8. Studies on the pH dependence of catalysis by IMP-1 revealed that (i) enzyme inactivation at low pH is a slow process with presumably two groups with a pK_a of ~5.2 in the protein being responsible for the loss of activity, (ii) inhibition by thiols is independent of pH between pH 5 and 9, and (iii) an apparent enhancement of the catalytic activity of IMP-1 by thiols occurs at pH <5. The last mentioned phenomenon is explained by a model in which mercaptans retard the proton-dependent isomerization of the enzyme. Studies on the thiol-mediated inhibition of the binuclear forms of *Bacteroides fragilis* (CcrA) and *Bacillus cereus* (BcII strain 5/B/6) metallo-β-lactamase have revealed that while CcrA was instantaneously albeit moderately inhibited by mercaptans, BcII mimicked IMP-1 in its interaction with thiols. These differences are proposed to be due partly to the structural divergence of these proteins in the vicinity of Zn2.

β-Lactamases, enzymes capable of deactivating β-lactam antibiotics by hydrolysis of their endocyclic amide bond, have been identified as one of the major factors contributing to the emergence of microbial resistance to β-lactam antibiotics (*1*−*3*). To date, four distinct classes of these enzymes, designated A−D, have been identified (*4*). While enzymes of the A, C, and D families rely on the function of a serine residue as a nucleophile in their catalytic mechanism (*5*), the members of class B are metalloproteins requiring one or two Zn(II) ions for their function (*6*, *7*).

Initial efforts directed toward the identification of either naturally occurring or synthetic inhibitors of class A enzymes resulted in the selection of penicillanic acid sulfones (e.g., sulbactam and tazobactam) and clavulanic acid (*1*, *8*), for achieving the extended use of β-lactam antibiotics in clinical therapy (*9*). More recently, metallo-β-lactamases (MBLs)[1] have received considerable attention in view of their ability to utilize most β-lactams, including clavulanic acid, penicillanic acid sulfones (*10*), and carbapenems (*11*, *12*), β-lactam antibiotics resistant to most class A, C, and D enzymes, as their substrates.

While most MBLs isolated to date are encoded by chromosomes, some, such as the IMP and VIM enzymes found in certain strains of *Pseudomonas aeruginosa*, *Serratia marcescens*, and other Gram-negative bacteria, are of plasmidic origin (*12*). On the basis of differences in their primary structures, MBLs have been classified recently into three subgroups (*13*). While the B1 subfamily comprises some of the most extensively investigated enzymes (the commonly employed abbreviations given in parentheses) such as those from *Bacteroides fragilis* (CcrA), *Bacillus cereus* (BcII), *P. aeruginosa* (IMP-1 and VIM-1), *Chryseobacterium meningosepticum* (BlaB), and *Chryseobacterium indologenes* (IND-1), subclass B2 enzymes are found only in certain strains of *Aeromonas* (CphA and ImiS) and *Serratia* (Sfh-1). The most

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[1] Abbreviations: ACV, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine; AMT, buffer consisting of 50 mM acetic acid, 50 mM MES, and 100 mM Tris; BAL, British Anti-Lewisite; BSA, bovine serum albumin; DMPS, 2,3-dimercaptopropanesulfonic acid; DMSA, *meso*-2,3-dimercaptosuccinic acid; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MBL, metallo-β-lactamase; MES, 2-(*N*-morpholino)-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCEP, tris(2-carboxyethyl)phosphine; Tris, tris(hydroxymethyl)aminomethane; μ, ionic strength; v_0, initial rate of substrate hydrolysis; v_S, steady state rate of substrate hydrolysis.

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prominent members of subfamily B3 enzymes are those from *Stenotrophomonas maltophilia* (L1) and *Legionella gormanii* (FEZ-1). Crystal structures of several MBLs have been reported, including those of CcrA, BcII, IMP-1, and L1, revealing the overall protein fold to be similar (*14*−*18*).

A common feature to all MBLs investigated so far concerns the presence of two distinct Zn(II) binding sites (*6*). In CcrA (*14*), IMP-1 (*17*), and the binuclear form of BcII (*16*), one of the two Zn(II) ions is coordinated by three histidine residues [His116, His118, and His196, numbering according to the recently proposed class B β-lactamase numbering scheme (*13*)], constituting the His, H_3, or Zn1 site. The second Zn(II) ion is ligated by Asp120, Cys221, and His263 as well as a water molecule (DCH or Zn2 site). In addition, a second water molecule is shared by both Zn(II) ions (presumably as a bridging hydroxide ion), resulting in a distorted tetrahedral coordination sphere for Zn1 and a trigonal bipyramidal arrangement for Zn2. This bridging hydroxide ion appears to serve as the nucleophile in the cleavage of the β-lactam bond. In the structure of the mononuclear form of the BcII enzyme, the Zn(II) ion resides in the His site, tetrahedrally coordinated by its three histidine residues and the nucleophilic hydroxide ion (*19*).

The number of Zn(II) ions involved in the protein's catalytic mechanism appears to depend on its origin. For instance, the enzyme from *Aeromonas hydrophila* (CphA) is catalytically competent only with the metal's presence in one of these locations (*20*). In contrast, other MBLs such as CcrA, BcII, IMP-1, and L1 are capable of functioning as binuclear metalloenzymes. Although the mononuclear counterparts of these enzymes have been shown to be catalytically competent as well, the binding of a second Zn(II) ion is accompanied by a moderate enhancement of catalytic activity (*21*−*26*). Despite the noted similarities among CcrA, IMP-1, L1, and BcII with respect to their metal requirement−activity relationship, only the former three have been classified as "binuclear" enzymes since they are capable of binding both Zn(II) ions with high affinity (*18*, *25*, *27*, *28*). In contrast, BcII binds one of the two Zn(II) ions much more tightly than the other (*23*), and thus has been categorized as a "mononuclear" enzyme.

A number of compounds have been examined for their ability to serve as inhibitors of MBLs, and these include trifluoromethyl alcohols and ketones (*29*), hydroxamates (*30*), thiols (*31*−*35*), thioester derivatives (*34*, *36*−*38*), cysteinyl peptides (*39*), biphenyl tetrazoles (*40*, *41*), mercaptocarboxylates (*9*, *35*), 1β-methylcarbapenem derivatives (*42*, *43*), 2,3-disubstituted succinic acid derivatives (*44*), tricyclic natural products (*45*), and sulfonyl hydrazones (*46*). Most of these compounds have been shown to exert their adverse effects only on a limited number of MBLs. Thus, there is a critical need for the development of broad-spectrum inhibitor(s) of MBLs with potential application in clinical therapy, an objective which may be more readily achievable with an insight into the mechanism of interaction of small molecule inhibitors with these enzymes.

Current investigations concern an examination of a variety of structurally distinct mercaptans for their ability to function as inhibitors of MBLs, with IMP-1 serving as the prototype of this family of enzymes. The choice of IMP-1 was motivated by its rapid dissemination via facile horizontal gene transfer (*12*), a feature contributing to the ever-growing incidence of microbial resistance to β-lactam antibiotics. Besides confirming the importance of the thiol function for effecting the inactivation of IMP-1, these studies have revealed the kinetics of the inhibition process to be governed by other functional groups present in its vicinity.

EXPERIMENTAL PROCEDURES

Materials. Nitrocefin was obtained from Oxoid (Basingstoke, U.K.). 1,2-Ethanedithiol, *meso*-2,3-dimercaptosuccinic acid (DMSA), methyl thioglycolate, and tris(2-carboxyethyl)-phosphine (TCEP) hydrochloride were purchased from Fluka (Buchs, Switzerland). ACV was purchased from Bachem (King of Prussia, PA). All other reagents were analytical-grade and were obtained from Sigma-Aldrich (St. Louis, MO). All buffers and solutions were prepared with Milli Q water (Millipore, Bedford, MA). L-Leucinethiol and 4-mercaptobutyric acid were generated by reduction of their oxidized forms (both purchased from Sigma-Aldrich) with TCEP (*47*). pCIP4 was kindly provided by M. Galleni (Laboratoire d'Enzymologie et Centre d'Ingénierie des Protéines, Institut de Chimie, Université de Liège, Liège, Belgium). Purified metallo-β-lactamase from *B. fragilis* (CcrA) was a gift from B. Rasmussen (Wyeth-Ayerst, Pearl River, NY).

Purification of IMP-1 and BcII Metallo-β-lactamases. IMP-1 was expressed in *Escherichia coli* BL21(DE3) carrying pCIP4 and purified as reported by Laraki et al. (*28*). The homogeneity of the protein was confirmed by SDS−PAGE according to the method of Laemmli (*48*). The molecular mass of purified IMP-1 ($M_r = 25\,112 \pm 1$ Da, as determined by ESI-MS) was in agreement with that deduced from its amino acid sequence (*49*). The concentration of the enzyme was determined from its absorbance at 280 nm ($\epsilon_M = 44\,380\ M^{-1}\ cm^{-1}$). BcII [from strain 5/B/6 (*50*)] was expressed as a glutathione *S*-transferase fusion protein in *E. coli* DH5α and was purified as described previously (*46*).

Metallo-β-lactamase Assays. The spectrophotometric procedure involving the use of the chromophoric substrate, nitrocefin (*51*), was employed for the measurement of β-lactamase activity with the aid of a Cary5 spectrophotometer (Varian, Mississauga, ON). The following stock solutions of reagents were prepared: (i) nitrocefin (2 mM) in Hepes (25 mM, pH 7.3) containing DMSO [5% (v/v)] and (ii) IMP-1 (0.4 μM) in Hepes containing $ZnCl_2$ (100 μM) and BSA (100 μg/mL). Inclusion of BSA in the latter solution was found to minimize the denaturation of IMP-1 when present at low concentrations, thus allowing for a reliable and reproducible assessment of its catalytic activity (*28*). A typical assay procedure was as follows. An aliquot (950 μL) of IMP-1 in Hepes (50 mM, pH 7.3) was placed in a cuvette and allowed to equilibrate at 25 or 30 °C (depending on the experiment) for 1 min. The reaction was initiated by the addition of an aliquot (50 μL) of a nitrocefin stock solution. The final concentrations of substrate, IMP-1, and BSA in the assay were 100 μM, 4 nM, and 1 μg/mL, respectively. The progress of nitrocefin hydrolysis was monitored by recording the increase in the absorbance at 482 nm.

In the experiments designed to assess the pH dependence of enzymatic activity, either in the presence or in the absence of thiols, Hepes was replaced in the assays with AMT, a three-component buffer system consisting of acetic acid (50

mM), MES (50 mM), and Tris (100 mM), to achieve a constant ionic strength μ of 0.1 over the pH range of 4.5−9.0 (*52*). IMP-1 was found to be stable in this pH range. Thus, the enzyme preexposed to pH 4.5 for 10 min was found to exhibit, after being assayed at pH 7.3, the same activity (±7%) as that maintained at pH 7.3.

Estimates of steady state kinetic parameters for the hydrolysis of nitrocefin (K_M and k_{cat}) were determined by fitting the initial velocity data to the Michaelis−Menten equation using the software package Grafit 4.0 (Erithacus Software Ltd., Staines, U.K.).

Determination of IC_{50} Values. IC_{50} values (concentration required to effect 50% inhibition of enzyme activity) were determined by preincubating IMP-1 (4 nM) in Hepes (50 mM, pH 7.3) with the desired thiol for 1 min at 30 °C prior to the initiation of the assay by the addition of nitrocefin. Since it was necessary to use TCEP (0.25 mM) in experiments involving L-leucinethiol and 4-mercaptobutyric acid, this reagent was routinely employed in the assays with other thiol compounds. TCEP, which not only generates the thiol function from a disulfide precursor but also ensures its integrity (*47*), was found to have no adverse effect on IMP-1's catalytic activity. For the determination of IC_{50} values, the steady state rate of hydrolysis of nitrocefin was determined in the presence of several concentrations of the desired thiol. A plot of activity (steady state rate) versus inhibitor concentration provided the basis for the assessment of the IC_{50} values.

Estimation of Kinetic Parameters for Slow-Binding Inhibitors. A model, analogous to that proposed for the action of slow-binding inhibitors (*53*, *54*), was employed to analyze the thiol-induced inhibition of nitrocefin hydrolysis by IMP-1 (Scheme 1).

On the basis of the assumption that (i) ES reaches the steady state instantaneously, (ii) the magnitudes of k_1S, k_{-1}, k_3I, and k_{-3} are much greater than those of k_4 and k_{-4}, (iii) $k_4 \gg k_{-4}$, and (iv) the depletion of free substrate is negligible, Morrison and Walsh (*54*) have shown that the progress curve is defined by the following equation:

$$P = v_S t + \frac{(v_0 - v_S)(1 - e^{-kt})}{k} \quad (1)$$

where v_0 is the initial and v_S the steady state velocity of the reaction and k is the exponential decay constant. When the reaction is started by the addition of enzyme, the expressions of v_0, v_S, and k are

$$v_0 = \frac{V_{max}S}{S + K_M(1 + I/K_3)} \quad (2)$$

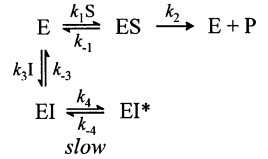
$$v_S = \frac{V_{max}S}{S + K_M(1 + I/K_i^*)} \quad (3)$$

$$k = k_{-4} + k_4\frac{I/K_3}{1 + S/K_M + I/K_3} \quad (4)$$

where K_3 ($=k_{-3}/k_3$) is the dissociation constant of the EI complex and K_i^* [$=k_{-4}K_3/(k_{-4} + k_4)$] is the overall dissociation constant.

Progress curves of substrate hydrolysis were recorded at 25 °C in AMT buffer (pH 7.3) in the presence of thiol at various concentrations. The assays were initiated by the addition of enzyme (0.5 nM) to a mixture of nitrocefin (200 μM) and thiol (no preincubation). K_3, k_4, and k_{-4} were determined with Grafit 4.0 by fitting the progress curves to eqs 1−4 using Marquardt's algorithm for nonlinear regression (*55*). V_{max} and K_M were determined as described above.

Scheme 1: Isomerization Model for Slow-Binding Inhibition[a]



[a] E represents the free enzyme, S the substrate, P the product, and I the inhibitor, and ES, EI, and EI* are the enzyme−substrate, enzyme−inhibitor, and isomerized enzyme−inhibitor complexes, respectively. The slow step in this model is the final isomerization step.

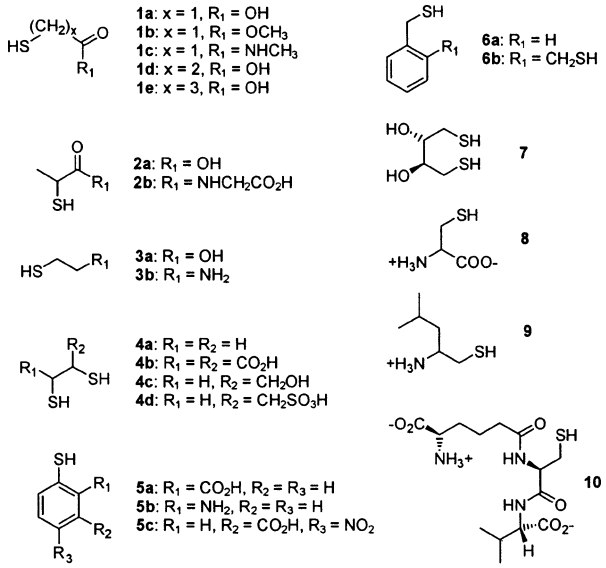


FIGURE 1: Structures of thiol compounds.

Progress curves were also fitted by numerical solutions of the set of simultaneous differential equations that mathematically describe the model presented in Scheme 1. The computations were performed with the aid of the freeware program Gepasi 3.21 (*56*, *57*), which is available on the Internet (http://www.gepasi.org). The isomerization model was entered into the program in simple chemical terms (e.g., E + S = ES, ES → E + P, etc.) along with initial values for the rate constants and concentrations of all species. Fitting by nonlinear regression was achieved using the Marquardt−Levenberg minimization algorithm (*55*).

ESI-MS. Electrospray ionization mass spectrometry (ESI-MS) studies were performed on a Micromass Q-Tof2 mass spectrometer (Micromass, Manchester, U.K.) equipped with a Z-spray electrospray ionization source as described previously (*25*).

RESULTS

Effect of Thiols on the Catalytic Function of IMP-1. The interaction of IMP-1 with mercaptans, structures of which are shown in Figure 1, was investigated by monitoring the effect on its catalytic activity. In these studies, IMP-1 was preincubated with the desired thiol compound for 1 min prior

Table 1: Inhibition of IMP-1 by Thiols in Hepes (50 mM, pH 7.3) at 30 °C

	thiol compound	feature[a]	IC_{50} (μM)[b]	ν_0/ν_S[c]
1a	mercaptoacetic acid	a	1.3	1.0
1b	methylmercaptoacetate	n	12	0.85−0.9
1c	*N*-(methyl)mercaptoacetamide	n	12	0.85−0.9
1d	3-mercaptopropionic acid	a	170	0.95
1e	4-mercaptobutyric acid	a	128	0.55
2a	2-mercaptopropionic acid	a	4.5	1.0
2b	α-mercaptopropionyl glycine	a	15	0.7
3a	mercaptoethanol	n	200	0.6
3b	2-aminoethanethiol (cysteamine)	c	120	1.0
4a	1,2-ethanedithiol	n	6	0.5
4b	*meso*-2,3-dimercaptosuccinic acid (DMSA)	a	20	1.0
4c	2,3-dimercapto-1-propanol (BAL)	n	13	0.7
4d	2,3-dimercaptopropanesulfonic acid (DMPS)	a	11	1.0
5a	*o*-mercaptobenzoic acid	a	200	1.0
5b	2-aminothiophenol	n	200	1.0
5c	2-nitro-5-thiobenzoic acid	a	>500	1.0
6a	benzylmercaptan	n	5	0.25
6b	1,2-benzenedimethanethiol	n	2.3	0.25
7	DTT	n	10	0.5
8	L-cysteine	a,c	1050	1.0
9	L-leucinethiol	c	75	0.9−0.95
10	ACV	a,c	20	0.85
11	Na_2S	n	20	1.0

[a] Associated charged functional group(s) present in the thiol compound at neutral pH: a, anionic function(s) such as carboxylate or sulfonate; c, cationic function(s) such as an amino group; and n, neutral (uncharged) functional group. [b] IC_{50} values were determined from the recorded steady state rates (ν_S) of substrate hydrolysis. The precision of the IC_{50} determinations was within the range of ±10%. [c] Ratio of the initial to steady state rate of substrate hydrolysis.

to the determination of the protein's catalytic activity by the introduction of the substrate, nitrocefin. The reaction was monitored in an effort to assess the steady state rate (ν_S) of hydrolysis of the substrate. Such measurements performed in the presence of the thiol compound at several fixed concentrations provided the basis for the estimation of the IC_{50} value recorded in Table 1.

Among the compounds listed in Table 1, mercaptoacetic acid (**1a**), 3-mercaptopropionic acid (**1d**), 2-mercaptopropionic acid (**2a**), mercaptoethanol (**3a**), and DTT (**7**) have been previously shown to serve as inhibitors of IMP-1 from *S. marcescens* (*33*). The observations recorded in the current investigations are in general agreement with the above findings, with compounds **1a**, **2a**, and **7** serving as more effective inhibitors than **1d** and **3a**. Interestingly, the simplest thiol compound, hydrogen sulfide (Na_2S serving as its source), functioned as a potent inhibitor of IMP-1, while thiocyanate was devoid of such activity. Benzylmercaptan (**6a**) proved to be one of the most potent inhibitors among the thiols investigated in this study.

Although compounds with two thiol functions are capable of serving as bidentate ligands for Zn(II) and other ions, a feature that has been exploited in the case of BAL, DMSA, and DMPS to alleviate heavy metal poisoning (*58*, *59*), their potency as inhibitors of IMP-1 was not superior to that noted in the case of monothiol counterparts (see Table 1). Furthermore, inhibition of IMP-1 by dithiols does not appear to involve the removal of the metal ion from the protein since (i) the inhibition could be reversed with full restoration of catalytic function upon removal of the inhibitor by centrifugal

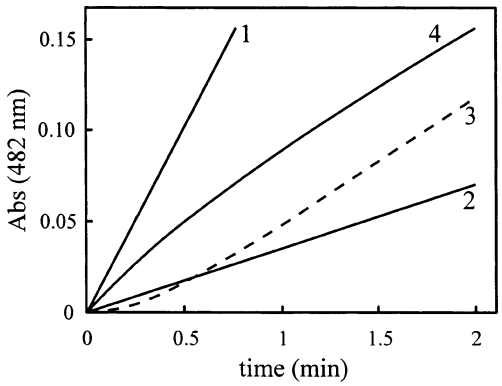


FIGURE 2: Influence of mercaptoacetic acid (**1a**) and benzylmercaptan (**6a**) on the enzymatic activity of IMP-1. All assays were performed in Hepes (50 mM, pH 7.3) at 25 °C with nitrocefin (0.2 mM) as a substrate and IMP-1 (0.8 nM). The progress of nitrocefin hydrolysis was monitored at 482 nm: (1) control with no mercaptan present and (2) in the presence of **1a** (10 μM). The profile is the same whether the enzyme is preincubated with **1a** or exposed to it in the presence of substrate. (3) The enzyme was preincubated with **6a** (10 μM) for 1 min prior to addition of substrate. (4) The enzyme was exposed to **6a** (10 μM) in the presence of substrate.

filtration and (ii) ESI-MS analysis of the protein after removal of the inhibitor under nondenaturing conditions (*25*) revealed the presence of both of its Zn(II) ions. A noted diminution in the extent of inhibition exerted by dithiols in the presence of Zn(II) in the assay (data not shown) appears to be related to their inherent ability to scavenge the extraneous divalent metal ions, a feature that will result in a concomitant decrease in their effective concentration.

A notable aspect of thiol-induced inhibition of IMP-1 concerns the time needed to attain the steady state rate (ν_S) of hydrolysis of nitrocefin. In the case of thiols of high inhibitory potency (with the exception of **1a**, **2a**, **4b**, and **4d**), the progress curve of nitrocefin hydrolysis was characterized by a "lag" phase prior to the attainment of ν_S (Figure 2). For the sake of clarity, the quotient ν_0/ν_S, where ν_0 is the initial rate of substrate hydrolysis, is proposed to provide a quantitative measure of the lag noted in the case of each of the thiol compounds, and these values are presented in Table 1. Thus, a value of 1.0 for this quotient denotes the absence of a lag in the progress curve of substrate hydrolysis (Figure 2), and this situation is observed in the case of such potent inhibitors as **1a**, **2a**, **4b**, **4d**, and **11**. The lower the value of the quotient, the longer the period required to attain the steady state rate of hydrolysis. On the basis of this criterion, compounds **6a** and **6b** exhibited the most pronounced lag among the compounds that were examined. The phenomenon of a lag in IMP-1-catalyzed reactions performed in the presence of these and other thiols (see Table 1) is not restricted to nitrocefin serving as a substrate. This feature is also noted in reactions with either benzylpenicillin (PenG) or cephalosporin C as the substrate for the enzyme (data not shown).

Scrutiny of the features of the various thiols and their inhibitory effect reveals the following. (i) In the case of thiol compounds with an α-carboxylate or anionic function as in **1a**, **2a**, **4b**, and **4d**, the steady state was reached immediately after the initiation of the assay (i.e., $\nu_0 = \nu_S$). (ii) In other instances, ν_0 was found to deviate from ν_S, the extent of which was influenced by the location of the anionic function (if present) relative to that of the thiol in the compound.

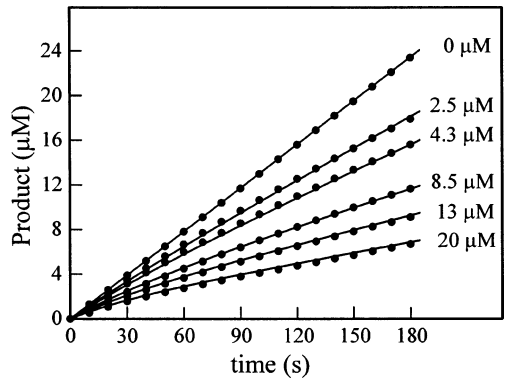


FIGURE 3: Inhibition of IMP-1 as a function of the concentration of benzylmercaptan. All assays were performed in AMT buffer (pH 7.3) at 25 °C. The concentrations of the substrate and enzyme were 0.2 mM and 0.5 nM, respectively. The reaction was initiated by the addition of enzyme to a mixture of substrate and thiol at the concentration indicated in the figure. Following rapid mixing (5 s), data points were collected every 0.5 s. For clarity of presentation, only every 20th data point (10 s interval) is shown as a filled circle. The continuous curves represent the best fit of the progress curves to eqs 1−4 (see Experimental Procedures).

Thus, ν_0 was found to decrease relative to ν_S with the enhanced distal character of the anionic function (as in the case of **1e** compared to **1a** or **1d**, Table 1), a feature reflected in a more pronounced lag in the substrate hydrolysis profile. (iii) In accordance with the above-mentioned criterion, neutral thiols were found to exhibit the most pronounced lag in the substrate hydrolysis profile as demonstrated in the cases of **4a**, **6a**, **6b**, and **7** (Table 1). (iv) Masking of the negative charge of the α-carboxylate function in **1a** or **2a**, as in the case of ester derivatives **1b** and **2b** or amide derivative **1c**, resulted in both a diminution of inhibitory potency and the appearance of a lag. (v) Amino group(s) tended to diminish the inhibitory potency of the compound (as is the situation with **3b** and **8**). (vi) Aromatic compounds with thiol substituents (**5a**−**c**) were found to be poor inhibitors of IMP-1.

Mode of Inhibition. As shown in Figure 2, the progress curves of nitrocefin hydrolysis in the presence of some thiols with α-anionic substituents (e.g., **1a**) were linear [i.e., the initial rate of hydrolysis being the steady state rate ($\nu_0 = \nu_S$)], while those recorded with neutral thiols (e.g., **6a**) exhibited a pronounced lag prior to attainment of the steady state rate ($\nu_0 < \nu_S$). Such a phenomenon in the latter cases required the preincubation of the enzyme with the inhibitor prior to the introduction of the substrate. In contrast, when the enzyme was exposed to the inhibitor in the presence of the substrate, a "burst" phase, in which the initial rate progressively declined toward the steady state rate, was noted (Figure 2, line 4). These results are typical of those expected from slow-binding inhibitors of the enzyme (*53*, *54*). In view of the relatively high concentrations (several orders of magnitude over that of the enzyme) required to achieve detectable inhibition, thiols do not appear to be tight-binding inhibitors of IMP-1.

To determine the validity of the mechanism depicted in Scheme 1 in accounting for the inhibitory action of neutral thiols, IMP-1-mediated hydrolysis of nitrocefin was examined in the presence of several concentrations of **6a**. The results are shown in Figure 3. In all instances, the initial burst in the rate of the reaction was followed by a slower steady state rate ($\nu_0 > \nu_S$). Both ν_0 and ν_S were found to decrease with the increments in the concentration of the mercaptan, a feature consistent with the proposed isomerization model (see eqs 2 and 3). The kinetic parameters k_4, k_{-4}, and K_3, the dissociation constant for the enzyme−inhibitor complex (EI), were obtained by fitting the progress curves to eq 1 (see Experimental Procedures), and the values are given in Table 2.

Table 2: Parameters for Inhibition of IMP-1-Mediated Nitrocefin Hydrolysis by Compound **6a**

		numerical solution	
parameter[a]	fit to eqs 1−4	start	end (fitted)[g]
[E] (nM)	0.5	0.5	0.5 (fixed)
[S] (μM)	200	200	200 (fixed)
K_M (μM)[b]	12.0 (fixed)	−	(13.6)
V_{max} (μM/s)[b]	0.138 (fixed)	−	(0.138)
k_1 (μM^{-1} s^{-1})	−	100[d]	100 (fixed)
k_{-1} (s^{-1})	−	1000[e]	1078 ± 6
k_2 (s^{-1})[b]	276	276	276 (fixed)
k_3 (μM^{-1} s^{-1})	−	100[d]	100 (fixed)
k_{-3} (s^{-1})	−	122[f]	133.3 ± 2.3
k_4 ($\times 10^2$ s^{-1})	3.24 ± 0.32	3.24	2.75 ± 0.12
k_{-4} ($\times 10^2$ s^{-1})	1.35 ± 0.08	1.35	1.11 ± 0.04
K_3 (μM)	1.22 ± 0.04	−	(1.33)
K_i^* (μM)[c]	0.36 ± 0.05	−	(0.38)

[a] The kinetic parameters were obtained from best fits of the recorded progress curves (shown in Figure 3) to eqs 1−4 using Grafit 4.0 and by numerical solution of sets of differential equations with the aid of Gepasi 3.21 as outlined in Experimental Procedures. [b] Experimentally observed values. [c] The overall inhibitor dissociation constant was calculated from the relation $K_i^* = k_{-4}K_3/(k_{-4} + k_4)$. [d] The rates of formation of the enzyme-substrate (ES) and the enzyme-inhibitor (EI) complexes were assumed to be diffusion-controlled [$k_1 = k_3 = 10^8$ M^{-1} s^{-1} (*60*). [e] Value estimated from the relation $k_{-1} = K_M k_1 - k_2$. [f] Value estimated from the relation $k_{-3} = K_3 k_3$. [g] Values in parentheses were calculated from the determined kinetic parameters.

A good fit of the data (Figure 3, solid lines) was obtained. However, an examination of the observed values for k_4 and k_{-4} revealed the former to be only 2.4 times as large as the latter (Table 2). This feature is not in full conformity with the premise that $k_4 \gg k_{-4}$, which formed the basis for the model (see eqs 1−4). Hence, progress curves were also analyzed by fitting the data to a set of differential equations that describe the isomerization model (see Experimental Procedures). The salient feature of this approach concerns its ability to fit the data to the model without the imposition of any constraints on the relationship between k_4 and k_{-4}. The rate constants k_4 and k_{-4} as well as the calculated values for K_3 and K_i^* obtained by numerical solution are very similar to those determined by fitting the progress curves to eqs 1−4 (Table 2).

In summary, progress curve fitting, both to eqs 1−4 and with numerical methods, yield similar results with relatively low standard deviations, suggesting that benzylmercaptan is a slow-binding inhibitor of IMP-1 and that the slow step is the isomerization of the initially formed EI complex as depicted in Scheme 1.

pH Dependence of Reactions Catalyzed by IMP-1. A study of the influence of pH on the catalytic activity of IMP-1 may provide an insight into the nature of functional group(s) that may play a pivotal role in the catalytic mechanism of the protein. Furthermore, since mercaptans are weak acids, their interaction with the enzyme may depend not only on their ionization state(s) but also on those of the functional

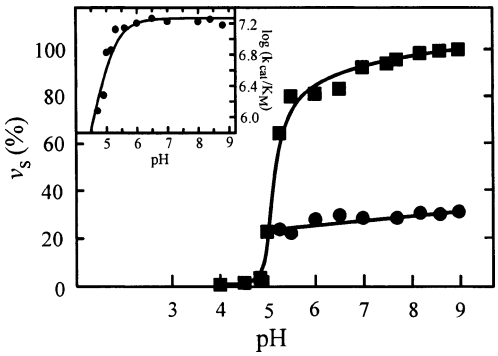


FIGURE 4: Influence of pH on IMP-1-catalyzed hydrolysis of nitrocefin, both in the absence and in the presence of benzylmercaptan. Assays were performed in AMT buffer at the desired pH and 25 °C. The reaction was initiated by the addition of nitrocefin (0.1 mM) to IMP-1 (4 nM), which had been preexposed to the buffer in the absence (■) or presence (●) of benzylmercaptan (10 μM) for 1 min. The data shown represent the steady state rates of substrate hydrolysis recorded at 482 nm. The inset shows the relationship between log k_{cat}/K_M (the ratio of kinetic parameters given in M^{-1} s^{-1}) and pH in the absence of thiol (Dixon−Webb plot). The continuous curve represents the best fit (with Grafit 4.0) of the observed data to a model in which two functional groups, essential for IMP-1's activity, are being protonated (*32*), with an apparent pK_a of 5.15 ± 0.07 for both groups.

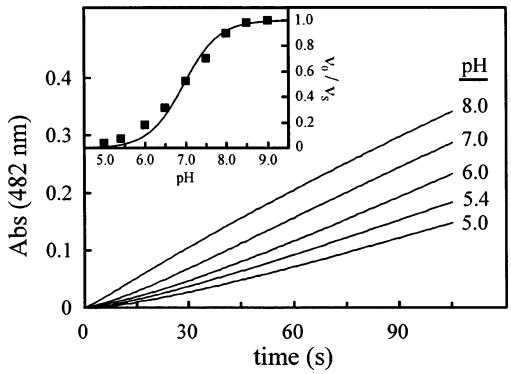


FIGURE 5: Inhibition of IMP-1 by benzylmercaptan. Influence of pH on the lag phase. Assays were performed in AMT buffer at the desired pH and 25 °C. The reaction was initiated by the addition of nitrocefin (0.2 mM) to IMP-1 (4 nM), which had been preexposed to the thiol (10 μM) for 1 min. The progress of nitrocefin hydrolysis was followed at 482 nm at the indicated pH. The profile formed the basis for the estimation of the initial (v_0) and steady state (v_S) rates of hydrolysis. The inset shows a plot of v_0/v_S as a function of pH. The continuous curve represents a best fit (with Grafit 4.0) of the data to the Henderson−Hasselbalch equation.

group(s) of IMP-1. In light of these considerations, the catalytic activity of the enzyme was examined over the pH range of 4.5−9.0, both in the absence and in the presence of the thiol inhibitor, with nitrocefin serving as the substrate. In the latter instances, **1a** and **6a** served as the prototypes of thiol inhibitors. The results that were obtained are presented below.

A study of IMP-1-catalyzed hydrolysis of nitrocefin in the absence of thiol inhibitors revealed the enzyme to be maximally active between pH 6 and 9, with only a marginal enhancement being noted with increments in pH in this range. At pH <6, a sharp decrease in catalytic activity was noted, with a total loss of activity at pH ≤4.5 (Figure 4). These findings are in general agreement with those recorded for IMP-1 derived from *S. marcescens* (*33*, *61*). Analysis by the Dixon-Webb method (*62*) confirmed that the catalytic efficiency, k_{cat}/K_M, of the protein remains unaffected by pH over the range of pH 6−9 (Figure 4, inset). A plot of log-(k_{cat}/K_M) was characterized by an acidic limb (pH <6) with a slope of approximately 2, consistent with the simultaneous protonation of two functional groups of IMP-1. Thus, the pK_a values of these two functional groups appear to be close to each other. Since a similar phenomenon has also been reported with BcII (*32*), such a dependence of the enzyme's catalytic function on the ionization state of two functional groups is not unique to IMP-1, although it has been pointed out that caution is warranted in the interpretation of the pH dependence of the catalytic function of enzymes (*63*).

As noted earlier, thiol compounds are weak acids [pK_a ∼ 9−11 (*64*)] and hence can be expected to remain protonated at pH <8. On the basis of this feature, one could expect the ability of thiols to inhibit IMP-1 to remain unaffected over the pH range of pH 4.5−8. However, a study of the interaction of IMP-1 with mercaptans over this pH range has revealed a unique feature, which becomes apparent when experiments are performed at pH <5.0. This aspect can be described as follows. Mercaptans, which serve as inhibitors of IMP-1 over the pH range of 5−9, appear to enhance the enzyme's activity at pH <5.0. This phenomenon appears to be an inherent feature of all thiol compounds (be they neutral or charged), since it was not observed in studies with benzylpenicillin, used as a competitive inhibitor, and sulfonyl hydrazones, recently shown to function as mixed-type inhibitors of IMP-1 (*46*). The extent of enhancement of the enzyme's catalytic function caused by the thiol appears to be related to its inhibitory potency at pH >5.0. The above facets of the interaction of IMP-1 with thiols are presented below.

Interaction of IMP-1 with Thiols at pH 5−9. At a final concentration of 10 μM in the assay, thiol **6a** (prototype of neutral thiols, which show a lag in the substrate hydrolysis profile at pH <8) was found to effect an ∼70% decrease in IMP-1's catalytic activity. This estimate of the extent of inhibition was obtained by a comparison of the steady state rate of substrate hydrolysis observed in the presence of the inhibitor with that noted in its absence. The **6a**-mediated inhibition of IMP-1 appeared to be independent of pH over the range of pH 5−9 (Figure 4). As noted in an earlier section, the IMP-1-catalyzed hydrolysis of nitrocefin in the presence of **6a** or other neutral thiols is characterized by an initial lag at pH <8.0 (i.e., $v_0 < v_S$). In an effort to determine the influence of the pH on the magnitude of the lag, both v_0 and v_S were recorded as a function of pH. At pH >8.0, the initial rate represented the steady state rate ($v_0/v_S = 1$), indicating the absence of a lag in the substrate hydrolysis profile (Figure 5).

A decrease in the pH of the assay medium was accompanied by a progressive diminution in both v_0 and v_S, as well as a concomitant decrease in the quotient v_0/v_S. Thus, the estimated values of v_0/v_S at pH 5.0 and 8.0 were found to be ∼0.05 and 1.0, respectively. A plot of v_0/v_S versus pH (Figure 5, inset) yielded a sigmoidal profile reminiscent of that of the ionization state of a functional group with an apparent pK_a of 6.9. To the best of our knowledge, no such correlation between v_0/v_S and the ionization state of the enzyme's functional group has been documented. Similar studies with **1a**, the prototype of thiol compounds with an α-anionic substituent, revealed the substrate hydrolysis profile to be linear over the range of pH 5−8, observations in conformity with those documented in the previous section.

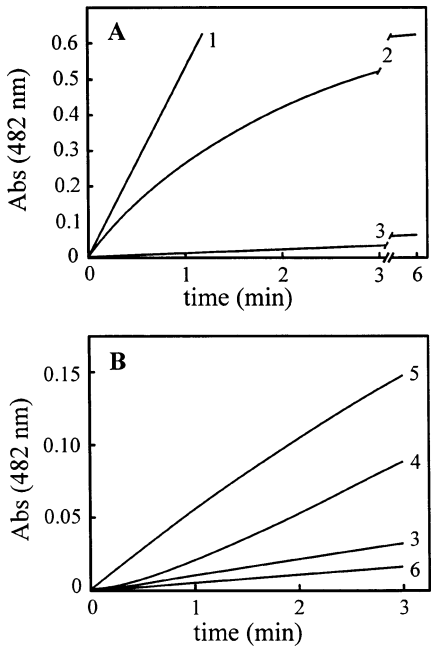


FIGURE 6: Interaction of IMP-1 with thiols at pH <5.0. Assays were performed in AMT buffer (pH 4.7) at 25 °C, using nitrocefin (0.1 mM) as the substrate and IMP-1 (4 nM). The stock solution of the enzyme (0.4 μM) was maintained in AMT or Hepes buffer at pH 7. The progress of substrate hydrolysis was followed at 482 nm: (A) (1) IMP-1 at pH 7.0 (control), (2) IMP-1 (initially at pH 7.0) added to nitrocefin in AMT buffer at pH 4.7 (protocol A), and (3) enzyme exposed to AMT buffer (pH 4.7) for 1 min prior to the addition of nitrocefin (protocol B) and (B) (3) same as in panel A, (4) IMP-1 incubated with **6a** (10 μM) for 1 min in AMT buffer prior to the addition of substrate (protocol C), (5) same as 4, except for **1a** (10 μM) in the place of benzylmercaptan, and (6) IMP-1 exposed to AMT buffer (pH 4.7) for 1 min, followed by incubation of the enzyme with **1a** (10 μM) for an additional 1 min, prior to the addition of substrate (protocol D).

Interaction of IMP-1 with Thiols at pH <5.0. When studies were extended to slightly below pH 5.0, mercaptans were found to be devoid of an adverse effect on IMP-1's catalytic activity, a phenomenon which has also been noted previously (*33*). However, as the pH was further lowered, thiols appeared to enhance the activity of IMP-1, since the steady state rate of substrate hydrolysis was significantly higher than that noted in the assay performed under identical conditions except for the omission of the thiol compound. This beneficial effect was restricted to a very narrow pH range of 4.5−4.9, since it was not observed at pH <4.5 (conditions which led to the total loss of catalytic activity), and it was stringently dependent on the experimental conditions that were employed. The four distinct protocols, A−D, used in these studies are described below. While the first two protocols served as controls (in the absence of thiol), the latter two involved the participation of mercaptans. A thiol-induced enhancement of IMP-1's catalytic action could be observed only with procedure C.

In protocol A, IMP-1, initially at pH 7.0 (Hepes of AMT buffer medium), was introduced to nitrocefin (100 μM) at pH 4.7 and the progress of the reaction was monitored. In protocol B, IMP-1 was exposed to pH 4.7 for 1 min prior to the initiation of the assay by the addition of the substrate. In protocol C, IMP-1 was exposed to pH 4.7 in the presence of the thiol compound for 1 min prior to the addition of nitrocefin. In protocol D, the enzyme was initially exposed to pH 4.7 for 1 min, followed by its incubation with the thiol for an additional 1 min, prior to the initiation of the assay by the introduction of the substrate.

Table 3: Influence of the Experimental Protocol and Substrate Concentration on the Catalytic Activity of IMP-1 at pH 4.7

protocol[a]	[nitrocefin] (μM)	activity[b]
B	100	1.0
B	25	2.0 ± 0.2
C	100	2.8 ± 0.2
C	25	1.1 ± 0.1
D	100	0.5 ± 0.05

[a] Experimental protocols: (B) IMP-1 at pH 4.7 for 1 min prior to initiation of the assay by addition of substrate, (C) IMP-1 exposed to pH 4.7 in the presence of thiol compound **6a** (10 μM) for 1 min prior to addition of nitrocefin, and (D) IMP-1 at pH 4.7 for 1 min, followed by incubation with **6a** (10 μM) for an additional 1 min prior to the assay described above. The concentration of nitrocefin in the assay was as indicated above. [b] Activity values shown are relative to that observed in the assay using 100 μM nitrocefin and experimental protocol B.

The results obtained under protocols A and B are shown in Figure 6A. In the former instance, the initial rapid rate of substrate hydrolysis, which was close to that observed with the enzyme at pH 7.0, declined slowly toward the steady state rate that can be attained 6 min after the initiation of the assay (Figure 6A, curve 2). The final steady state rate corresponded closely to that expected in the assay at pH 4.7 (identical with that shown in Figure 4). The observations recorded under protocol B demonstrate that, in the absence of substrate, the pH-dependent diminution of catalytic function of the enzyme is rapid, as indicated by the very low rate of hydrolysis (Figure 6A, curve 3). In protocol C, the rate of hydrolysis observed in the presence of the thiol is considerably higher (with increases of approximately 3- and 5-fold in the presence of **6a** and **1a**, respectively) than that noted in its absence (Figure 6B, curves 4 and 5). Furthermore, the initial lag, recorded with **6a** at pH <8, was also observed at pH <5. Similar studies with **3a**, a very weak inhibitor, resulted in only a marginal enhancement of IMP-1's activity (data not shown). Thus, the ability of thiol compounds to enhance the activity of IMP-1 at pH <5.0 would appear to be correlated with their inhibitory potency at physiological pH. As stated earlier, the presence of the thiol at the time of the enzyme's exposure to pH 4.7 was an absolute requirement for observing the phenomenon, since it was not apparent in protocol D. Under the latter experimental conditions, mercaptans were found to serve as inhibitors, with a 50% decrease in activity being noted (compare curves 3 and 6 of Figure 6B).

In an effort to identify the basis for the thiol-mediated enhancement of IMP-1's catalytic activity, the steady state rate of hydrolysis of nitrocefin was examined as a function of its concentration at pH 4.7 both in the absence (according to protocol B) and in the presence (as per protocol C) of **6a**. In the former instance, IMP-1 was found to be prone to substrate inhibition, a feature not prevalent at pH >5 (data not shown). The observed 2-fold increase in the activity of IMP-1 upon reducing the substrate concentration from 100 to 25 μM in the assay (Table 3) would appear to lend further support for the phenomenon of substrate inhibition at pH <5. When similar experiments were performed in the presence of **6a** (the enzyme preexposed to the thiol as per protocol C), the results were as follows. (i) Substrate

inhibition was not observed in the assays carried out at a nitrocefin concentration of 100 μM, and (ii) at lower substrate concentrations (25 μM), the thiol reverted to its inhibitory function (Table 3).

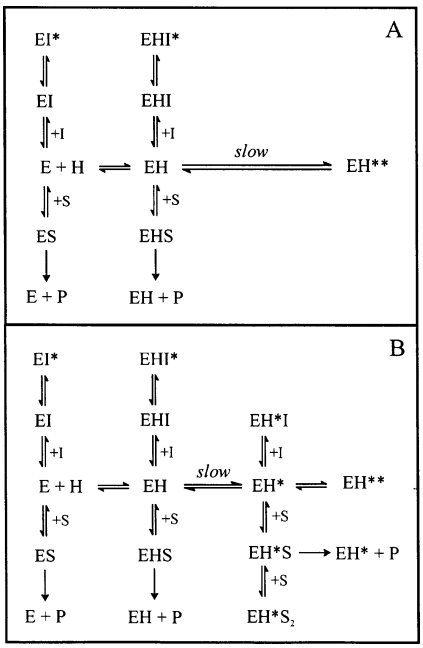
A qualitative "minimal" model that may account for the above-noted observations is depicted in Scheme 2. For the sake of clarity, this scheme is presented in two parts (panels A and B), with the latter representing an overall view of the phenomenon. The vertical pathway from E in panel A corresponds to the isomerization process proposed earlier (Scheme 1). Upon exposure of IMP-1 to pH 4.7 in the presence of nitrocefin, as per protocol A, the enzyme is presumed to become rapidly protonated [presumably at a location distant from the active site of the protein, as has been suggested for the protonation of carbonic anhydrase (*65*)], to yield EH. Since the enzyme's activity immediately after its exposure to pH 4.7 is close to that observed at pH 7, EH is suggested to be catalytically competent. The progressive decline of this initial activity toward the steady state value (reached after ~6 min) appears to be indicative of a slow isomerization of EH to EH**. In view of the loss of enzymatic activity upon protonation of either of the two functional groups in the protein (see Figure 4), the EH** species would be catalytically inert. The observation that the steady state rate of nitrocefin hydrolysis after exposure of IMP-1 to low pH in the absence of the substrate, as per protocol B, is achieved instantaneously is in agreement with this proposal since the initial presence of the substrate (protocol A) or thiol (protocol C) would lead to a diminution of the concentration of free EH and thus to a decrease in the rate of isomerization to EH**. The "retardation" of the isomerization of EH to EH** in the presence of thiols (protocol C) may thus provide the basis for the observed enhancement of the enzyme's activity when exposed to the inhibitor prior to the addition of nitrocefin.

The model presented in panel A of the above scheme, however, fails to accommodate the basis for the enzyme becoming susceptible to substrate inhibition. This phenomenon, which could be observed only under protocol B (see Table 3), points to the existence of an additional intermediate, EH*, in the isomerization process, with a branched pathway that accounts for the noted substrate inhibition. Finally, observations recorded with protocol D suggest that preincubation of IMP-1 at pH 4.7 prior to the exposure to thiols would generate EH* and EH**, the former species being inhibited not only by substrate but also by thiol compounds (vertical upper pathway originating from EH*).

Thiol-Mediated Inhibition of CcrA and BcII (strain 5/B/6). Studies were extended to include CcrA and recombinant BcII (strain 5/B/6) to determine if the above recorded facets of the interaction of IMP-1 with thiols are typical features of MBLs. It is pertinent to note that ESI-MS analysis of the latter enzyme under nondenaturing conditions revealed that the protein contains two Zn(II) ions (data not shown). Thus, BcII, like CcrA and IMP-1, appears to be binuclear.

The IC_{50} values for the inhibition of both enzymes by thiols **1a** and **6a** (prototypes for negatively charged and neutral mercaptans, respectively) were recorded under conditions analogous to those in experiments with IMP-1. These studies revealed that **1a** and **6a** are fairly potent inhibitors of BcII, with IC_{50} values of 30 and 25 μM, respectively. In sharp contrast, both compounds were found to be weak inhibitors of CcrA, with IC_{50} values of 0.95 and 0.17 mM being noted for **1a** and **6a**, respectively. Furthermore, when **6a** served as the inhibitor, the lag phase in the substrate hydrolysis profile was observed in the case of BcII, but not with CcrA. The influence of pH on **6a**-mediated inhibition of nitrocefin hydrolysis by both BcII and CcrA was assessed at pH 6−8. These studies revealed that the substrate hydrolysis profile in the case of CcrA exhibits linear kinetics (no lag) regardless of the pH of the assay medium. In contrast, the situation with BcII was similar to that noted in the case of IMP-1, with the lag in the substrate hydrolysis profile becoming more pronounced with the decrease in the pH of the assay medium.

Scheme 2: Proposed Pathway for the pH Dependence of Catalytic Function of IMP-1[a]



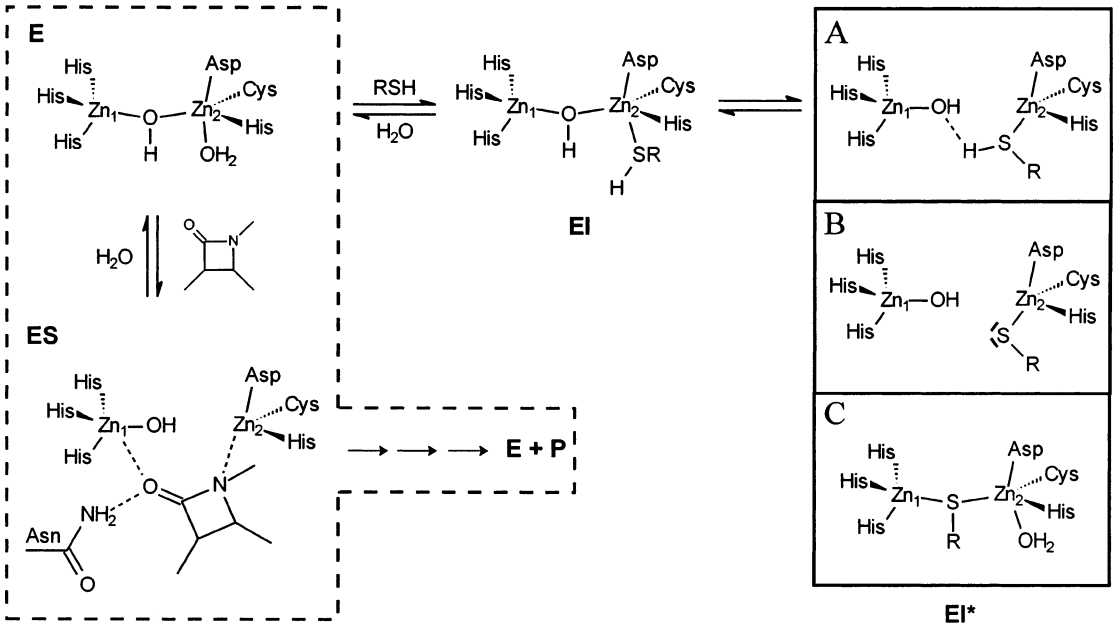
[a] For clarity, the pathway is presented in two panels (A and B). Panel B, an extension of the version in panel A, represents the complete pathway, which also accommodates the phenomenon of substrate inhibition (see the text).

DISCUSSION

The rapid dissemination of some MBLs such as IMP and VIM via facile horizontal gene transfer to organisms inherently incapable of producing such enzymes poses a serious problem to the continued use of β-lactam antibiotics in clinical therapy (*28*, *66*). Furthermore, both the recent production of an Asn233Ala mutein of IMP-1 with superior catalytic efficiency (*66*) and the emergence of spontaneous IMP-1 variants [the latest being IMP-10 at the time of submission of this report (*67*)] with just one or two amino acid substitutions underscore the imminent threat of MBLs such as IMP-1 to the efficacy of β-lactam antibiotics in combating bacterial infections.

Compounds with thiol function(s) have received considerable attention as inhibitors of MBLs in view of their affinity for divalent metal ions. The thiol inhibitors used in this study were found to fall into two distinct categories. In the case of the first type, the inhibition was instantaneous and the substrate hydrolysis profile was linear with time and unchanged over the pH range of 5−9. Mercaptans in this category are characterized by the presence of a proximal

Scheme 3: Proposed Pathways in the Isomerization of the IMP-1−Thiol Inhibitor Complex[a]



[a] The initial step of substrate hydrolysis, based on the mechanism proposed for CcrA (*72*−*74*), is given in the dashed box at the left.

anionic function as in **1a** or **2a**. In contrast, the mode of inhibition of IMP-1 by the second group of thiols, which comprise neutral thiols (e.g., **6a,b**) and compounds in which the anionic function is farther from the thiol group (e.g., **1e** and **2b**), was found to be pH-dependent. While at pH >8 inhibition was instantaneous (linear substrate hydrolysis profile), it was found to be time-dependent at pH <8.

Analysis of the kinetics of inhibition by this latter type of mercaptan revealed the process to be consistent with a model which involves the rapid formation of an enzyme−inhibitor complex (EI), followed by its isomerization to a relatively more stable species, EI* (see Scheme 1). This phenomenon is analogous to that documented in the case of captopril (*68*), a potent thiol inhibitor of Zn(II)-containing angiotensin converting enzyme, and some thiol inactivators of leucine aminopeptidase (*69*, *70*), a binuclear Zn(II) protein. Isomerization has also been reported to occur in the inhibition of other metalloproteins such as arginase. In the case of this binuclear Mn(II) enzyme, boronic acid inhibitors have been shown to exhibit slow binding kinetics at pH >9 (*71*), in contrast to the above-noted situation with the thiol-mediated inhibition of IMP-1, which acquires slow binding character at pH <8.

The proposals advanced herein to account for the "slow binding" of neutral thiols to IMP-1 rely on precedents invoked not only in the catalytic mechanisms of other relevant metalloenzymes but also in the initial stages of substrate hydrolysis by CcrA, a dizinc MBL. Thus, it is appropriate to examine the early events of the mechanism proposed for CcrA-mediated hydrolysis of nitrocefin (*72*−*74*), which, briefly stated, are as follows. (i) In the resting state, the two Zn(II) ions of the enzyme are bridged by a hydroxide ion. (ii) The binding of the substrate occurs by the replacement of the apical water molecule on Zn2 with the nitrogen of the β-lactam ring. (iii) The interaction of Zn1-bound hydroxide, formed as a consequence of a rapid substrate-induced rupture of the μ-OH bridge, with Asp120 results in its appropriate orientation for nucleophilic attack on the carbonyl function to form a tetrahedral intermediate. The catalytic cycle is completed by the cleavage of the C−N bond of the substrate leading to the generation of an anionic intermediate, its subsequent protonation by solvent water, and finally the release of the product with the concomitant restoration of the enzyme to its resting state. The initial events of substrate hydrolysis, i.e., the binding of the substrate on Zn2 with concomitant rupture of the μ-OH bridge, serve as a precedent for proposing an analogous event in the interaction of IMP-1 with thiol inhibitors as described below (Scheme 3).

One could envisage the mercaptan inhibitor to bind to Zn2 by displacing its apical water ligand without perturbing the μ-OH bridge between the metal ions, generating the initial EI complex. The displacement of the apical water molecule by an inhibitor molecule can be expected to be rapid, since the process is analogous to that involved in the binding of the substrates (*72*, *73*). The slow isomerization of EI to EI*, after the rupture of the μ-OH bridge, may involve a conformational change of the enzyme accompanying the orientation of the un-ionized thiol inhibitor toward the Zn-bound hydroxide ion so as to facilitate an energetically favorable H bond (Scheme 3, panel A). Such a rearrangement would result in a tighter binding of the inhibitor to IMP-1, a feature that is likely to retard its dissociation from the enzyme at pH <8.

Alternatively, the apparent pK_a of 6.9, noted in the experiments on the pH dependence of the slow-binding process, may indeed be a reflection of a change in the ionizability of the Zn-bound thiol function of **6a** relative to that noted in its free form [p$K_a \sim 9.4$ (*64*)]. Such diminution in pK_a values of mercaptans upon ligation to Zn(II) or other metal ions has been widely noted in both model compounds (*75*, *76*) and proteins (*77*, *78*). Furthermore, the deprotonation of a Zn-bound thiol has been suggested to be associated with the slow step in the inhibition of aminopeptidase by mercaptans (*69*). Analogously, the deprotonation of the Zn-bound thiol in IMP-1 may follow rapidly a slow rearrangement of the enzyme to facilitate such an event, with the concomitant rupture of the μ-OH bridge (Scheme 3, panel B).

Another alternative to be considered is based on observations of the crystal structure of IMP-1 in complex with a mercaptocarboxylate inhibitor, which indicate a displacement of the hydroxide moiety bridging the protein's two Zn(II) ions by a μ-SR bridge (*17*). It is conceivable that the rapid displacement of the apical water molecule by the thiol inhibitor is accompanied by a slow step in which the μ-OH bridge is replaced with a μ-SR bridge (Scheme 3, panel C). Since the formation of a μ-SR bridge is likely to involve the nucleophilic attack of the thiol in its deprotonated form on Zn(II), such a process might be rapid at high pH.

Thiols with proximal anionic groups such as **1a** or **2a** fail to exhibit slow-binding kinetics, but behave as potent reversible competitive inhibitors, regardless of the pH of the assay medium. It is possible that the proximal anionic function (either a carboxylate as in **1a** or **2a** or a sulfonate moiety as in **4d** in the thiol inhibitor) serves as an additional binding determinant, allowing for an acceleration of the isomerization process. Thus, the anionic thiol inhibitors may be considered similar to normal substrates, which possess an analogous carboxylate group and appear to induce a rapid rearrangement to facilitate the formation of the ES complex (Scheme 3). Consequently, the anionic group of the inhibitor may interact with a positively charged amino acid residue and/or with one of the Zn(II) ions in the protein. While the interaction with the protonated ϵ-NH_2 group of Lys224 has been observed in the crystal structure of IMP-1 in complex with a mercaptocarboxylate (*17*), crystallographic analysis of succinic acid derivatives bound to the enzyme indicates the interaction of one of the inhibitor's carboxyl functions with both the side chain of Lys224 and Zn2 (*44*).

To explore the generality of the above-noted features in the inhibition of IMP-1 by thiol compounds, studies were extended to include two other dizinc MBLs, BcII (from *B. cereus* strain 5/B/6) and CcrA (from *B. fragilis*). The former enzyme was found to mimic IMP-1 in its interaction with thiol inhibitors (either neutral or with proximal anionic substituents). In contrast, CcrA was found to be essentially insensitive to mercaptoacetic acid, **1a**, and only moderately inhibited by benzylmercaptan, **6a**. Furthermore, the inhibition of CcrA by **6a**, a neutral thiol, failed to exhibit slow binding kinetics, a feature observed in similar studies with BcII and IMP-1.

In considering the basis for the variation in response of MBLs to thiol inhibitors, we found it was instructive to examine the structural differences of these enzymes, especially those around the Zn2 locale, which is presumed to be the initial site of substrate (or inhibitor) interaction. Examination of crystal structures has revealed that all three enzymes incorporate a positively charged entity in the vicinity of Zn2 (*14*, *16*, *17*), the nature of which is distinct to each protein.

In both IMP-1 and BcII, the positive charge, provided by a flexible side chain of an amino acid residue (ϵ-NH_3^+ group of Lys69 in IMP-1, guanidinium group of Arg121 in BcII), may interact with the anionic substituent of the thiol inhibitor triggering a rapid isomerization of the EI complex. In the case of neutral thiol inhibitors devoid of a proximal anionic substituent, hydrophobic interactions (between the enzyme and the inhibitor) may trigger the isomerization of EI, however, at a slower rate as indicated by a lag phase in the substrate hydrolysis profile.

In the case of CcrA, the positive charge does not arise from the side chain of an amino acid residue, but instead is provided by a sodium ion, which appears to be inflexible by being bound to five oxygen atoms derived from one carboxylate oxygen atom of Asp84, two main chain carbonyl oxygen atoms, and two water molecules (*14*). It is conceivable that the inflexibility of the sodium ion binding site prevents optimal electrostatic interactions between the enzyme and the inhibitor. This feature may account for thiols such as **1a** and **6a** not serving as effective inhibitors of CcrA.

Studies on the interaction of MBLs with substrates have focused on the later stages of the catalytic cycle, which influence the overall rate of the catalytic process. The initial events (formation of ES), however, have not been investigated in detail. The analogous interactions between MBLs and inhibitors, which mimic, at least initially, those between the enzymes and normal substrates, are also worthy of detailed consideration. The observations recorded herein suggest that such interactions may be much slower and may therefore play an important role in the overall inhibition process (e.g., classical vs slow-binding). Thus, the identification of the initial stages of MBL−thiol inhibitor interactions may constitute a first step in unraveling the subtle complexities of such processes.

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