SHORT COMMUNICATION

Studies on Ternary Metallo-β Lactamase-Inhibitor Complexes Using Electrospray Ionization Mass Spectrometry

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Metallo-β-lactamases (MBLs) are targets for medicinal chemistry as they mediate bacterial resistance to β-lactam antibiotics. Electrospray-ionization mass spectrometry (ESI-MS) was used to study the inhibition by a set of mercaptocarboxylates of two representative MBLs with different optimal metal stoichiometries for catalysis. BcII is a dizinc MBL (Class B1), whilst the CphA MBL (Class B2) exhibits highest activity with a single zinc ion in the active site. Experimental parameters for the detection of the metallo-enzyme and the metallo-enzyme-inhibitor complexes were evaluated and optimized. Following investigations on the stoichiometry of metal binding, the affinity of the inhibitors was investigated by measuring the relative abundance of the complex compared to the metalloprotein. The results for the BcII enzyme were in general agreement with solution assays and demonstrated that the inhibitors bind to the dizinc form of the BcII enzyme. The results for the CphA(Zn^{II}) complex unexpectedly revealed an increased affinity for the binding of a second metal ion in the presence of thiomandelic acid. The results demonstrate that direct ESI-MS analysis of enzyme:inhibitor complexes is a viable method for screening inhibitors and for the rapid assay of the enzyme:metal:inhibitor ratios. (J Am Soc Mass Spectrom 2006, 17, 1000–1004) © 2006 American Society for Mass Spectrometry

The hydrolysis of β-lactam antibiotics is employed by both Gram-positive and -negative bacteria to enable antibiotic resistance. β-Lactamases may be divided into the serine and metallo-β-lactamase (MBL) classes. The MBLs can be further subdivided into three subclasses, B1, B2, and B3, on the basis of their structures and substrate specificity [1]. Subclasses B1 and B3 display a broad substrate selectivity profile, whereas the B2 enzymes are more selective for carbapenems [2, 3]. MBLs utilize one or two zinc ions as a cofactor, but there is uncertainty as to whether one or two zinc ions are involved in catalysis in vivo [4, 5]. The presence of a second zinc ion inhibits the B2 class enzymes non-competitively but increases the rate of catalysis of enzymes from the other subclasses [6]. The substrate profile of the family, coupled with the different metal stoichiometries, poses a challenge for the design of clinically useful inhibitors. Mercaptocarboxylates have been identified as competitive inhibitors of the MBLs [7] and structures of the MBLs reveal that the thiol group of inhibitor is chelated by both zinc ions [8, 9].

The aim of this work was to investigate the utility of ESI-MS for the screening of MBL inhibitors, by direct analysis of enzyme:metal:inhibitor complexes. Precedents for the use of ESI-MS to directly investigate

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ternary metalloprotein-ligand/inhibitor complexes are, however, limited [10–13]. We studied the interaction of a set of mercaptocarboxylate inhibitors with representative B1 and B2 MBLs: BcII from *Bacillus cereus* and CphA from *Aeromonas hydrophilia*. The results reveal that screening for MBL inhibitors by direct ESI-MS not only has the potential to be applied to high throughput analyses for the identification of MBL inhibitors, but can rapidly provide information on changes in enzyme: metal:inhibitor stoichiometry.

Experimental

Enzymes

The BcII MBL from *Bacillus cereus* 569/H/9 and CphA MBL from *Aeromonas hydrophilia* AE036 were prepared as described [14, 15]. Protein concentrations were determined spectrophotometrically using extinction coefficients λ_{280} = 30,500 M^{-1} cm^{-1} (BcII) and λ_{280} = 38,000 M^{-1} cm^{-1} (CphA). Apo-BcII was prepared as described [5].

Chemicals

Reagents were from Sigma-Aldrich, Riedel-de-Häen or Fluka. Thiomandelic acid was synthesized in a racemic form from α-bromophenylacetic acid, which was first thioacylated using potassium thioacetate, then hydrolyzed using a diluted solution of sodium hydroxide. Subsequent purification using a preparative TLC afforded the desired compound [7].

Nano-ESI-MS

Before analyses, the MBLs were desalted using Microcon YM-10 (cut off = 10,000 Da) centrifugal filters (Millipore, Bedford, MA) in 15 mM NH_4Ac (pH 7.5). Seven dilution/concentration steps were performed at 4 °C and 14,000 g. Enzymes were diluted in the NH_4Ac buffer to a final concentration of 15 μM. Inhibitors were dissolved in ethanol at 100 mM, diluted in 15 mM NH_4Ac, and the pH adjusted to 7.5. Unless stated, for the inhibitor analyses the MBL was mixed with 1.3 equivalents inhibitor at room temperature before analysis.

Analyses used an ESI Q-TOF mass spectrometer (Q-TOFmicro, Micromass, Altrincham, UK) interfaced with a NanoMate chip-based nano-ESI source (Advion Biosciences, Ithaca, NY). Typically, a spraying voltage of 1.68 kV and a sample pressure of 0.25 psi were applied. The instrument was equipped with a standard Z-spray source block. Each well was loaded with 5 μl of sample and was infused to the mass spectrometer (estimated flow rate ca. 100 nL/min). Clusters of $Cs_{(n+1)}I_n$ (1 mg/ml CsI in 100% methanol) were used for calibration. Calibration and sample acquisitions were performed in the positive ion mode in the range of *m/z* 500 to 5000. Operating conditions for the mass spectrometer were: sample cone voltage (varied) between 15 to 200 V, source temperature 40 °C. Acquisition and scan time were 2 min and 1 s, respectively. The pressure at the interface between the atmospheric source and the high vacuum region was fixed at 6.7 mbar (measured with the roughing pump Pirani gauge) by throttling the pumping line using an Edwards Speedivalve (Sussex, UK) to provide collisional cooling. Data were smoothed by the Savitzky Golay method (smooth windows: 20, number of smooth: 4), the background subtracted, and the masses finally calculated by centering. The standard deviation reported for all the calculated masses represents the precision of the mass calculation from *m/z* values reported from the ESI mass spectrum. Data were processed using MassLynx software versions 4.0 and 3.5 (Micromass, Altrincham, UK).

Results and Discussion

Analysis of the BcII Metalloenzyme-Inhibitor Stoichiometry

Initially the BcII apoenzyme was analyzed under "denaturing" (MeOH/0.2% HCO_2H) and "nondenaturing" (aq. NH_4OAc, pH 7.5) conditions. The ESI mass spectrum from the former revealed a charge state distribution between +16 and +32, with a maximum at +27 at low *m/z* values (*m/z* 800 and 1600) and gave a mass identical to the predicted value (24,960 ± 0.6 Da). Under nondenaturing conditions, an identical mass but with a lower charge state distribution (between +8 and +10 with a maximum at +9) was observed. Upon addition of 2 equivalents of Zn(II) at pH 7.5, a mass of 25,087 ± 0.2 was observed, corresponding to the dizinc species; the mass difference (127 Da) suggested that for each bound Zn(II) ion, two protons are lost (see Supplementary Material Figure 1, which can be found in the electronic version of this article).

Since it co-purifies with 1 equivalent of tightly bound zinc, the CphA enzyme was measured directly after purification under denaturing and nondenaturing conditions without addition of exogenous zinc. The observed mass for the CphA enzyme under nondenaturing conditions (25,252 ± 0.3 Da) was in agreement with the predicted value with binding of one zinc ion and loss of two protons. As for the BcII enzyme, a shift from a high (+32 until +20) to a low (+9 until +10) charge state distribution was observed for denaturing compared with nondenaturing conditions. The monozinc-protein complex was stable up to a cone voltage of 200 V.

Although both mono- and dizinc versions of the BcII enzyme are catalytically active [14, 16], most spectroscopic and inhibition studies on the B1 class MBLs have been performed in the presence of two zinc ions [7, 17], hence the $BcII(Zn^{II})_2$ enzyme was used for inhibition studies. Figure 1 shows the mass spectrum for $BcII(Zn^{II})_2$ with 1.3 equivalents of thiosalicylic acid (in EtOH; final concentration <0.05%) at

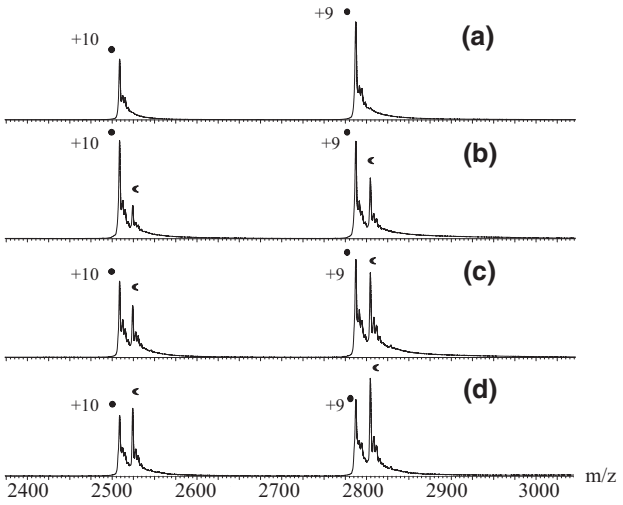

Figure 1. ESI-MS Spectra of the BcII MBL with 1.3 equivalents of thiosalicylic acid at a cone voltage of 120 V (**a**), 70 V (**b**), 50 V (**c**), and 20 V (**d**). Experiments were performed in aq. NH_4Ac (pH 7.5), pressure at interface 6.7 mbar; (circle) Metalloenzyme; (crescent) metalloenzyme-inhibitor-complex.

different cone voltages. Controls indicated that EtOH had no significant influence on the charge state distribution but slightly increased the relative intensity of the +10 compared with the +9 charge state. At 20 V cone voltage, two peaks were observed; the first at 25,087 ± 0.2 Da corresponds to the predicted protein mass (24,960 Da) binding two zincs with loss of four protons. The second peak had a mass increment of 154 Da relative to $BcII(Zn^{II})_2$, corresponding to the mass of thiosalicylic acid. No further signals were observed when the molar excess of the inhibitor to the protein was increased to 4-fold (data not shown), consistent with specific formation of a complex with an equimolar E:I stoichiometry.

By increasing the cone voltage from 20 to 120 V, the intensity of $BcII(Zn^{II})_2$ inhibitor complex decreased and disappeared at 120 V (Figure 1), whereas the $BcII(Zn^{II})_2$ complex was apparently stable. Together with solution data, these observations exclude the formation of a covalent disulfide bridge between the inhibitor sulfhydryl group and the Cys-residue present in the active site of the BcII enzyme [18], since a covalent interaction would not be expected to be disrupted by increased cone voltage.

Comparison of Different Thiol Inhibitors for BcII Enzyme

The relative abundance of the $BcII(Zn^{II})_2$-enzyme-thiomandelate complex was constant (70.7 ± 2.5%) from cone voltages of 20 to 50 V but decreased as the cone voltage was increased to 90 V, where only 20% of the complex relative to that at 20 V was detected. Similar behavior was observed for thiosalicylic acid and 4-mercaptobenzoic acid; the effect was more apparent for 4-mercaptobenzoic acid where the complex was not observed at 90 V (Supplementary Material Figure 2). Due to the effect of cone voltage on the relative stability of the metalloprotein-inhibitor complexes, measurements for a comparison of the binding of different inhibitors were performed at a constant cone voltage of 50 V, thus enabling observation of both complexes. Use of lower cone voltages led to an inefficient desolvation resulting in a significantly lowered degree of mass accuracy (data not shown).

Figure 2 shows mass spectra for $BcII(Zn^{II})_2$ enzyme complexes in the presence of thiomandelic acid (1.3 equivalents) (Figure 2a), thiosalicylic acid (Figure 2b), and 4-mercaptobenzoic acid (Figure 2c). The first peak detected in presence of each inhibitor corresponds to $BcII(Zn^{II})_2$, the second peak to the particular $BcII(Zn^{II})_2$-inhibitor complex. The ratio of $BcII(Zn^{II})_2$ to the $BcII(Zn^{II})_2$-inhibitor complex differed only by a maximum of 5% between the +9 and +10 charge states. To account for this difference, the abundance of the complex at the +9 and +10 charge states were averaged in the analyses [19]. Due to its low intensity, the data for the +8 charge state were not used. Comparison of the spectra for the three inhibitors (Figure 2a, b, and c) implies that ca. 70% of enzyme$(Zn^{II})_2$ is associated with (±)-thiomandelic acid, compared with ca. 43% for thiosalicylic acid and 27% for 4-mercaptobenzoic acid. In solution experiments [7], K_i-values of 0.34, 29, and 346 μM were found for the racemic thiomandelic acid, thiosalicylic acid and 4-mercaptobenzoic acid, respectively. An apparent dissociation constant K_d was determined for binding of thiosalicylic acid to the BcII enzyme by titration of the MBL with the inhibitor (from 10 to 35 μM) using data from the +9 and +10 charge states (Supplemen-

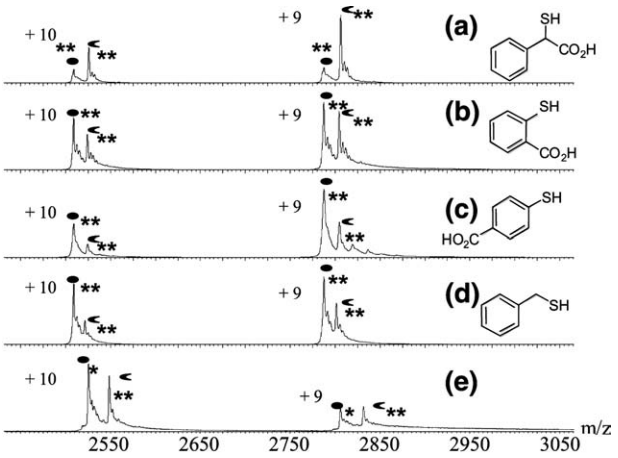

Figure 2. ESI-MS spectra of the BcII dizinc MBL with 1.3 equivalents of (**a**) racemic thiomandelic acid (168 Da), (**b**) thiosalicylic acid (154 Da), (**c**) 4-mercaptobenzoic acid (154 Da), (**d**) benzylmercaptan (124 Da), and (**e**) CphA monozinc MBL with 2.6 equivalents of racemic thiomandelic acid. Experiments were performed in aq. NH_4Ac (pH 7.5), cone voltage 50 V, interface pressure 6.7 mbar. (circle) Metalloenzyme, (crescent) metalloenzyme-inhibitor-complex, (two asterisks) dizinc species, (one asterisk) monozinc species.

tary Material Figure 3). A linear correlation between inhibitor concentrations of 10 to 35 μM was observed and a K_d value of 35.3 $\pm$ 4 μM obtained in good agreement with the solution studies (Supplementary Material Figure 3) [7].

The relative affinity of this set of inhibitors for the BcII enzyme is proposed to increase as the separation of the thiol and carboxylate groups decreases [7]. The MS analyses support this conclusion since thiomandelic acid was observed to bind more tightly than thiosalicylic acid or 4-mercaptobenzoic acid. 4-Mercaptobenzoic acid displays also some affinity for the BcII dizinc enzyme in solution (K_i 346 μM), but exhibited a relatively high abundance for the metalloprotein-inhibitor complex (27%) in the ESI mass spectrum (Figure 2c); With 1.3 equivalents of inhibitor to metalloenzyme, two further molecules of 4-mercaptobenzoic acid were observed to bind to the dizinc enzyme at a lower abundance than the first, suggesting that more than one molecule of 4-MBA can be accommodated by the enzyme.

A relative abundance of 33% was observed for the inhibitor complex with the BcII$(Zn^{II})_2$-enzyme for benzylmercaptan (K_i 9.2 μM) (Figure 2d), which is about half that for thiomandelic acid, demonstrating that the presence of an appropriately placed carboxylate results in increased complex stability. Replacing the thiol of thiomandelate by an alcohol, i.e., with mandelate, revealed that less than 5% of the enzyme$(Zn^{II})_2$-inhibitor complex was formed in the presence of 5.3 equivalents of inhibitor to metalloenzyme, demonstrating that the presence of the thiol is important. Analogous results were obtained with solution spectrophotometric experiments [7].

Effect of Thiomandelic Acid on the CphA Metal Stoichiometry

Now thiomandelic acid is probably the only available broad spectrum MBL inhibitor with a submicromolar K_i value (except for CphA, where it has a micromolar K_i value) [7]. To investigate this difference, monozinc CphA was analyzed with thiomandelic acid (2.6 equivalents) under the same conditions used for the dizinc BcII enzyme. Given that for the CphA enzyme K_d value for binding of the first zinc is 6 pM and for the second zinc 10 μM [20], the observation of the second peak corresponding to the *dizinc* form of the CphA enzyme binding one molecule of thiomandelate (25,483 $\pm$ 0.65 Da), was unexpected (Figure 2e). The apo-CphA enzyme was not detected, which is probably caused by the presence of Zn(II) in the inhibitor solution or by Zn(II) bound to the CphA not detected by MS. The abundance of the dizinc form binding thiomandelate was 52% relative to the inhibitor-free monozinc form at 50 V. A relatively low intensity peak (<10% of the dizinc complex at 50 V cone voltage) corresponding to the monozinc form of the CphA enzyme complexed with thiomandelate was observed. Raising the cone voltage to 80 V caused the peak intensity of the dizinc:enzyme:inhibitor complex to be considerably decreased; instead, a peak at 25,314 Da appeared, corresponding to the inhibitor free dizinc enzyme was observed. These observations suggest that thiomandelate induces a higher affinity for binding of the second metal ion. The presence of two metals thus seems to be required for efficient binding of thiomandelic acid, which is also confirmed by the drop in the K_i value for the monozinc CphA enzyme (144 μM) compared to the dizinc BcII enzyme (0.34 μM).

Conclusions

When coupled to multi-well plate sample delivery, such as chip-based nano-ESI [19], the speed and sensitivity of direct analyses of enzyme-inhibitor complexes by ESI-MS make it an attractive tool for the direct high-throughput screening for enzyme inhibitors, and more generally for small-molecule protein interactions. The technique is particularly suited to the rapid determination of the enzyme:inhibitor ratios, and in particular the determination of the stoichiometry of metal binding. This information can be difficult to obtain by other methods and is often ignored in high-throughput studies on the inhibition of metallo-enzymes. The results obtained by ESI-MS for the binding of the mercaptocarboxylate inhibitors to two MBLs via analysis of the relative abundance of the ternary or quaternary enzyme:metal:inhibitor complexes were in general agreement with the inhibition data obtained in solution. An exception was for benzylmercaptan, where the relative abundance of the enzyme-inhibitor complex was lower than that for thiosalicylate complex when determined by ESI MS, whereas in solution the affinity of benzylmercaptan (9.2 μM) was slightly higher than for thiosalicylate (29 μM). This observation may reflect proposals that electrostatic and hydrogen bonding interactions are emphasized during ion transfer from solution to the gas-phase, whereas hydrophobic interactions are reduced [11]. The results for thiomandelate with the CphA enzyme also provided unexpected new information on inhibition of the CphA enzyme. This inhibitor induced an increase of the affinity for binding of a second zinc ion in the complexed compared to uncomplexed enzyme, analogous to observations on the inhibition of the mono-cadmium form of the BcII enzyme [8], where thiomandelate induced conversion of the mono- to the dicadmium enzyme. Thus, the present work indicates that the differences in the stoichiometry of metal binding by the MBLs may be more subtle than previously appreciated.

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