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Catalytic Inactivation of Human Carbonic Anhydrase I by a Metallopeptide−Sulfonamide Conjugate is Mediated by Oxidation of Active Site Residues

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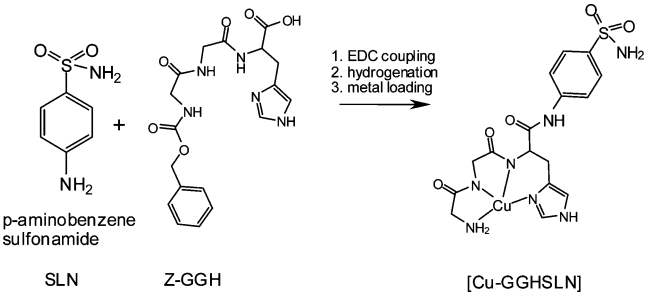
Zinc-dependent metalloproteases are a well-recognized drug target for treatment of a variety of disease states.[1] Inhibitors are often substrate-based, involving recognition of the active site coupled with coordination to the zinc cofactor.[2] Potent drug inhibitors typically show dissociation constants in the nanomolar or submicromolar range.[2] Recently, we have reported an alternative strategy to the classical design of enzyme inhibitors,[3−5] exploring the development of catalytic drugs that are selective for a therapeutic target and mediate the irreversible inactivation of that target. Such an approach is complementary to traditional concepts underlying drug development but has the potential to lower both dosage and dose frequency.

Our prior reports of catalytic inactivation of ACE and ECE-I described metallopeptide-mediated time-dependent inactivation in the presence of a physiologically relevant co-reactant such as ascorbate.[3,4] The mechanism of catalytic inactivation presumably involves metal-associated reactive oxygen species that promote either protein cleavage[3] and/or modification of active site residues. Herein we report the results of experiments designed to elucidate the mechanism of enzyme inactivation by metallopeptide-promoted formation of reactive oxygen species. This work also establishes a non-peptide moiety as a targeting agent when coupled to a copper−ATCUN motif,[8] using a metallopeptide−drug conjugate to catalytically inactivate the zinc-dependent enzyme, human carbonic anhydrase I (CA-I).

Carbonic anhydrases (CAs, EC 4.2.1.1) carry out a number of biosynthetic reactions including the reversible hydration of CO_2 to form bicarbonate.[9] Possessing a $(His)_3$-coordinated active site Zn^{2+}, these enzymes are inhibited by sulfonamides,[9−12] and numerous enzyme−sulfonamide complexes have been crystallographically characterized.[11,12] The simplest of these sulfonamides, sulfanilamide (Scheme 1), represents the active pharmacophore and the building block for several generations of carbonic anhydrase inhibitors (CA-Is).[9] We have utilized a sulfanilamide derivative as a targeting motif for CA-I in a Gly−Gly−His (GGH)-tagged conjugate (GGHSLN) (Scheme 1) that directs a Cu^{2+}-bound GGH complex to mediate oxidative damage within the active site of the enzyme.[13] The amino terminal copper/nickel binding motif (ATCUN) is naturally found in serum albumins, demonstrating high affinity toward Cu^{2+} and Ni^{2+} metal ions under physiological conditions.[8] The metal is redox active in the $Cu^{3+/2+}$ states, with high affinity to the ATCUN ligand in both states. Accordingly, and as evidenced elsewhere,[4,6,14] the metal ion is not released during catalytic turnover. The most likely active species is a Cu^{2+}-associated hydroxyl radical, or Cu^{3+}═O (copper oxone species).[3,4,14−17]

Under hydrolytic conditions (no reducing agent), Cu−GGHSLN inhibited CA-I ($K_I = 5.00\ \mu M$) in a dose-dependent manner (relative to $K_I = 8.20\ \mu M$ for the free sulfanilamide motif) and comparable to the Cu^{2+}-free ligand (GGHSLN, $K_I = 5.20\ \mu M$). Neither Cu^{2+}(aq) nor Cu−GGH alone demonstrated significant inhibition

Scheme 1. Synthetic Route to a Metallopeptide−Sulfonamide Conjugate as a Catalytic Inactivator of Human Carbonic Anhydrase I[a]



[a] The coupling of sulfanilamide (SLN) to Z-GGH (Z-Gly−Gly−His, where Z is carboxybenzyl) in the presence of EDC ((1-ethyl-3-[3-dimethyl aminopropyl] carbodiimide hydrochloride) resulted in the N-terminally protected ligand that upon hydrogenation yielded the zero-linker-length bifunctional ligand GGHSLN. THE Cu^{2+} complex of GGHSLN demonstrated the characteristic absorbance signature for GGH-bound Cu^{2+} ($\lambda_{max} = 525$ nm)[6] as well as a $Cu^{3+/2+}$ redox couple of 1.23 V (vs NHE).[7]

at the concentration used. Under oxidative conditions and subsaturating concentrations of Cu−GGHSLN (5 μM), time-dependent inactivation of CA-I activity was observed (Figure 1), yielding $k_{obs} \sim 0.038\ min^{-1}$ ($k_2 \sim 7600\ M^{-1}\ min^{-1}$). The initial activity of ~76% reflects the inhibitory influence of the complex prior to initiation of irreversible chemistry in the active site. Within 3 h, CA-I activity was eliminated. The residual 20% activity reflects the intrinsic hydrolytic activity of the Cu−GGHSLN complex toward the substrate. Inactivation by Cu^{2+}(aq) under oxidative conditions was considerably slower ($k_{obs} \sim 0.003\ min^{-1}$). No evidence of protein cleavage was found by SDS-PAGE gel electrophoresis, but several residues were found to be susceptible to oxidation following a detailed mass spectrometric analysis (Supporting Information).

Following incubation of CA-I with Cu−GGHSLN for 3 h under oxidative conditions, the Q-TOF2 mass spectrum yielded clear evidence for oxidation of 1 or 2 residues per protein molecule. More extensive oxidation (at least 12 residues) was observed with Cu^{2+}(aq), although the relatively slow inactivation chemistry suggests that surface residues are the most probable target sites for free copper ion. Control reactions with Cu−GGH and ascorbate also did not show any evidence of protein oxidation, consistent with a lack of binding by the latter (as noted earlier). Characterization of oxidized residues was achieved by enzymatic digestion using both chymotrypsin and trypsin, and the modified residues were mapped by mass spectrometric analysis (nano LC/MS/MS) (Supporting Information). Clear evidence for specific amino acid residue modification in the proximity of the enzyme active site was obtained (Supporting Information). The most prominent oxidation was observed for select histidine residues (H40, H64, H67, H94, H96, H103, H200, and H243). Prior reports indicate that histidine residues

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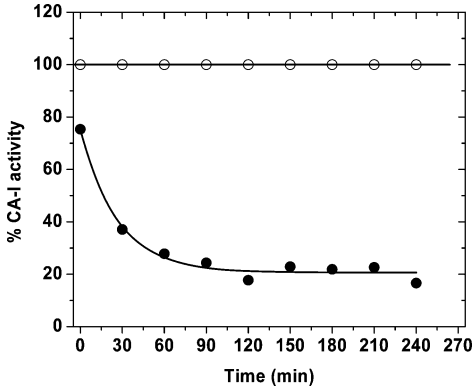

Figure 1. Human carbonic anhydrase I (CA-I) activity was measured at the specified time intervals. Reactions were set up as individual reactions (0.3 mL final volume) containing 1.1 μM CA-I, with zero time started at the simultaneous incubation of all reactions with Cu−GGHSLN (5 μM) and ascorbate (1 mM) in Tris buffer (12.5 mM, 75 mM NaCl, pH 8.0). The inhibitor, Cu−GGHSLN, concentration was employed at the K_I concentration (5 μM). The activity at each time interval was determined under initial velocity conditions with 1 mM substrate (4-nitrophenyl acetate). Control reactions containing no inhibitor but only ascorbate were run simultaneously. The initial velocity data were converted to % CA-I activity with respect to control and plotted as a function of time. The figure shows (○) control reaction with ascorbate only and (●) the time-dependent inactivation in the presence of Cu−GGHSLN and ascorbate.

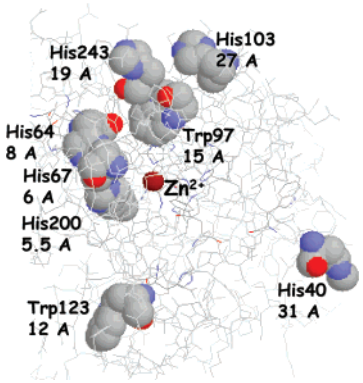

Figure 2. Summary of residues that are susceptible to oxidative modification (H40, H64, H67, H103, H200, H243, W97, W123) in human CA-I (PDB: 1CZM) following interaction with Cu−GGHSLN in the presence of ascorbate. A maximum of two residues are modified per protein. The catalytic zinc ion is coordinated by three histidines that are not oxidized. Overall, the figure shows modified amino acids to be localized in the proximity of the enzyme active site (except H40 and H103). Distances from the oxidized residues to the catalysts are listed under each label as detailed in the main text.

are effectively converted to the 2-oxo-histidine upon exposure to Cu−ATCUN-derived oxygen-based radicals.[18−20] Trp was also found to be selectively oxidized (W97, W123).[21,22] Significantly, none of the zinc-bound histidine residues were oxidized (Supporting Information), and so the time-dependent inhibition of CA-I by Cu−GGHSLN does not involve a random oxidation pathway but seems to be specific to residues that lie close to the Cu−GGH domain of the metallopeptide−drug conjugate.

Figure 2 shows the location of oxidized residues with respect to the active site and a crystallographically determined bound sulfonamide inhibitor.[10] Using this structural data (PDB 1CZM), distances to the oxidized residues from the terminal *para*-amino nitrogen of the sulfanilamide inhibitor to the presumed site of reaction on the His and Trp rings [C2, respectively, assuming 2-oxo-His and (formyl)kynurenine formation] were calculated. These distances are listed in Figure 2 under each residue label. Taken together, the results showed two nonspecific long-range residue oxidations (H40 and H103) that could be attributed to the diffusion of metal-associated radicals or nonspecific surface reactions. Neither residue lies close to a secondary inhibitor binding site that has been structurally characterized.[23] Overall, the oxidation of the amino acid residues was restricted to the proximity of the active site (5−20 Å) where the inhibitor tail with the Cu−GGH motif would be oriented. The pattern of oxidation also reveals a certain degree of freedom to the bound inhibitor that can transfer metal-bound oxygen species to neighboring residues. Localization of modified residues around the active site supports the nondiffusive nature of the oxygen species and association with the catalytic copper center. Three of the modified residues sites (His64, His67, and His200) have been implicated in rate-limiting proton transfer within the active site.[24,25] Enzyme inactivation through targeted catalytic modification of active site residues represents a novel mechanism for enzyme inhibition. Such an approach is not only limited to the design of catalytic inhibitors that, to the best of our knowledge, have not been explored but also would give deeper insights on protein−substrate interactions.

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Supporting Information Available: Synthetic procedures, enzyme assays, UV−vis and electrochemical characterization of copper complexes, mass spectrometric data, and control assays for Cu^{2+}(aq). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hooper, N. M. *Zinc Metalloprot. Health Dise.* **1996**, 1−21.
(2) Babine, R. E.; Bender, S. L. *Chem. Rev.* **1997**, *97*, 1359−1472.
(3) Gokhale, N. H.; Cowan, J. A. *J. Biol. Inorg. Chem.* **2006**, *11*, 937.
(4) Gokhale, N. H.; Cowan, J. A. *Chem. Commun.* **2005**, 5916−5918.
(5) Jin, Y.; Cowan, J. A. *J. Am. Chem. Soc.* **2006**, *128*, 410−411.
(6) Jin, Y.; Cowan, J. A. *J. Am. Chem. Soc.* **2005**, *127*, 8408−8415.
(7) Jin, Y.; Lewis, M. A.; Gokhale, N. H.; Long, E. C.; Cowan, J. A. *J. Am. Chem. Soc.* **2007**, *129*, 8353−8361.
(8) Harford, C.; Sarkar, B. *Acc. Chem. Res.* **1997**, *30*, 123−130.
(9) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146−189.
(10) Chakravarty, S.; Kannan, K. K. *J. Mol. Biol.* **1994**, *243*, 298−309.
(11) Di Fiore, A.; De Simone, G.; Menchise, V.; Pedone, C.; Casini, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1937−1942.
(12) Khadikar, P. V.; Sharma, V.; Karmarkar, S.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 923−930.
(13) Stadtman, E. R. *Am. J. Clin. Nutr.* **1991**, *54*, 1125S−1128S.
(14) Gokhale, N. H.; Bradford, S.; Cowan, J. A. *J. Biol. Inorg. Chem.* **2007**, *12*, 981−987.
(15) Bossu, F. P.; Margerum, D. W. *J. Am. Chem. Soc.* **1976**, *98*, 4003−4004.
(16) Margerum, D. W.; Owens, G. D. *Met. Ions Biol. Syst.* **1981**, *12*, 75−132.
(17) McDonald, M. R.; Fredericks, F. C.; Margerum, D. W. *Inorg. Chem.* **1997**, *36*, 3119−3124.
(18) Schoneich, C. *J. Pharm. Biomed. Anal.* **2000**, *21*, 1093−1097.
(19) Uchida, K.; Kawakishi, S. *Arch. Biochem. Biophys.* **1990**, *283*, 20−26.
(20) Hovorka, S. W.; Biesiada, H.; Williams, T. D.; Huhmer, A.; Schoneich, C. *Pharm. Res.* **2002**, *19*, 530−537.
(21) Uchida, K.; Enomoto, N.; Itakura, K.; Kawakishi, S. *Arch. Biochem. Biophys.* **1990**, *279*, 14−20.
(22) Griffiths, H. R. *Free Radical Res.* **2000**, *33*, S47−S58.
(23) Jude, K. M.; Banerjee, A. L.; Haldar, M. K.; Manokaran, S.; Roy, B.; Mallik, S.; Srivastava, D. K.; Christianson, D. W. *J. Am. Chem. Soc.* **2006**, *128*, 3011−3018.
(24) Engstrand, C.; Jonsson, B.-H.; Lindskod, S. *Eur. Biochem, J.* **1995**, *229*, 696−702.
(25) Mohanty, A. K.; Kannan, K. K.; Mahajan, S. K. *J. Biosci.* **1998**, *23*, 235−246.

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