Protease Activity of 1,10-Phenanthroline−Copper(I). Targeted Scission of the Catalytic Site of Carbonic Anhydrase†

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ABSTRACT: To investigate the mechanism of scission of proteins by the chemical cleaving agent 1,10-phenanthroline−copper, the active sites of human carbonic anydrase I and bovine carbonic anhydrase II have been targeted for cleavage by a tight binding sulfonamide inhibitor tethered to the metal complex. The inhibitor−phenanthroline−copper conjugate binds to the carbonic anhydrases with sub-micromolar K_d's and, upon addition of a reducing agent, causes scission specifically within the active site of the enzymes to yield a discrete set of cleavage fragments. N- and C-terminal sequencing and mass spectrometric analysis of several fragments indicate that the C-terminal cleavage fragments have free amino groups at their N termini, thereby allowing facile location of the cut sites through standard Edman degradation. The N-terminal cleavage fragments do not have a free carboxyl group at their C termini. It is proposed that scission occurs by abstraction of H at C_α, followed by oxidation at C_α by the neighboring cupric ion and cleavage of the C_α−C(O) bond to give an N-terminal fragment containing a C-terminal acyl amide, and an unstable C-terminal fragment containing an N-terminal isocyanate group which undergoes hydrolysis to a free amino terminus. Modeling of the inhibitor−phenanthroline−copper conjugate within the active site of human carbonic anhydrase I shows that the sites of cleavage that have been identified are fully consistent with the available structural data.

Redox active coordination complexes which cleave DNA and RNA, in particular 1,10-phenanthroline−copper(I) (OP−Cu[1]) and ferrous−EDTA (Fe−EDTA), are well-established tools for the analysis of ligand−nucleic acid interactions. Unlinked to a targeting ligand, the free coordination complexes serve as footprinting reagents (*1*, *2*). Tethered to a nucleic acid-binding ligand, highly selective scission reagents targeted to the ligand binding site can be generated (*3*, *4*). Recently, the redox chemistry of these coordination complexes has been redirected to the cleavage of the polypeptide backbone of proteins (*5*−*12*). While cleavage by naturally occurring proteases is determined by the sequence positions of particular amino acid residues, cleavage by these artificial proteases appears to be independent of the residues involved and instead is governed either by accessibility or by proximity to the metal chelate. Protein cleavage by tethered metal chelates has been used to establish proximity relationships among interdomainal residues in two membrane proteins, the lactose permease (*5*) and cytochrome *bd* quinol oxidase (*13*) of *Escherichia coli*, and to study non-native conformations of staphylococcal nuclease during unfolding (*14*). Protein cleavage by untethered metal chelates has been used to map protein domains involved in the cAMP−cAMP receptor protein−DNA complex (*15*), to map interactions between subunits of *E. coli* RNA polymerase (*16*), and to locate metal and metal−substrate binding sites of actin (*17*) and of isocitrate dehydrogenase (*18*).

The ability of 1,10-phenanthroline−copper(I) to efficiently cleave peptide bonds was first exploited in analyzing the structural topology of *E. coli* lactose permease (lac permease), a paradigm for membrane transport proteins (*5*). The method entailed covalently linking 5-(iodoacetamido)-1,10-phenanthroline to a functional lac permease mutant via a single cysteine residue situated within one of the 12 membrane-spanning α-helices. In the presence of Cu(II), O_2, and a reductant, efficient, localized scission (up to 30%) of the protein backbone was observed in adjacent helices. Since membrane proteins such as lac permease are intrinsically difficult to crystallize, structural information is generally obtained by spectroscopic, genetic, and chemical methods. In the case of lac permease, a helix-packing model had been proposed on the basis of site-directed fluorescence studies and later supported by ESR studies of engineered metal-binding sites (*19*, *20*). However, establishing proximity relationships between interhelical residues by such methods required prior evidence that the residues might interact. That had hindered extending the helix-packing model to the N-terminal helices (I−VI), for which such evidence was

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[1] Abbreviations: OP−Cu, 1,10-phenanthroline−copper(I); HCA I, human carbonic anhydrase I; BCA II, bovine carbonic anhydrase II; CBSA, 4-carboxybenzenesulfonamide; DNSA, 5-(dimethylamino)-1-naphthalenesulfonamide or dansyl amide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; ESR, electron spin resonance; NMR, nuclear magnetic resonance; DCC, 1,3-dicyclohexylcarbodiimide; HRMS, high-resolution mass spectrometry; EI, electron ionization; ESI, electrospray ionization; KOTMS, potassium trimethylsiloxide.

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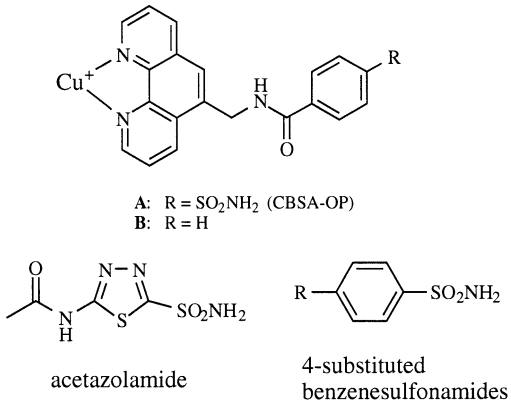


FIGURE 1: Ligands for the active site of carbonic anhydrase. Acetazolamide and 4-substituted benzenesulfonamides are known inhibitors of the enzyme. Compound A is the inhibitor−1,10-phenanthroline−copper(I) conjugate used to cleave the enzyme in its active site. Compound B is a control lacking the essential sulfonamide moiety which coordinates to Zn.

lacking. In contrast, determining which residues were located near a given site which had been mutated to a cysteine and derivatized with phenanthroline only required locating the cleavage sites. This approach was used successfully to determine the proximity and facial orientation of helix V relative to the C-terminal helices.

Neither the nature of the end groups generated by OP−Cu scission of lac permease nor the precise location of the cut sites was identified in the study. The approximate cleavage sites were located after gel electrophoresis with appropriate size markers by immunoblotting. Given the planar, hydrophobic nature of OP−Cu, characteristics not shared by Fe−EDTA, site-directed proteolytic scission by OP−Cu could be uniquely and generally suited to mapping the tertiary structure of membrane proteins if fragments with free amino termini were generated, thereby permitting rapid, precise location of the cut sites by standard protein sequencing techniques.

Fe−EDTA linked to proteins can act via an iron peroxide intermediate to efficiently hydrolyze the protein backbone and thereby generate fragments with free N termini in some cases (*11*, *21*) yet is known to produce diffusible hydroxyl radicals which presumably lead to multiple products which are difficult to analyze in other cases (*9*). Similarly, affinity cleavage of proteins by ferrous ion at metal or metal−substrate binding sites has given cleavage fragments with both free N termini (*17*, *18*) and blocked N termini (*18*). It is unclear which factors govern the outcome of protein scission by ferrous chelates.

OP−Cu reacts with nucleic acids via a nondiffusible copper−oxo intermediate according to a mechanism fundamentally different from that of Fe−EDTA. Presumably, the mechanism of protein scission by OP−Cu also differs. In the work reported here, we have investigated some aspects of the mechanism of the proteolytic activity of OP−Cu through analysis of the products derived from human carbonic anhydrase I (HCA I) and bovine carbonic anhydrase II (BCA II) after cleavage by an inhibitor−1,10-phenanthroline−copper(I) conjugate (CBSA−OP−Cu^+, Figure 1). The carbonic anhydrases are a family of zinc metalloenzymes which catalyze the interconversion of bicarbonate and carbon dioxide and are involved in the transport of metabolic CO_2 and the transfer and accumulation of H^+ and HCO_3^- (*22*−*24*). Inhibitors of the enzymes include a variety of aromatic sulfonamides such as acetazolamide and 4-substituted benzenesulfonamides (Figure 1). High-resolution crystal structures of several of the human isozymes and of their complexes with sulfonamide drugs have been determined (*25*−*27*). In the study presented here, CBSA−OP−Cu^+ is shown to cleave specifically within the active site cavity, the cleavage products are identified, a mechanism is proposed, and the selectivity is explored through molecular modeling. We chose to investigate the chemistry using carbonic anhydrase because of successful prior studies involving ferrous−EDTA and the availability of a wealth of structure/function information.

Two important results have emerged from these studies. The first is that an amino terminus reactive to the Edman degradation is generated in the cleavage reaction. The identification of the C terminus has not yet been achieved, but our initial results indicate that it is not a sequenceable C-terminal end as generated in a proteolytic cleavage. Characterization of its structure may allow development of an analytical method for isolating and identifying a product uniquely formed in this new alternative to protein cross-linking.

MATERIALS AND METHODS

General

All chemicals were purchased from Fisher, Sigma, or Aldrich. 4-Carboxybenzene-1-sulfonamide (CBSA), acetazolamide, 5-(dimethylamino)-1-naphthalenesulfonamide (DNSA), bovine carbonic anhydrase II (product no. C 2522), and human carbonic anhydrase I (product no. C 4396) were all from Sigma. Silica gel 60 (Merck, 230−400 mesh) was used for flash chromatography. NMR spectra were recorded on Bruker AC200 or AM360 instruments. Trans Blot poly(vinyl difluoride) (PVDF) blotting membrane was purchased from Bio-Rad. Zitex membrane was provided by the Department of Immunology at the Beckman Research Institute of the City of Hope. Amino acid analyses and N-terminal sequencing were performed by the UCLA Protein Microsequencing Facility. C-Terminal sequencing and mass spectrometric analyses were carried out at the Division of Immunology of the Beckman Research Institute of the City of Hope.

Synthesis

CBSA−OP (A). Dry dimethylformamide (DMF, 0.5 mL) was added to 5-(aminomethyl)-1,10-phenanthroline (27.4 mg, 0.13 mmol) (*28*) and 4-carboxybenzenesulfonamide (39.5 mg, 1.5 molar equiv) in a flask under argon, and the resulting slurry was cooled to 0 °C. Next, 1,3-dicyclohexylcarbodiimide (DCC, 40.5 mg, 1.5 molar equiv) in DMF (0.5 mL) was added, and the mixture was stirred and allowed to warm to room temperature overnight. The crude reaction mixture was concentrated to approximately 0.5 mL and purified by flash chromatography on silica gel [CH_2Cl_2/CH_3OH/$(CH_3)_2$-$CHNH_2$, gradient from 98:2:0.5 to 80:10:0.5]. The product was obtained as a white powder in 10% yield (not optimized). Unreacted 5-(aminomethyl)-1,10-phenanthroline (30%) was also recovered: ^{1}H NMR (DMSO-d_6, 360 MHz) δ 9.38 (t,

J = 5.5 Hz, 1H), 9.11 (d, J = 3.0 Hz, 1H), 9.05 (d, J = 2.8 Hz, 1H), 8.70 (dd, J = 8.3, 1.3 Hz, 1H), 8.48 (dd, J = 8.1, 1.5 Hz, 1H), 8.08 (d, J = 8.4 Hz, 2H), 7.93 (s, 1H), 7.90 (d, J = 8.4 Hz, 2H), 7.81 (dd, J = 8.4, 4.2 Hz, 1H), 7.74 (dd, J = 8.1, 4.3 Hz, 1H), 7.51 (broad s, 2H), 5.74 (d, J = 5.5 Hz, 2H); HRMS (EI) *m/e* 393.1008 (MH^+, calcd for $C_{20}H_{17}N_4O_3S$, 393.1021).

5-(Benzamidomethyl)-1,10-phenanthroline (B). Chloroform (3.4 mL) was added to 5-(aminomethyl)-1,10-phenanthroline (34.6 mg, 0.17 mmol), benzoic acid (24.9 mg, 1.2 molar equiv), and DCC (41.9 mg, 1.2 molar equiv) in a flask under argon, and the resulting slurry was stirred at room temperature for 19 h. Next, some insoluble tarry substance was removed by filtration. The product was extracted into 0.1 N HCl; the pH of the aqueous extract was then adjusted to pH 12 with 1 N NaOH, and the product was extracted back into CH_2Cl_2 and finally purified by flash chromatography on silica gel: yield 87%; ^{1}H NMR (CD_3OD, 200 MHz) δ 9.06 (dd, J = 4.4, 1.6 Hz, 1H), 9.01 (dd, J = 4.4, 1.7 Hz, 1H), 8.68 (dd, J = 8.4, 1.5 Hz, 1H), 8.34 (dd, J = 8.2, 1.6 Hz, 1H), 7.85−7.93 (m, 3H), 7.75 (dd, J = 8.4, 4.3 Hz, 1H), 7.69 (dd, J = 8.0, 4.4 Hz, 1H), 7.41−7.58 (m, 3H), 5.10 (s, 2H); HRMS (EI) *m/e* 313.1218 (M^+, calcd for $C_{20}H_{15}N_3O$, 313.1215).

Binding Assay

Fluorescence measurements were performed on a Kontron Instruments SFM 25 spectrofluorometer equipped with a magnetic stirrer and a temperature bath set at 25 °C. Excitation was at 290 nm. The fluorescence of bound DNSA was monitored at 460 nm.

Carbonic anhydrase (25 μM, 6 μL) was added to KH_2PO_4 buffer at pH 7.6 (20 mM, 2.994 mL) to give a final concentration of 50 nM in the cell. Stock solutions of the inhibitors (10−105 mM) were prepared in DMSO-d_6 containing 19.7 mM dimethylformamide (DMF). The concentrations of the stock solutions were determined by comparing the integrals of the peaks due to inhibitor and to DMF. The DNSA stock solution was diluted with DMF to 2 mM (for the BCA II titrations) or to 0.2 mM (for HCA I titrations), and 6.0 μL of this was added to the cell containing carbonic anhydrase in buffer to give a concentration of 4 or 0.4 μM DNSA, respectively. The stock solutions of the other inhibitor solutions were diluted into 1:3 DMSO/H_2O to obtain titrant solutions for the fluorescence experiments of 0.04−10 mM. Aqueous $CuSO_4$ was added to CBSA−OP for the CBSA−OP−Cu^{2+} titrant solution immediately prior to the experiment. The decrease in the fluorescence of bound DNSA was recorded as a function of the amount added of one of the other inhibitors. After correction for dilution by the titrant volume, the data were analyzed using Scatchard plots (*29*). The dissociation constants for CA·DNSA needed for the analysis were determined to be 0.38 μM for BCA II and 0.032 μM for HCA I.

Cleavage of Carbonic Anhydrase by CBSA−OP−Cu^+

In a typical cleavage experiment, 1 μL of 100 mM ascorbate was added to 9 μL of a solution containing 100 μM carbonic anhydrase and 100 μM CBSA−OP−Cu^{2+} (freshly prepared from stock solutions of CBSA−OP and $CuSO_4$) in 20 mM KH_2PO_4 (pH 7.6). After 30 min at 22 °C, the cleavage was stopped by addition of 10 μL of loading buffer [200 mM sucrose, 6% SDS, 125 mM Tris·HCl (pH 6.9), 4 mM EDTA, 0.01% bromophenol blue, and 5% 2-mercaptoethanol]. After this mixture was heated to 90 °C for 5 min, the cleavage products were separated by electrophoresis at 25 mA on SDS−PAGE, according to the method of Laemmli (*29*), using 4% stacking and 18% resolving layers in a Mini-Protean unit (Bio-Rad). The peptide bands were visualized by silver staining or staining with Coomassie Blue R-250. For LC−electrospray ionization mass spectrometric analysis, the fragment of interest was isolated after the cleavage reaction by HPLC on a Nucleosil 5 C8 column (Phenomenex) using a water/acetonitrile/trifluoracetic acid gradient.

Electroblotting to PVDF and Zitex Membranes

Gel electrophoresis was performed as described above with the following modifications. First, the gel was cast 24−72 h prior to use. Second, the samples were heated at 37 °C (not at 90 °C) for 10−15 min prior to loading the gel. Finally, ca. 10 mg/L thioglycolic acid was added to the upper running buffer, and the gel was pre-run for 15 min prior to loading.

For amino acid analysis and N-terminal sequencing, the SDS−PAGE-separated fragments were transferred to PVDF. The membrane, cut to the exact size of the gel, was bathed in 100% methanol and then equilibrated in prechilled Towbin buffer [25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.1−8.5)]. The gel and membrane were then assembled in a sandwich between two pieces of filter paper, also pre-equilibrated in Towbin buffer, and placed in the blotting cassette (Bio-Rad Trans Blot Cell). Electroblotting was conducted at 200 mA for ca. 14 h at 4 °C. After the transfer, the membrane was washed thoroughly with deionized water to remove all Tris and glycine. The membrane was stained with 0.025% Coomassie Blue R-250 in 40% aqueous methanol for 2 h at room temperature, and destained in 50% methanol. The membrane was allowed to air-dry, and the bands of interest were cut out and stored at −20 °C until they were analyzed.

For C-terminal sequencing, the SDS−PAGE-separated fragments were electroblotted onto Zitex membrane as described above but with the following changes. First, the Zitex membrane was bathed in 100% ethanol and was *not* equilibrated in Towbin buffer prior to assembling the transfer cassette. Second, the blotting apparatus was the TE Series Transphor unit (Hoffer Scientific Instruments), and the transfer was carried out at 70 mA for 2.5 h at 4 °C. Finally, the transferred bands were stained with 0.5% Ponceau S in 0.1% aqueous acetic acid for 2 min at room temperature, and destained with several changes of deionized water.

RESULTS

Binding of the Inhibitor−OP−Cu^+ Conjugate to Carbonic Anhydrase. The phenanthroline-substituted arenesulfonamide (CBSA−OP) was prepared by carbodiimide coupling of 4-carboxybenzene-1-sulfonamide (CBSA) with 5-(aminomethyl)-1,10-phenanthroline. The dissociation constants for the binding of the inhibitor conjugate to bovine carbonic anhydrase II (BCA II) and human carbonic anhydrase I (HCA I) were determined using a competitive fluorescence-

Table 1: Dissociation Constants of Arenesulfonamide Ligands from Bovine Carbonic Anhydrase II Determined by a Scatchard Analysis of Data from a Competitive Fluorescence-Based Binding Assay Using Dansyl Amide[a]

inhibitor	K_d (μM) for BCA II	K_d (μM) for HCA I
CBSA	0.40	2.1
CBSA−OP	0.0095	0.071
CBSA−OP−Cu^{2+}	0.023	0.33
acetazolamide	0.010	0.024

[a] λ_{excit} = 290 nm; λ_{emis} = 460 nm. Titrations of BCA II·DNSA with CBSA, CBSA−OP, CBSA−OP−Cu^{2+}, and acetazolamide were carried out in 20 mM KP_i (pH 7.6) at 25 °C. Uncertainties in these values are ±30%.

based binding assay (*30*, *31*). The assay is based on the large increase in the quantum yield for fluorescence of 5-(dimethylamino)-1-naphthalenesulfonamide (DNSA, dansyl amide) upon entering the hydrophobic binding pocket of carbonic anhydrase relative to that of free dansyl amide in aqueous solution. Light with a wavelength of 290 nm, an absorption minimum for dansyl amide, was used to excite the 10 tryptophan residues of the protein. Energy transfer from the excited tryptophans [λ_{max}(emission) = 320 nm] occurred selectively to the bound dansyl amide [λ_{max}(absorption) = 326 nm] so that the emission signal detected at 460 nm corresponded only to bound dansyl amide. The binding of the nonfluorescent sulfonamide inhibitors to carbonic anhydrase was monitored by recording the decrease in fluorescence of bound dansyl amide upon their addition. The dissociation constants shown in Table 1 were calculated by a Scatchard analysis of the titration data. The K_d's for the inhibitor−OP−Cu^{2+} conjugate are 23 nM for BCA II and 330 nM for HCA I. By way of comparison, the K_d's for acetazolamide, a well-known inhibitor of carbonic anhydrase, are 10 and 24 nM, respectively.

Cleavage of HCA I and BCA II by CBSA−OP−Cu^{2+}. In Figure 2, the cleavage of bovine CA II (lanes 2−5) and human CA I (lanes 6−9) by the inhibitor−1,10-phenanthroline−copper(I) conjugate (CBSA−OP−Cu^{+}) is shown. A limited number of discrete cleavage fragments is observed, and the cleavage patterns for the two carbonic anhydrases (lanes 2 and 6) are similar, as expected. The cleavage is dependent upon the presence of copper and a reducing agent; no cleavage occurs without both (data not shown). The cleavage is caused by the action of OP−Cu. The cleavage is not due to free cuprous ion or copper bound to side chain residues, since no cleavage occurs in the presence of $CuSO_4$ and ascorbate without CBSA−OP (lanes 3 and 7). Addition of radical scavengers such as thiourea, D-mannitol, and *tert*-butanol has no effect, which suggests that free hydroxyl radicals are not involved in the cleavage events (data not shown). Rather, the oxidative intermediate is likely a metal-oxo species, as is implicated in the cleavage of DNA (*32*). Little cleavage occurs when the phenathroline derivative **B**, which lacks the sulfonamide substituent, is substituted for CBSA−OP (lanes 5 and 9). This is consistent with the cleavage being directed specifically to the active site cleft of carbonic anhydrase by the arenesulfonamide moiety of the inhibitor. Furthermore, addition of a competitive binding sulfonamide ligand, the known inhibitor acetazolamide, almost completely protects the enzyme from cleavage by CBSA−OP−Cu^{+}, which demonstrates that cleavage is taking place within the cleft (lanes 4 and 8).

Although changes in variables such as the concentrations of the enzyme and/or the reagents, reaction time, temperature, buffer, pH, and the addition of H_2O_2 were investigated, the optimal conditions found are those shown in the legend of Figure 2. The scission yields for fragments b1, b2, h1, h2, and h3 are 2−4% per fragment, as determined by amino acid analysis and/or HPLC using a calibration curve.

Identification of Major Fragments Obtained after Cleavage with CBSA−OP−Cu^{+}. Three discrete fragments which migrate at positions corresponding to molecular masses of approximately 14, 15, and 22 kDa were produced upon cleavage of both BCA II and HCA I (Figure 2). We concentrated our efforts on identifying fragments b1 and b2, or alternately h1 and h2, since the apparent molecular masses of these fragments (14 and 15 kDa, respectively) suggested that they could be the N- and C-terminal fragments generated from a single cleavage event of the 29 kDa enzymes. The 22 kDa fragment h3, which appears to result from cleavage at the same site in both HCA I and BCA II though the higher scission yield is consistently observed with HCA I, was also investigated. These peptides were identified by amino acid analysis, N- and C-terminal sequencing, and LC−ESI mass spectrometry. The results are summarized in Table 2.

The five cleavage fragments were separated by SDS−PAGE, electroblotted onto a poly(vinyl difluoride) membrane, and submitted for amino acid and N-terminal sequencing analysis. The N-terminal sequence of the peptide b1, Thr-Ala-Ala-Gln-Gln-Pro, is uniquely located at residues 131−136 of BCA II, while that of peptide h1, Glu-Ala-Ala-Ser-Lys-Ala, corresponds to residues 133−138 of HCA I (Table 2, column 4). Therefore, cleavage occurred just before residues 131 and 133, respectively. It is noteworthy that residue 131 in BCA II aligns with residue 133 in HCA I, and that these residues lie within the central hydrophobic cleft. N-terminal sequencing of peptide h3 yielded the unique sequence Gly-His-Ser-Phe-His-Val, corresponding to residues 63−68. Therefore, cleavage occurred just before residue 63, which is situated in the outer wall of the binding pocket opposite Glu133. More than a single residue was found in many cycles of the analyses of peptides b1 and h3, although no minor sequences consistent with CA sequences were apparent. The native enzymes are acetylated at their N termini. The amino acid analysis of peptide b2 indicated it was an N-terminal fragment, so N-terminal sequencing was not performed. The N terminus of the analogous peptide h2 was found to be blocked when sequencing was attempted, thereby suggesting that it too was an N-terminal fragment.

A fragment with a molecular mass of approximately 6 kDa is apparent in the cleavage of BCA II (Figure 2, lane 1). A similar fragment also was produced from HCA I, although the gel electrophoresis conditions did not lead to resolution of this band in most cases. These fragments may be complementary to the 22 kDa fragments. The fragment from HCA I with apparent molecular mass of approximately 20 kDa had only a very weak counterpart in the cleavage of BCA II. Upon attempted sequencing of the 6 kDa fragment from BCA II and the 20 kDa fragment from HCA I, no sequences were obtained, suggesting both may be N-terminal fragments. They were not investigated further.

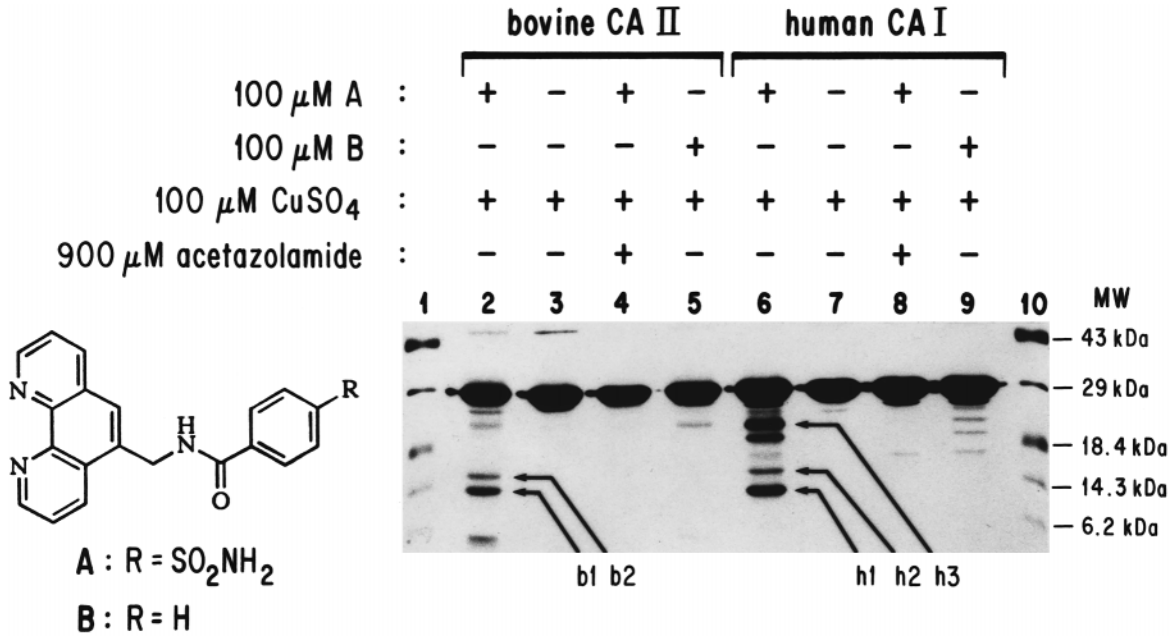


FIGURE 2: Cleavage of bovine CA II and human CA I by the CBSA−OP−Cu^+ conjugate. The scission reaction was allowed to proceed for 30 min at 22 °C in 20 mM KP_i (pH 7.6) and 10 mM sodium ascorbate, and was halted by the addition of an excess of EDTA. The reaction products were separated by SDS−PAGE (Tris-glycine, 4% stacking gel, 18% resolving gel) and silver stained.

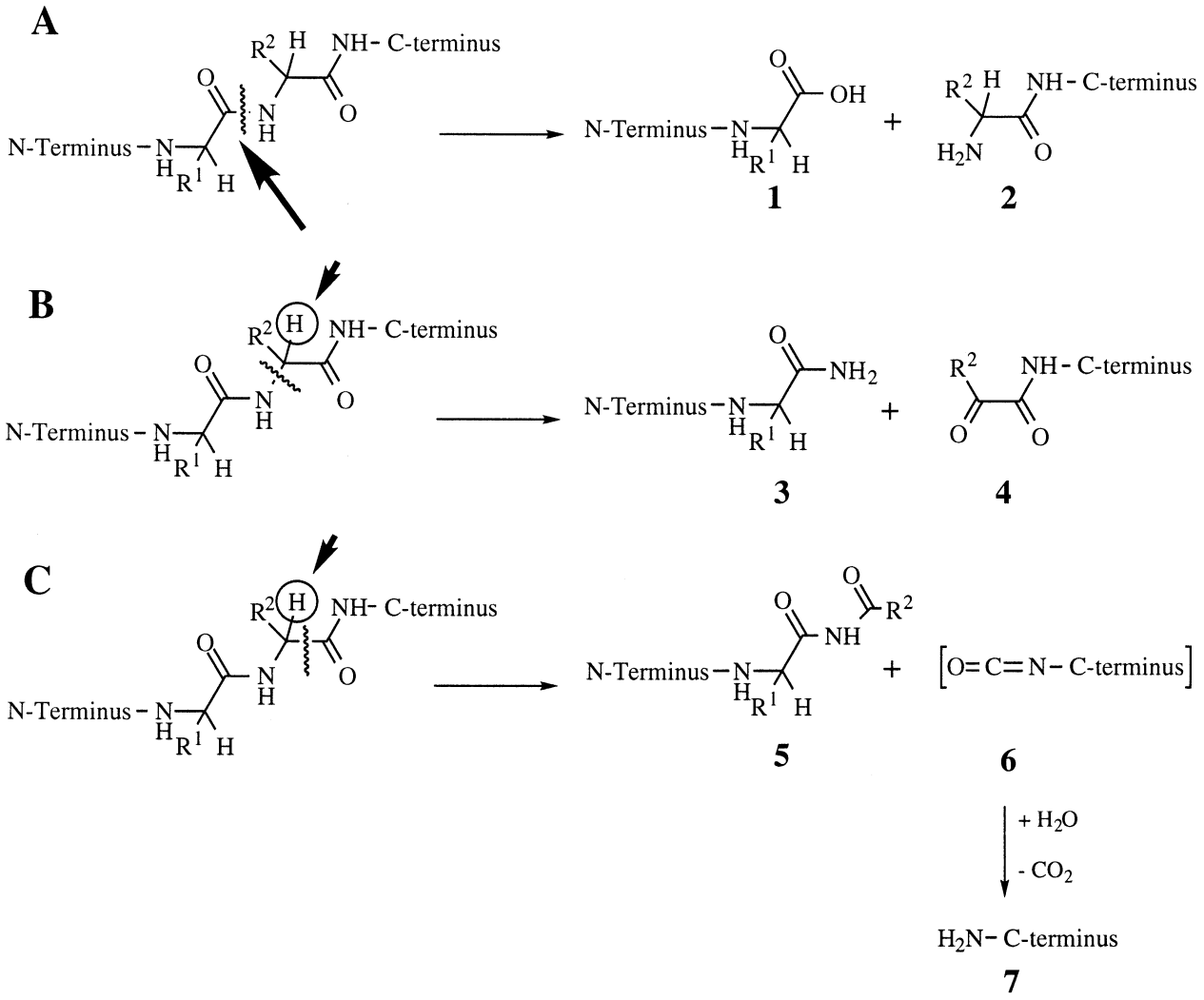
Table 2: Analysis of the Cleavage Products of Bovine CA II and Human CA I by the CBSA-OP-Cu^+ Conjugate

Fragment	Approx. MW	C- or N-term. fragment, based on amino acid analysis	N-terminal sequencing analysis	C-terminal sequencing analysis	MW via LC-ESI mass spectrometry
b1	14 kDa	C-terminal fragment	Thr-Ala-[Ala][a]-Gln-[Gln][b]-Pro[c] yield:[d] 7.0 5.1 -- 9.7 8.9 8.5 residues: 131·····136	•••••••	•••••••
b2	15 kDa	N-terminal fragment	•••••••	1) X^1-Asp-Gly-Tyr yield:[d] 24 130 41 residues: 128·····126 2) X^2-Gly-Tyr yield:[d] 117 28 residue: 127-126	•••••••
h1	14 kDa	C-terminal fragment	Glu-Ala-Ala-Ser-Lys-Ala yield:[d] 7.0 9.2 8.7 4.9 5.4 5.4 residues: 133·····138	•••••••	•••••••
h2	15 kDa	(not conclusive)	blocked N-terminus	•••••••	14,796 ± 8
h3	22 kDa	(not conclusive)	Gly-His-Ser-Phe-His-Val[c] yield:[d] 4.4 4.7 6.7 4.2 5.3 6.3 residues: 63·····68	•••••••	•••••••

[a] Interference prevented determination of this residue. [b] Expected from the sequence of the enzyme but not determined conclusively because of the low yields of this amino acid. [c] Other residues were also found in many cycles. [d] Amount in picomoles.

Since peptide fragments b2 and h2 appeared to be N-terminal fragments, they were analyzed by C-terminal chemical sequencing (*33*, *34*) and electrospray ionization (ESI) mass spectrometry. In the first cycle of C-terminal sequencing of peptide b2, no known amino acid thiohydantoin derivative was found, although subsequent cycles did yield identifiable amino acid derivatives (Table 2, column 5). The sequence Tyr-Gly-Asp-X^1 was found, where X^1 is the unknown moiety at the C terminus of the fragment which was cleaved in the first cycle but which presumably does *not* have a free carboxyl group. The sequence Tyr-Gly-Asp corresponds to residues 126−128 of BCA II, demonstrating that this fragment is complementary to fragment b1. Since N-terminal sequencing of peptide b1 indicated that cleavage occurred just before residue 131, X^1 is presumably derived from residues 129 and 130, Phe-Gly. The analysis of b2 indicated that frayed ends were produced since a secondary sequence, Tyr-Gly-X^2, was also observed.

Scheme 1: End Products from Three Proposed Mechanisms of Metal-Catalyzed Cleavage of the Polypeptide Backbone of Proteins



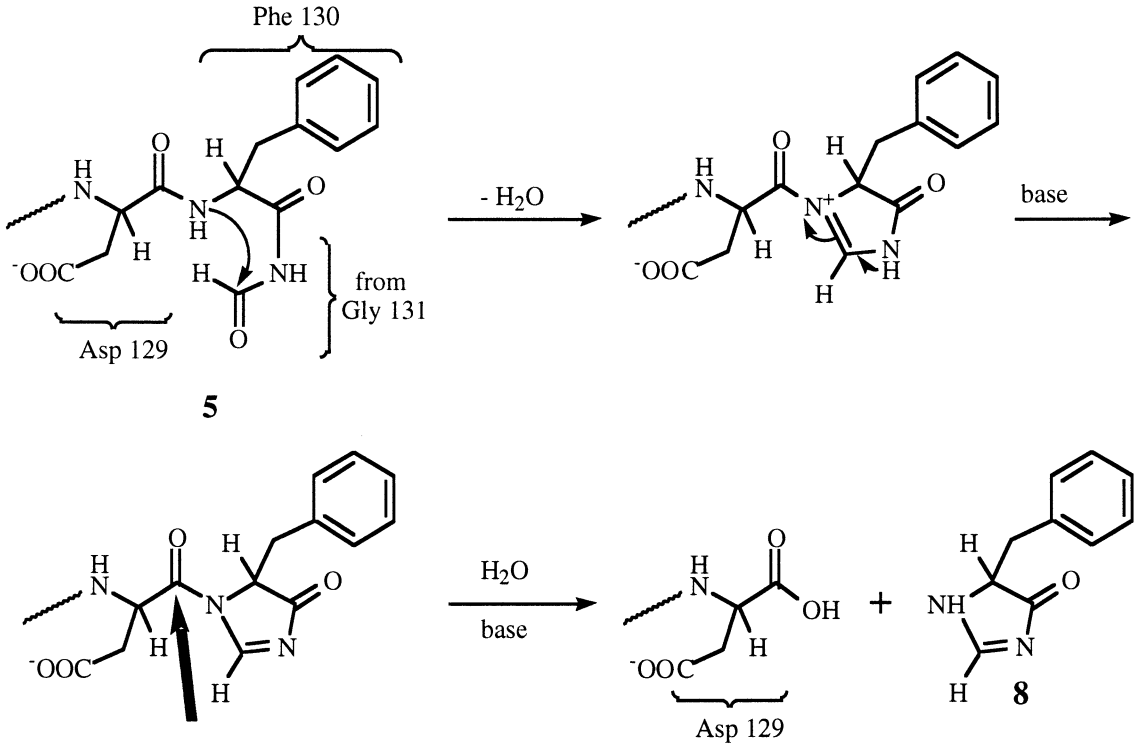
To investigate further the nature of the modified C terminus of the N-terminal fragments produced by OP−Cu cleavage, LC−ESI-MS analysis of peptide h2 was carried out. After cleavage of HCA I by CBSA−OP−Cu^+, fragment h2 was isolated by HPLC on a C-8 column. The average mass of the peptide was determined to be 14 796 ± 8 Da (Table 2, column 6). Since the complementary C-terminal fragment h1 begins with residue Glu133, the C terminus of peptide h2 could be expected to end with Ala132 or a modified alanyl residue. Indeed, the mass of 14 796 Da is larger than that expected for a fragment composed of residues 1−131 (14 763 Da), yet smaller than that for a fragment consisting of residues 1−132 (14 834 Da), which indicates that the Ala132 residue has been modified.

DISCUSSION

Metal ion-catalyzed cleavage of the polypeptide backbone of proteins is implicated in the pathogenesis of a number of diseases as well as in the accumulation of altered forms of enzymes during aging (*35*). In the presence of O_2 and a reducing agent, chelated Cu(I) or Fe(II) catalyzes the formation of H_2O_2. The reduced metal reacts with H_2O_2 to produce a reactive oxygen species, such as hydroxyl radical or a metal-coordinated oxo or peroxo species, which in turn can initiate cleavage of the peptide backbone nearby. Three mechanisms of cleavage, giving rise to three distinct sets of products, have been proposed to explain the variety of products obtained with iron−EDTA linked to proteins (*8*, *10*, *11*, *14*) and to protein affinity-binding ligands (*6*, *7*) (Scheme 1): (A) hydrolysis of the amide bond to give an N-terminal fragment **1** with a free carboxy terminus, and a C-terminal fragment **2** with a free amino terminus; (B) abstraction of H at C_α followed by oxidation at C_α and cleavage of the C_α−NH bond to give an N-terminal fragment **3** with a C-terminal amide, and a C-terminal fragment **4** with a blocked N terminus; and (C) abstraction of H at C_α followed by oxidation at C_α and cleavage of the C_α−C(O) bond to give an N-terminal fragment **5** containing a C-terminal acyl amide, and a (putative) unstable C-terminal fragment **6** which is hydrolyzed to fragment **7** having a free amino terminus.

In this study, a sulfonamide ligand has been used to direct protein cleavage by 1,10-phenanthroline−copper(I) specifically to the active site of carbonic anhydrase. Copper, phenanthroline, a reducing agent, and the sulfonamide functional group are all necessary to effect the observed cleavage, and the cleavage can be suppressed by addition of another ligand which competes for the same binding site.

Scheme 2: Proposed Degradation of Fragment b2 during the First Cycle of C-Terminal Sequencing



Freely diffusible hydroxyl radicals are *not* involved, thereby implicating a copper−oxo species as the active cleaving agent.

An analysis of the new termini generated during the cleavage leads us to propose a mechanism for the scission of the polypeptide backbone. We have shown that the fragments arising from the C terminus of carbonic anhydrase upon cleavage with CBSA−OP−Cu^+ have free amino termini, which would be consistent with cleavage via pathway A or C, but not B (Scheme 1). However, pathway A can also be excluded, since the C-terminal chemical sequencing of the fragment b2 arising from the N terminus of BCA II gave no identifiable thiohydantoin amino acid derivative in the first cycle. In other words, the terminal amino acid residue (X in Table 2) does not have a free carboxyl group. The observed products are only consistent with pathway C, abstraction of H at C_α, followed by oxidation at C_α and cleavage of the C_α−C(O) bond to give an N-terminal fragment **5** containing a C-terminal acyl amide, and an unstable C-terminal fragment **6** which undergoes hydrolysis to fragment **7** having a free amino terminus. According to this mechanism, the terminal group X^1 on fragment b2 would be the terminal acyl amide group in **5**, where R^1 is the Phe129 side chain and R^2 is the Gly130 side chain from BCA II. This moiety would not give one of the known amino acid thiohydantoin derivatives in the first cycle of the C-terminal sequencing, but might be cleaved under the mildly basic conditions [pyridine or KOTMS (*34*)] of the chemical sequencing method to give imidazolidinone **8** and a peptide containing a free carboxyl terminus which would yield to sequencing of the subsequent residues (Scheme 2).

That a terminal acyl amide group such as **5** may be produced is also supported by the ESI-MS analysis of the analogous fragment h2 from HCA I. Since cleavage occurred just before Glu133, the end group on fragment h2 should contain the acyl amide which would be derived from Ala132 (an acetamide group). The mass of fragment h2 (14 796 ± 8 Da) is consistent with that expected (14 804 Da) from HCA I if a fragment ending in an acylamide were produced.

One of the advantages of using the carbonic anhydrases to explore the chemical mechanism of the scission stems from the availability of high-resolution structural studies on this family of enzymes (*25*−*27*). The tight binding sulfonamide inhibitor has also provided the opportunity to confirm that a reversibly binding ligand, in addition to cysteines inserted by site-directed mutagenesis (*5*), can target scission. In Figure 3, we have docked CBSA−OP−Cu^+ to human carbonic anhydrase I and show that the sites of cleavage that we have identified from our analysis of the protein cleavage data are fully consistent with the available structural data. The conical active site cavity, approximately 15 Å deep and 15 Å wide at its mouth, is formed by portions of a 10-stranded twisted β-pleated sheet, two loops consisting of residues 121−138 and 198−204, and some of the amino-terminal residues. The essential zinc ion, situated near the bottom of the cavity 12 Å from the mouth, is coordinated to the protein through three histidine residues (94, 96, and 119) and a water molecule in the native structure. In crystal structures of sulfonamide drug complexes of HCA I, the nitrogen of the sulfonamide group replaces water as the fourth ligand. We have superimposed the benzenesulfonamide group of CBSA−OP−Cu^+ onto acetazolamide in its cocrystal structure with HCA I (*25*) and then have moved the phenanthroline ring by rotation about two of the bonds of the tether connecting this ring to the benzenesulfonamide group in order to determine if the observed sites of cleavage are accessible to phenanthroline−copper. Although the reactive copper-coordinated oxygen species responsible for

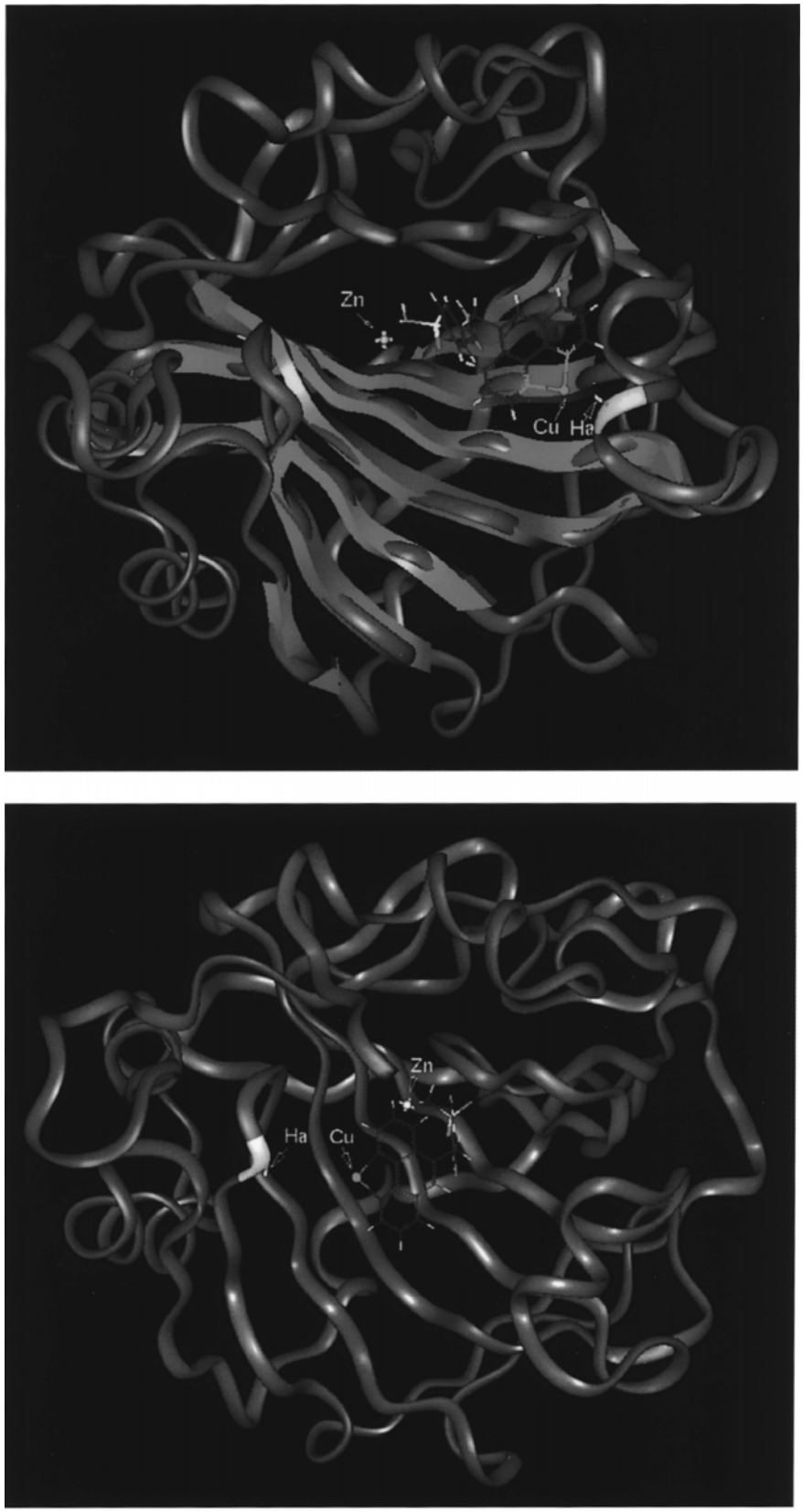


FIGURE 3: Modeling of the HCA I·CBSA−OP−Cu^+ complex. (A, top) Phenanthroline−copper is shown positioned within 2 Å of Hα of Ala132, the proposed site of initial abstraction leading to scission of HCA I into fragments h1 and h2. (B, bottom) Phenanthroline−copper is shown positioned within 6.3 Å of H_α of Val62, the proposed site of initial abstraction leading to generation of fragment h3. InsightII was used to generate the models.

initiating the cleavage events is unknown, the copper ion itself can be moved to within 2 Å of H_α of Ala132, the proposed site of initial abstraction leading to scission of HCA I into fragments h1 and h2 (Figure 3A). Similarly, fragment h3 is believed to result from initial abstraction of H_α of Val62 situated on the opposite side of the opening to the active site, and the copper ion can be moved to within 6.3 Å of this atom (Figure 3B). These results strongly suggest that

the structural information obtained using this new type of "virtual" cross-linking should provide a reliable method for mapping nearest neighbors in multicomponent biochemical systems.

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