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Transfer of oxygen from an artificial protease to peptide carbon during proteolysis

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ABSTRACT **Site-specific cleavage of proteins with metal chelates is an approach for designing artificial proteolytic reagents that are directed by proximity to a peptide bond rather than by an amino acid residue type. In the presence of ascorbate and H_2O_2, an iron chelate attached to Cys-212 of the enzyme human carbonic anhydrase I quickly cleaved the protein between residues Leu-189 and Asp-190 to produce two discrete fragments. The transfer of an ^{18}O atom from $[^{18}O]H_2O_2$ (or $[^{18}O]O_2$) to the carboxyl group of Leu-189 was demonstrated by mass spectrometry. Quantitative experiments revealed that one molecule of H_2O_2 and one molecule of ascorbate afforded the hydrolysis of one peptide bond (1:1:1 stoichiometry) and that the reaction required ascorbate and H_2O_2. The process is catalytic, since related experiments on the protein bovine serum albumin revealed two cleavage events for each polypeptide chain cleaved. Hydroxyl radical scavengers had no significant effect. These results may be explained by generation of a highly nucleophilic oxygen species, such as peroxide coordinated to the iron chelate, that attacks a carbonyl carbon nearby.**

Transition metals are known to catalyze a number of oxidative chemical reactions with deleterious effects in biological systems (1–3). Some of these reactions have been put to practical use in the laboratory to explore the properties of ligand–DNA complexes (4–9) or the properties of proteins (1, 2). Recently, we and others (10–13) have sought to extend this technology to develop reagents that cleave polypeptide chains at sites determined by proximity to a metal chelate. In doing so, we discovered a reaction by which a protein could be cut by an analog of iron–EDTA in the presence of ascorbate and H_2O_2 (10); surprisingly, the net reaction involved hydrolysis—not oxidation—of the peptide backbone. Here we report studies of the mechanism of the reaction indicating that the observed peptide cleavage results from generation of a powerful nucleophile that selectively attacks peptide carbon rather than the anticipated action of hydroxyl radical. The observed nucleophilic reaction is remarkable in light of the extensive literature (e.g., refs. 1–9) documenting the production of hydroxyl radicals and other electrophilic reagents by iron complexes.

EXPERIMENTAL PROCEDURES

All reagents and solvents were the purest available. Electrophoretically pure human carbonic anhydrase I (EC 4.2.1.1) was purchased from Sigma. $[^{18}O]O_2$ with 98% isotopic enrichment was purchased from Cambridge Isotope Laboratories [Cambridge, MA; lot no. F-3024 (7782-44-7)]. $[^{18}O]H_2O_2$ with 90% enrichment was purchased from Icon Services (lot no. 3521). $[^{18}O]H_2O$ with 90% enrichment was a gift from A. L. Balch of this department. Pure water (18 MΩ) was used throughout. All labware was acid-washed (14, 15).

Buffer A contained 0.1 M Tris·H_2SO_4 (pH 7.5); buffer B contained 0.02 M Tris·HCl (pH 8.0); buffer C contained 0.1 M sodium phosphate (pH 7.0); buffer D contained 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (pH 7.0); buffer E contained buffer D with 10 mM ascorbate; buffer F contained 0.5 ml of 25% (wt/vol) $NaDodSO_4$, 0.31 ml of 2-mercaptoethanol, 0.12 ml of glycerol, 0.215 ml of 2 M Tris·HCl (pH 6.8), and 0.1 ml of 0.2% (wt/vol) bromophenol blue.

Preparation of Iron–(*S*)-1-(*p*-Bromoacetamidobenzyl)-EDTA (Fe–BABE) Complex. BABE was synthesized as reported (16, 17). Complexation of iron was carried out as follows. Four hundred microliters of 9.4 mM BABE was used, with the pH adjusted to 1.0 by adding 8.0 μl of 6.0 M HCl. A trace amount of ^{59}Fe solution in 2.0 M HCl was added to the chelate solution and incubated at room temperature for 30 min. The pH of the solution was raised to 3.0 by the addition of 1.0 M ammonium acetate, and 300 μl of *freshly prepared* 20 mM ferrous ammonium sulfate was added. After 1 hr of incubation at room temperature, the chelation of ^{59}Fe with BABE was confirmed by thin-layer chromatography (15). All of the radioactivity moved with the same R_f (0.9) as the chelate, whereas control experiments showed that nonchelated ^{59}Fe would have stayed at the origin.

Site-Specific Conjugation of Human Carbonic Anhydrase I (HCAI) to Fe–BABE. Alkylation of the unique cysteine (Cys-212) of HCAI with Fe–BABE was carried out according to the method of Carlsson *et al.* (18, 19) with the following modifications. The enzyme (35 μM) was denatured in buffer A plus 5 M guanidine hydrochloride (3.5 ml), and an equimolar amount of 2-mercaptoethanol was added. After 5 min of stirring at room temperature, a 30-fold molar excess of Fe–BABE was added. To scavenge free metal ions, 100 μl of 20 mM EDTA was added to the reaction mixture. The course of alkylation of the sulfhydryl groups was monitored by the 5,5'-dithiobis(2-nitrobenzoic acid) test (20); the alkylation reaction was complete in 30 min. A 30-fold molar excess of 2-mercaptoethanol was added to quench the BABE.

The modified enzyme was reactivated by dilution to 0.5 M guanidine hydrochloride, and a final protein concentration of 0.025 mg/ml, in buffer A (18). The number of chelates per enzyme molecule was 0.83 ± 0.2 by ^{59}Fe analysis. Carbonic anhydrase was assayed by measuring CO_2 hydration activity in buffer B (21, 22).

Cleavage with H_2O_2. Cleavage with H_2O_2 was performed by mixing (in order) 7 μl of 0.91 mM modified HCAI in buffer C with 55 μl of buffer E and 1.3 μl of 5 mM H_2O_2 (in buffer D) at 25°C. The reaction was quenched by adding 35 μl of buffer F, 10 sec after the addition of H_2O_2. The quenched reaction mixture was heated at 95°C for 5 min and analyzed by

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Abbreviations: BABE, (*S*)-1-(*p*-bromoacetamidobenzyl)-EDTA; HCAI, human carbonic anhydrase I; PVDF, poly(vinylidene difluoride).

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electrophoresis. For the experiments with different radical scavengers, thiourea, mannitol, or *tert*-butyl alcohol (at a final concentration of 0.5 M in buffer D) was added to the reaction mixture before addition of H_2O_2.

Cleavage also was carried out under 1 atm (1 atm = 101.3 kPa) of O_2 by incubating 100 μM modified HCAI in a sealed container at 37°C for 30 hr in buffer E. The reaction was quenched and analyzed as described above.

H_2O_2 titrations were carried out under the conditions described above, except that H_2O_2 was added to final concentrations of 10–120 μM in 10 μM increments. Ascorbate titrations were carried out in the presence of 5 mM H_2O_2 in buffer D, and ascorbate was added to final concentrations of 10–120 μM in 10 μM increments.

The protein gels used in these experiments employed $NaDodSO_4$/polyacrylamide (23), 20 cm × 0.75 mm with a 7.5–20% acrylamide gradient. Gels were run for 1 hr at 80 V and then at 50 mA until the bromophenol blue tracking dye had eluted from the bottom (≈4 hr). Products were visualized by Coomassie blue or silver stain (24). Band intensities were quantified on a Visage-60 bioimage analyzer (Kodak).

For amino-terminal sequencing of the cleaved fragments, the proteins were transferred from polyacrylamide gels to poly(vinylidene difluoride) (PVDF) membrane (25). Amino-terminal sequence analyses were performed by automated Edman degradation, using an Applied Biosystems 470A gas phase sequencer.

For carboxyl-terminal sequence analysis, the 21-kDa fragment was recovered from the gel by electroelution (26). The 8-kDa fragment was eluted from the gel by the method of Tolan *et al.* (27) and recovered by acetone precipitation (28). Concentrations of eluted proteins were measured by the micro-bicinchoninic acid method (29). Carboxyl-terminal sequence analyses were performed by carboxypeptidase Y digestion (30, 31).

Cleavage Reaction in the Presence of $[^{18}O]O_2$. The chelate-conjugated HCAI (≈250 μg) at a final concentration of 100 μM in buffer E was transferred to an Eppendorf tube; the tube was purged with argon for 2–3 hr and capped, and $[^{18}O]O_2$ was injected with a needle. The sealed tube was incubated at 37°C for 30 hr. To check for ^{18}O in the new carboxyl terminus, the cleavage product with apparent molecular mass of 21 kDa was digested with carboxypeptidase Y. After 2 min, the reaction was stopped and the proteins were precipitated with 50 μl of trichloroacetic acid solution (20%, wt/vol). The supernatant of the digestion reaction was analyzed by fast atom bombardment mass spectrometry to measure the incorporation of ^{18}O into the carboxyl terminus of the cleaved peptide. The results of incorporation of ^{18}O into Leu-189 are shown in Table 1.

Cleavage Reaction in the Presence of $[^{18}O]H_2O_2$. Cleavage of the enzyme was performed by mixing modified HCAI (100 μM) at 25°C for 10 sec with buffer E and 100 μM $[^{18}O]H_2O_2$. Products were isolated and analyzed as described above.

RESULTS

The experimental strategy for site-specific chelate conjugation and selective cleavage of HCAI is outlined in Fig. 1. Cys-212 specific conjugation of HCAI to Fe–BABE was performed in 5 M guanidine hydrochloride (18). The course of the conjugation reaction was followed carefully to ensure that only the single free sulfhydryl group of HCAI would be alkylated. Correlation of the loss of −SH with the gain in chelate groups indicated that conjugation of Fe–BABE with HCAI was restricted to the free cysteine residue of the protein. After reactivation and concentration of modified HCAI, the CO_2 hydration activity was restored to practically the same level (≈97%) as obtained for the native enzyme. The circular dichroism spectra of unmodified and chelate-conjugated enzymes were very similar, each with a sharp minimum near 210 nm ($[\theta] = -3.9 \times 10^5$ deg·cm^2·dmol^{-1}) and a broad maximum near 240 nm ($[\theta] = +2.3 \times 10^5$ deg·cm^2·dmol^{-1}).

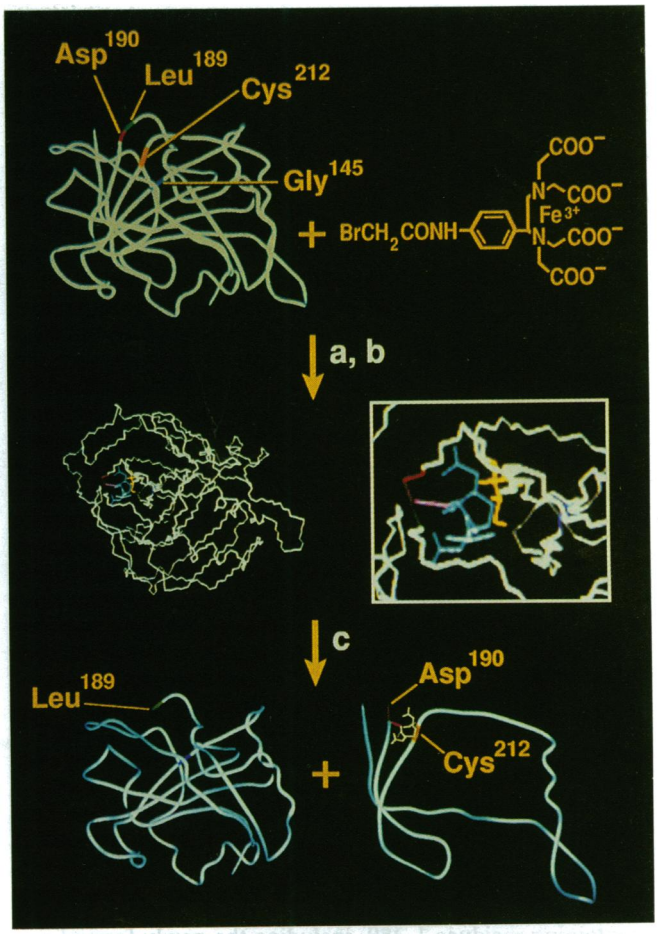

FIG. 1. Experimental outline, showing the structures of HCAI and Fe–BABE. Structures of HCAI (32) and Fe–EDTA (33) were visualized using MIDAS PLUS (University of California, San Francisco) software on an IRIS 4D/70GT work station. Reaction a, conjugation of HCAI Cys-212 to Fe–BABE under denaturing conditions in the presence of 5 M guanidine hydrochloride (pH 7.5). Reaction b, reactivation of the modified enzyme by dilution to 0.5 M guanidine hydrochloride and a final protein concentration of 0.025 mg/ml. Reaction c, site-specific hydrolysis of the polypeptide chain in the presence of ascorbate and H_2O_2 (or O_2).

Table 1. Incorporation of an ^{18}O atom into the carboxyl group of Leu-189

Labeled reagent	% ^{18}O incorporation efficiency*
$[^{18}O]O_2$	93.0, 91.2
$[^{18}O]H_2O_2$	93.6, 92.1
$[^{18}O]H_2O$	≈3

Reaction conditions are described in the text.
*Values are from independent experiments.

At the beginning of this study, we expected that the cleavage would be initiated in some way by hydroxyl radical. To explore the effect of radical scavengers, the cleavage reaction was carried out in the presence of thiourea, mannitol, and *tert*-butyl alcohol (7, 34). In Fig. 2, the products of the cleavage reaction in the presence and the absence of radical scavengers are shown (lanes 3–6). As can be seen from the similarity of the cleavage products and their relative intensities, high concentrations of radical scavengers (0.5 M) had no effect on the cleavage reaction. This suggested the possibility that the Leu-189/Asp-190 peptide bond in HCAI

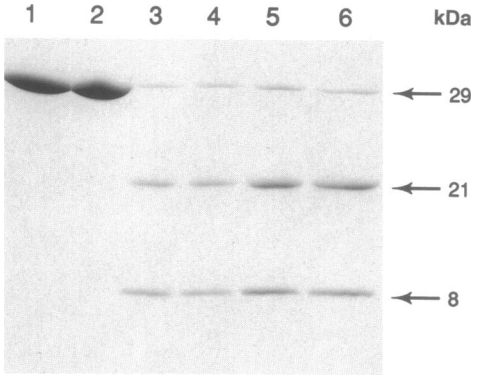

FIG. 2. Separation of the cleavage products by NaDodSO$_4$/polyacrylamide gel electrophoresis. Lanes 1 and 2, chelate-conjugated HCAI in the absence of ascorbate/H_2O_2 (lane 1) or with 100 μM H_2O_2 but in the absence of ascorbate (lane 2). Lanes 3–6, cleavage of chelate-conjugated HCAI with 10 mM ascorbate and 100 μM H_2O_2 in the absence of radical scavengers (lane 3) in the presence of 0.5 M thiourea (lane 4), 0.5 M mannitol (lane 5), or 0.5 M *tert*-butyl alcohol (lane 6).

was cleaved through the direct attack of an oxygen-activated iron–EDTA complex.

The stoichiometry of the protein cleavage reaction was determined by either titrating with H_2O_2 in the presence of excess ascorbate or titrating with ascorbate in the presence of excess H_2O_2, as shown in Fig. 3. These results indicate that one molecule of H_2O_2 and one molecule of ascorbate afford the hydrolysis of one peptide bond (1:1:1 stoichiometry) and that H_2O_2 and ascorbate are required for cleavage.

We carried out the cleavage reaction of chelate-conjugated HCAI using [^{18}O]H_2O_2 or [^{18}O]O_2. The 21-kDa fragment (containing residues 1–189, including the newly formed carboxyl terminus) was digested with carboxypeptidase Y for 2 min, and the amino acids initially released were analyzed by fast atom bombardment mass spectrometry. This revealed that one atom of ^{18}O was incorporated into the carboxyl group of the carboxyl-terminal Leu-189, with ≈93% efficiency (Table 1). The carboxyl group of Leu-189 did not incorporate significant amounts of ^{18}O when the reaction was carried out in the presence of [^{18}O]H_2O.

DISCUSSION

HCAI contains a single cysteine, Cys-212, which, in the native conformation, is not accessible to alkylating agents (35). Thus, no modification of the sulfhydryl group was achieved after incubation of the enzyme with a 50-fold molar excess of BABE at 37°C, pH 7.5, for 48 hr. In favorable cases it is possible to unfold the protein, modify this buried group with a probe molecule, and subsequently refold the protein to a native (or native-like) active conformation (18, 35). The modified and reactivated enzymes have properties similar to the native enzyme.

The cleavage of HCAI was very efficient; with a concentration of H_2O_2 equal to the protein concentration, all of the HCAI molecules bearing chelates were cleaved in 10 sec. As had been previously observed with bovine serum albumin (10), the amino acids at the site of cleavage were detected unaltered. For bovine serum albumin, the yield of the amino-terminal amino acid in the first cycle of Edman degradation was roughly half that of the following residue obtained in the second cycle. For the 8-kDa fragment of HCAI the yield of the amino-terminal amino acid in the first cycle of Edman degradation was consistent with yields of the succeeding residues, implying that side reactions were insignificant (the amino terminus of native HCAI, which is also the amino

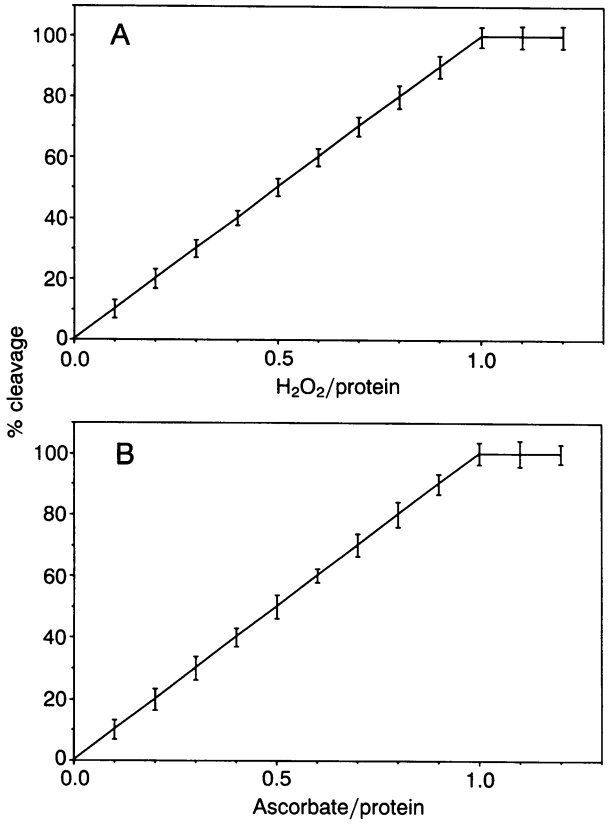

FIG. 3. Efficiency of protein cleavage plotted against the ratio of the concentrations of H_2O_2 and chelate-conjugated HCAI (*A*) or ascorbate and chelate-conjugated HCAI (*B*). Error bars show the range of two independent measurements. Results are normalized to 100% chelate–enzyme conjugation.

terminus of the 21-kDa fragment, is blocked and cannot be sequenced by these procedures).

One may ask whether *every* break in the polypeptide chain is accounted for by simple hydrolysis or whether other reactions also contribute. We compared the number of moles of protein added to a cleavage reaction with the number of moles of resulting fragments, as determined during Edman sequencing. In one experiment, at least 67% of the HCAI was converted into new amino termini; in another, at least 53%. These observations provide only a lower limit on the yield of the hydrolysis reaction; variable losses of material during the processing steps could easily account for the observed differences, and the true yield is almost certainly higher. Thus, most (if not all) of the HCAI molecules are cleaved by hydrolysis of a peptide bond.

The high efficiency and lack of side reactions observed with this conjugate led us to explore the mechanism of the cleavage reaction. The observation of ^{18}O atom transfer from H_2O_2 to the newly formed carboxylate is not what would be expected for a process mediated by the electrophilic hydroxyl radical (12). Rather, the attack of a nucleophile on the polypeptide backbone is indicated. In studies of cytochrome P-450, which contains a *porphyrin*-iron prosthetic group, several authors have suggested the occurrence of nucleophilic attack of iron-coordinated peroxo groups at carboxyl carbon (36–38).

Studies of the conversion of benzonitrile to benzamide by Wiberg (39) showed that the hydroperoxide anion is highly nucleophilic. Jencks and Carriuolo (40) compared the reactivity of nucleophiles toward carboxylic esters, finding that peroxide anions $HO\text{-}O^-$ and $CH_3O\text{-}O^-$ were $>10^2$ times more reactive than hydroxide. Edwards and Pearson (41) noted that the *alpha effect*, which arises from the presence of

unshared electron pairs on the atom adjacent to the nucleophilic atom, appears to be particularly important for the peroxides.

It is possible that the tethered iron complex studied here serves first to localize and orient a peroxo nucleophile with respect to the peptide bond. The oxidation states of iron involved in this process are not yet characterized, but it is known that ferric–EDTA is heptacoordinate in the crystalline state, having six atoms donated by EDTA and one by a water molecule (33). Nucleophilic ligands have been shown to bind this complex, presumably by replacing the water molecule (42). Fee and co-workers (43, 44) investigated the dismutation of superoxide by iron–EDTA and suggested that an intermediate peroxo complex [Fe^{3+}–EDTA–O_2^{2-}] was formed. This complex has been studied in detail; at pH >9, the peroxide ion binds to iron in a side-on, bidentate fashion (45), whereas at pH 7.6, it binds end-on, monodentate (46).

From models, the Fe–BABE reagent could span distances up to ≈12 Å and might be expected to cleave several peptide bonds in addition to 189–190. The 145–146 peptide bond is comparably close to Cys-212, but it was not cleaved. In the crystal structure of HCAI, the sulfur of Cys-212 lies above the plane of the Leu-189/Asp-190 peptide bond, 5.3 Å from the carbonyl carbon of Leu-189 and 5.4 Å from its carbonyl oxygen. Thus, the C=O group of Leu-189 is oriented such that nucleophilic attack on carbon by a reagent attached to Cys-212 could be quite favorable (47). On the other hand, the sulfur of Cys-212 is 5.1 Å from the carbonyl carbon of Gly-145 and 4.2 Å from the carbonyl oxygen. Hence, the oxygen points *toward* the Cys-212 residue, presumably making nucleophilic attack on carbon by a reagent attached to Cys-212 unfavorable.

All of the above observations are consistent with the proposal that in the presence of H_2O_2 (or molecular oxygen) and ascorbate, iron–EDTA may form an intermediate oxygen-activated complex that leads to nucleophilic attack by oxygen on the carbonyl carbon of the peptide bond. The outline of a reaction mechanism can be suggested (Fig. 4). In the first step, intermediate **B** is formed by the direct binding of H_2O_2 to the metal chelate. Intermediate **B** also could be formed after O_2 has been reduced to H_2O_2 by ascorbate in the presence of the iron chelate (2, 48). The oxidation state of Fe in **B** is not known; if it is Fe(II), **B** might be expected to decompose to form hydroxyl radical (48). In the next step, coordinated peroxide acts as a nucleophile to attack the peptide bond, forming the key intermediate **C**. It may be that the efficiency of this nucleophilic attack is such that it occurs before hydroxyl radical formation can take place, in which case the proximity and orientation of the reacting groups would be crucial to the observed selectivity of the reaction. The final step could be heterolytic cleavage of the C—N bond and of the peroxide to yield a new carboxyl terminus, or it could be a more complex series of steps leading to incorporation of an ^{18}O atom into Leu-189. Subsequently, the metal complex is presumed to be converted back to its original form; since peptide chains were cleaved *twice* during the cleavage of bovine serum albumin, the chelate evidently

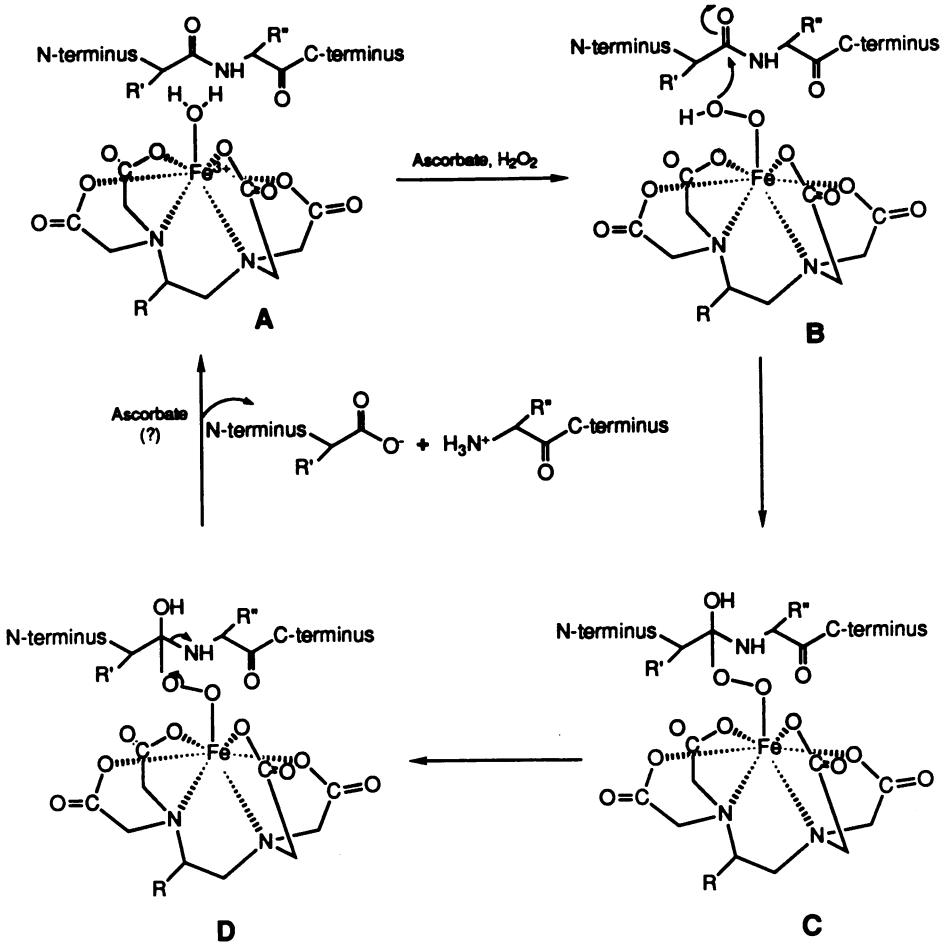

FIG. 4. Plausible mechanism for the observed proteolysis. Ascorbate may be involved in one step as a two-electron reductant or in two, one-electron steps. The oxidation state of iron in *A* is Fe(III); in the other species, it is unknown. The proposed mechanism satisfies the experimental observations of 1:1:1 ascorbate:H_2O_2:cleavage stoichiometry, O atom transfer to the new carboxyl group, extremely narrow geometric constraints, insensitivity to hydroxyl radical scavengers, and apparent regeneration of the starting metal species.

generates the reactive species at least twice and possibly many times (10).

The observed chelate-mediated proteolysis apparently does not depend on the chemical reactivity of the amino acid residue that is to be cleaved; besides the Leu/Asp hydrolysis of HCAI, the cleavage of bovine serum albumin occurred between Ala-150 and Pro-151 and between Ser-190 and Ser-191 (10).

The transformation of a bifunctional chelate into a site-specific protease provides a methodology to characterize structural features of proteins and their complexes under physiological conditions. Further developments of this technology could lead to the specific cleavage of fusion proteins or the mapping of ligand binding sites on proteins, analogous to currently available methods for mapping binding sites on nucleic acids (4–9).

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1. Tabor, S. & Richardson, C. C. (1987) *J. Biol. Chem.* **262,** 15330–15333.
2. Kim, K., Rhee, S. G. & Stadtman, E. R. (1985) *J. Biol. Chem.* **260,** 15394–15397.
3. Halliwell, B. & Gutteridge, J. M. C. (1984) *Methods Enzymol.* **105,** 47–56.
4. Mack, D. P. & Dervan, P. B. (1990) *J. Am. Chem. Soc.* **112,** 4604–4606.
5. Latham, J. A. & Cech, T. R. (1989) *Science* **245,** 276–282.
6. Chen, C. B. & Sigman, D. S. (1987) *Science* **237,** 1197–1201.
7. Tullius, T. D. & Dombroski, B. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5469–5473.
8. Francois, J.-C., Saison-Behmoaras, T., Barbier, C., Chassignol, M., Thuong, N. T. & Helene, C. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9702–9706.
9. Chow, C. S. & Barton, J. K. (1990) *J. Am. Chem. Soc.* **112,** 2839–2841.
10. Rana, T. M. & Meares, C. F. (1990) *J. Am. Chem. Soc.* **112,** 2457–2458.
11. Schepartz, A. & Cuenoud, B. (1990) *J. Am. Chem. Soc.* **112,** 3247–3249.
12. Hoyer, D., Cho, H. & Schultz, P. G. (1990) *J. Am. Chem. Soc.* **112,** 3249–3250.
13. Rana, T. M. & Meares, C. F. (1991) *J. Am. Chem. Soc.* **113,** 1859–1861.
14. Thiers, R. C. (1957) *Methods Biochem. Anal.* **5,** 273–335.
15. Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. I. & McTigue, M. (1984) *Anal. Biochem.* **142,** 68–78.
16. DeRiemer, L. H., Meares, C. F., Goodwin, D. A. & Diamanti, C. I. (1981) *J. Labelled Compd.* **18,** 1517–1534.
17. Mukkala, V. M., Mikola, H. & Hemmila, I. (1989) *Anal. Biochem.* **176,** 319–325.
18. Carlsson, U., Aasa, R., Henderson, L. E., Jonsson, B.-H. & Lindskog, S. (1975) *Eur. J. Biochem.* **52,** 25–36.
19. Carlsson, U., Henderson, L. E. & Lindskog, S. (1973) *Biochem. Biophys. Acta* **10,** 376–387.
20. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77.
21. Rickli, E. E., Ghazanfar, S. A. S., Gibbons, B. H. & Edsall, J. T. (1964) *J. Biol. Chem.* **239,** 1065–1078.
22. Wilber, K. M. & Anderson, N. G. (1948) *J. Biol. Chem.* **76,** 147–154.
23. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
24. Morrissey, J. H. (1981) *Anal. Biochem.* **117,** 307–310.
25. Matsudaira, P. (1987) *J. Biol. Chem.* **262,** 10035–10038.
26. Stackhouse, T. M. & Meares, C. F. (1988) *Biochemistry* **27,** 3038–3045.
27. Tolan, D. R., Lambert, J. M., Boileau, G., Fanning, T. G., Kenny, J. W., Vassos, A. & Traut, R. R. (1980) *Anal. Biochem.* **103,** 101–109.
28. Hager, D. A. & Burgess, R. R. (1980) *Anal. Biochem.* **109,** 76–86.
29. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85.
30. Kitabatake, N., Indo, K. & Doi, E. (1988) *J. Agric. Food Chem.* **36,** 417–420.
31. Chang, J.-Y., Knecht, R. & Braun, D. G. (1981) *Biochem. J.* **199,** 547–555.
32. Kannan, K. K., Notstrand, B., Fridborg, K., Ohlsson, A. & Petef, M. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 51–55.
33. Lind, M. D., Hamor, M. J., Hamor, T. A. & Hoard, J. L. (1964) *Inorg. Chem.* **3,** 34–43.
34. Rush, J. D. & Koppenol, W. H. (1988) *J. Am. Chem. Soc.* **110,** 4957–4963.
35. Bergenhem, N., Carlsson, U. & Karlsson, J. A. (1989) *Int. J. Pept. Protein Res.* **33,** 140–145.
36. Hamilton, G. A. (1974) in *Molecular Mechanism of Oxygen Activation*, Hayaishi, O., ed. (Academic, New York), pp. 438–440.
37. White, R. E. & Coon, M. J. (1980) *Annu. Rev. Biochem.* **49,** 315–356.
38. Sligar, S. J., Shastry, B. S. & Gunsalus, I. C. (1977) in *Microsomes and Drug Oxidations*, Ullrich, V., Roots, I., Hildebrandt, A., Estabrook, R. W. & Cooney, A. H., eds. (Pergamon, Oxford), pp. 202–209.
39. Wiberg, K. B. (1955) *J. Am. Chem. Soc.* **77,** 2519–2522.
40. Jencks, W. P. & Carriuolo, J. (1960) *J. Am. Chem. Soc.* **82,** 1778–1786.
41. Edwards, J. O. & Pearson, R. G. (1962) *J. Am. Chem. Soc.* **84,** 16–24.
42. Philip, C. V. & Brooks, D. W. (1974) *Inorg. Chem.* **13,** 384–386.
43. McClune, G. J., Fee, J. A., McClusky, G. A. & Groves, J. T. (1977) *J. Am. Chem. Soc.* **99,** 5220–5222.
44. Bull, C., McClune, G. J. & Fee, J. A. (1983) *J. Am. Chem. Soc.* **105,** 5290–5300.
45. Ahmad, S., McCallum, J. D., Shiemke, A. K., Appelman, E. H., Loehr, T. M. & Sanders-Loehr, J. (1988) *Inorg. Chem.* **27,** 2230–2233.
46. Fujii, S., Ohya-Nishiguchi, H. & Hirota, N. (1990) *Inorg. Chim. Acta* **175,** 27–30.
47. Groves, J. T. & Baron, L. A. (1989) *J. Am. Chem. Soc.* **111,** 5442–5448.
48. Haber, F. & Weiss, J. (1934) *Proc. R. Soc. London Ser. A* **147,** 332–351.