A New Strategy for Directed Protein Cleavage

Bernard Cuenoud, Theodore M. Tarasow and Alanna Schepartz* Department of Chemistry, Yale University New Haven, Connecticut 06511 USA

Abstract: Attachment of a Ni(II)-glyglyhis chelate to trifluoperazine results in a molecule capable of cleaving calmodulin at a single locus upon activation with a peracid.

We reported recently¹ a molecule (TFE-Fe) capable of oxidative cleavage of the protein calmodulin.² TFE-Fe consists of a trifluoperazine moiety tethered to the iron complex of EDTA. By using a known calmodulin antagonist³ to direct redox active iron to the protein's active site, TFE-Fe cleaves calmodulin to produce six fragments in modest yield (pH 7.4, 7 °C). The reaction is O₂-dependent, inhibited by catalase, and generates several fragments, consistent with the involvement of a diffusible radical such as HO•. In an effort to increase the specificity of protein cleavage, we synthesized a reagent likely to support protein cleavage through a *nondiffusible* oxidative mechanism. We report that glyglyhis-trifluoperazine (GGH•TFP, 1) induces specific protein cleavage as the nickel chelate. Protein cleavage occurs in the second EF hand of calmodulin.⁴



Figure 1

Two factors dictated our choice of tetraaza-Ni(II) complexes as non-diffusible oxidative protein cleavage reagents. One was the recognition that in contrast to free Ni(II) salts,⁵ tetraaza-Ni(II) complexes are reactive catalysts for hydrocarbon oxidation in the presence of single oxygen atom donors.⁶ More to the point, the products observed include those which arise from hydrogen atom abstraction. Secondly, certain oligopeptide complexes of Ni(II) are capable of autocatalytic self-cleavage in the presence of molecular oxygen⁷ and strikingly specific oxidative cleavage of DNA.⁸ These results suggested that tetraaza-Ni(II) complexes might

be suitable for the oxidation and cleavage of protein substrates, thereby mimicking the activity of peptidyl α -amidating monooxygenases.⁹ GGH•TFP•Ni (1•Ni) was synthesized¹⁰ (Figure 1) and shown to be a potent calmodulin inhibitor (IC₅₀ = 4.5 μ M).

Protein cleavage experiments were performed with pre-formed GGH•TFP•Ni and analyzed by SDS-PAGE (Figure 2).^{11,2} Incubation of GGH•TFP•Ni with calmodulin in the presence of magnesium monoperoxyphthalate (MMPP) results in the appearance of two discrete cleavage bands whose intensities depend on the concentration of GGH•TFP•Ni between 10 μ M and 40 μ M (lanes 3-5). At concentrations greater than 80 μ M, non-specific cleavage is observed. No cleavage is observed when GGH•TFP•Ni is incubated with calmodulin alone or with high concentrations of GGH•Ni and MMPP (lanes 1 and 2). There is an absolute requirement for both Ni(II) (lane 6) and MMPP (data not shown). As observed with TFE-Fe, cleavage occurs in a specific trifluoperazine binding pocket on activated calmodulin: GGH•TFP•Ni dependent cleavage is inhibited by 2 mM trifluoperazine (lane 8) or when Ca²⁺ is omitted from the reaction (lane 9). The extent of protein cleavage is independent of molecular oxygen and unaffected by added catalase (100 μ g/mL, 4560 U/reaction), consistent with initial hydrogen abstraction by a metal¹² or ligand-based radical¹³ and the lack of involvement of free hydrogen peroxide in the reaction mechanism.¹⁴ The yield of each cleavage fragment increases with time to a maximum of ~5% after three minutes but does not increase further, signaling competitive degradation of the reagent.¹⁵



Figure 2. Silver stained denaturing polyacrylamide gel illustrating protein cleavage by GGH•TFP•Ni. Unless otherwise noted, all lanes contain 10 μ M calmodulin, 10 mM Tris-Cl pH 7.2, 1 mM CaCl₂, 1mM MMPP, and the indicated reagent. Lane 1: MMPP alone; 2: 40 μ M GGH•Ni; 3: 10 μ M GGH•TFP•Ni; 4: 20 μ M GGH•TFP•Ni; 5: 40 μ M GGH•TFP•Ni; 6: 40 μ M GGH•TFP•Ni; 7: 40 μ M GGH•TFP•Ni, argon purge; 8: 40 μ M GGH•TFP•Ni, 2 mM TFP; 9: 40 μ M GGH•TFP•Ni, no calcium in buffer. The GGH•TFP•Ni complex was preformed using Ni(AcO)₂ at 250 μ M in water. Calmodulin and GGH•TFP•Ni were preincubated at 25 °C for 10 min. The reaction was initiated by addition of MMPP and stopped after 3 min. by addition of thiourea to a final concentration of 4.2 μ M. The samples were frozen, lyophilized, and dissolved in loading buffer. Stacking gel: 10% acrylamide, 4.8% bis-acrylamide, 10% glycerol; separating gel: 20% acrylamide, 0.5% bis-acrylamide, 10% glycerol.

The two fragments have approximate molecular weights of 5 and 12 kD. Since calmodulin has a molecular weight of 17 kD,¹⁶ and no other GGH•TFP•Ni dependent fragments are visible on the gel, we conclude that these two polypeptides arise from cleavage of calmodulin at a single locus. Neither fragment yields a sequence when subjected to automated Edman degradation, signaling chemically blocked amino termini and a non-hydrolytic cleavage pathway. Therefore, the cleavage site was identified from the amino acid composition and apparent molecular weights of the cleavage fragments. Calmodulin from bovine brain contains a unique trimethyllysine residue at position 115 which migrates with a distinctive retention time during amino acid analysis. Amino acid analysis of PVDF-blotted¹⁷ fragments reveals the presence of trimethyllysine in the 12 kD fragment, and significantly, the absence of trimethyllysine in the 5 kD fragment. Thus, the 12 kD fragment contains the C-terminus of calmodulin while the 5 kD fragment contains the N-terminus. In concert with the molecular weights of the fragments this indicates that protein cleavage has occurred in the the second EF hand⁵ of calmodulin. Experiments designed to identify the products of this protein cleavage reaction are underway.

In summary, we have described a new reagent for the localized protein cleavage, applied to the protein calmodulin. This reagent can also direct protein self-cleavage: we have shown that the tetrapeptide glyglyhiscys may be attached through a disulfide bond to a unique cysteine on calmodulin to generate a protein conjugate that cleaves itself in a highly specific reaction in the presence on Ni(II) and MMPP.¹⁸ It is noteworthy that GGH is the only protein cleaving reagent yet described that can be encoded at the DNA level to permit the expression of peptides carrying a small protein cleaving domain at their amino termini.⁸ We anticipate that attachment of GGH•Ni to protein ligands^{1,2} or to proteins themselves² will provide reagents capable of probing protein conformational changes and the relationship between protein structure and function in a variety of systems.

Acknowledgment. This work was supported by the PRF, Merck & Co., Inc., Hoffmann-LaRoche, Inc., the David and Lucile Packard Foundation (Fellowship in Science and Engineering) and the NSF (Presidential Young Investigator Award). We thank Dr. William Hait and Ms. Joan Gesmonde for assistance with the calmodulin assays. B.C. is a Bristol-Myers Squibb Fellow.

References

- 1. Schepartz, A.; Cuenoud, B. J. Am. Chem. Soc. 1990, 112, 3247-3249.
- 2. 0 Hoyer, D.; Cho, H.; Schultz, P.G. J. Am. Chem. Soc. 1990, 112, 3249-3250. Rana, T.M.; Meares, C.F. J. Am. Chem. Soc. 1990, 112, 2457-2458. Bayer, E.A.; Grootjans, J.J.; Alon, R.; Wilchek, M. Biochemistry 1990, 29, 11274-11279.
 - Rana, T.M.; Meares, C.F. J. Am. Chem. Soc. 1991, 113, 1859-1861. Ermácora, M.R.; Delfino, J.M.; Cuenoud, B.; Schepartz, A.; Fox, R.O., in preparation.
- 3. Levin, R.M.; Weiss, B. Mol. Pharm. 1976, 12, 581-589.
- 4. Babu, Y.S.; Sack, J.S.; Greenhough, T.J.; Bugg, C.E.; Means, A.R.; Cook, W.J. Nature 1985, 315, 37-40.
- 5. VanAtta, R.B.; Franklin, C.C.; Valentine, J.S. Inorg. Chem. 1984, 23, 4123-4125. Kinneary, J.F.; Albert, J.S.; Burrows, C.J. J. Am. Chem. Soc. 1988, 110, 6124-6129.
- 6. (Koola, J.D.; Kochi, J.K. Inorg. Chem. 1987, 26, 908-916. Kinneary, J.F.; Wagler, T.R.; Burrows, C.J. Tetrahedron Lett. 1988, 29, 877-880. Wagler, T.R.; Burrows, C.J. Tetrahedron Lett. 1988, 29, 5091-5094. Yoon, H.; Burrows, C.J. J.

Am. Chem. Soc. 1988, 110, 4087-4089. Yoon, H.; Wagler, T.R.; O'Connor, K.J.; Burrows, C.J. J. Am. Chem. Soc. 1990, 112, 4568-4570.

- 7. Bossu, F.P.; Paniago, E.B.; Margerum, D.W.; Kirksey, S.T., Jr.; Kurtz, J.L. Inorg. Chem. 1978, 17, 1034-1042.
- Mack, D.P.; Dervan, P.B. J. Am. Chem. Soc. 1990, 112, 4604-4606. Chen, X.; Rokita, S.; Burrows, C.J. J. Am. Chem. Soc. 1991, 113, 5884-5886. Mack, D.P.; Iverson, B.L.; Dervan, P.B. J. Am. Chem. Soc. 1988, 110, 7572-7574.
- 9. Bradbury, A.F.; Finnie, M.D.A.; Smyth, D.G. Nature 1982, 298, 686-688.
- 10. 1: ¹H-NMR (250 MHz, CD₃OD) δ 7.63 (d, J=1.0, 1H), 6.8-7.1 (m, 8H), 5.05 (t_{abp}, J=7.2, 1H), 4.02 (t, J=6.2, 2H), 3.89 (s, 2H), 3.67 (s, 2H), 3.42 (m, br, 4H), 2.86 (dd, J=7.3, 14.0, 1H), 2.74 (dd, J=7.2, 14.0, 1H), 2.44 (t, J= 7.0, 2H), 2.09-2.37 (m, 4H), 1.88 (m, 2H). IR (neat, cm⁻¹) 3253, 3091 2936, 1647, 1513, 1443, 1330, 1238, 1125. HRMS (FAB) calculated M+H 645.2583, observed 645.2590. 3: ¹H-NMR (250 MHz, CDCl₃) & 8.79 (d, J=2.5, 1H), 8.52 (dd, J=2.5, 8.7, 1H), 7.62 (d, J=8.7, 1H), 7.57 (d, J=1.3, 1H), 6.85-6.99 and 7.05-7.20 (m, 7H), 7.00 (s, 2H), 5.47 (d, br, J=8.6, 1H), 4.92 (dd, J=6.7, 14.8, 1H), 3.97 (t, J=6.6, 2H), 3.47 (m, bd, 4H), 2.98 (dd, J=6.2, 14.7, 1H), 2.85 (dd, J=6.9, 14.6, 1H), 2.45 (t, J=6.7, 2H), 2.33 (s, bd, 4H), 1.90 (m, 2H), 1.37 (s, 9H). IR (neat, cm⁻¹) 3416, 3288, 3096, 2975, 2815, 1707, 1642, 1607, 1537, 1337, 1244, 742. HRMS (FAB) calculated M+H 797.2692, observed 797.2691. 4: ¹H-NMR (250 MHz, CD₃OD) δ 8.91 (d, J=2.1, 1H), 8.62 (dd, J=2.7, 8.8, 1H), 7.92 (s, 1H), 7.88 (d, J=8.8, 1H), 6.9-7.3 (m, 8H), 4.18 (t_{app}, J=7.0, 1H), 4.03 (t, J=6.2, 2H), 3.48 (m, br, 4H), 2.83 (dd, J=6.9, 18.7, 1H), 2.71 (dd, J=6.9, 18.7, 1H), 2.48 (t, J=7.2, 2H), 2.35 (m, br, 4H), 1.90 (m, 2H). IR (neat, cm⁻¹) 3313, 3102, 2944, 1646, 1608, 1540, 1423, 1340, 1243, 1120, 744, HRMS (FAB) calculated M+H 697.2168, observed 697.2186. 5: ¹H-NMR (250 MHz, CD₃OD) δ 8.92 (d, J=2.5, 1H), 8.63 (dd, J=2.5, 8.8, 1H), 7.97 (D, J=8.7, 1H), 7.89 (s, 1H), 6.9-7.3 (m, 8H), 5.13 (t_{app}, J=7.2, 1H), 4.04 (t, J=6.3, 2H), 3.89 (d, J=16.8, 1H), 3.78 (d, J=16.9, 1H), 3.70 (s, 2H), 3.3-3.6 (m, 4H), 3.01 (dd, J=6.9, 14.6, 1H), 2.91 (dd, J=8.3, 14.7, 1H), 2.50 (t, J=7.0, 2H), 2.39 (m, br, 4H), 1.91 (m, 2H), 1.42 (s, 9H). IR (neat, cm⁻¹) 3284, 2975, 2927, 2816, 1677-1632 (br), 1537, 1453, 1421, 1334, 1239, 1165, 1120, 727. HRMS (FAB) calculated M+H 911.3122, observed 911.3109. 6: ¹H-NMR (250 MHz, CD₃OD) § 7.58 (d, J=1.1, 1H), 6.9-7.2 (m, 7H), 6.84 (s, 1H), 5.02 (t_{app}, J=7.2, 1H), 4.03 (t, J=6.2, 2H), 3.88 (d, J=16.6, 1H), 3.78 (d, J=16.6, 1H), 3.71 (s, 2H), 3.42 (m, 4H), 2.98 (dd, J=7.2, 14.5, 1H), 2.86 (dd, J=7.1, 14.5, 1H), 2.45 (t, J=7.0, 2H), 2.1-2.4 (m, 4H), 1.89 (m, 2H), 1.44 (s, 9H). IR (neat, cm-1) 3297, 3081, 2932, 2816, 1686 (br), 1637 (br), 1608, 1540, 1533, 1339, 1242, 1165, 1121, 910, 733. HRMS (FAB) calculated M+H 745.3107, observed 745.3157. Compound 2 was prepared as described in reference 1.
- 11. Laemmli, U.K. Nature (London) 1970, 277, 680-685.
- 12. Holm, R.H. Chem. Rev. 1987, 87, 1401-1449.
- Balch, A.L.; Chan, Y-W.; Olmstead, M.M. J. Am. Chem. Soc. 1985, 107, 6510-6514. Balch, A.L. Chan, Y-W.;
 Olmstead, M.M. J. Am. Chem. Soc. 1985, 107, 2393-2398.
- 14. Neither Cr³⁺, Zn²⁺, Mn²⁺, Fe²⁺, Co²⁺, Fe³⁺ nor Cu²⁺ promote significant cleavage of calmodulin when complexed to GGH-TFP. The cleavage yield does not increase when the reaction mixture is heated at 60 °C for 1 hour at pH 8.5.
- 15. Cleavage yields were determined by amino acid analysis.
- 16. Watterson, D.M.; Sharief, F.; Vanaman, T.C.; J. Biol. Chem. 1980, 255, 962-975..
- 17. Matsudaira, P.J. J. Biol. Chem. 1987, 262, 10035-10038.
- 18. Paolella, D.; Schepartz, A. unpublished results.

(Received in USA 5 November 1991)