confirmed by the ^1H NMR spectra.[9,10] When $AcrH^+$ has been replaced by a common NAD^+ analogue, 1-benzylnicotinamidium ion (BNA^+), which is a much weaker oxidant than $AcrH^+$,[2a,11] however, no reduction of BNA^+ by Me_3SnMMe_3 has occurred in deaerated MeCN at 333 K. On the other hand, the reduction of $AcrH^+$ by Me_3SnMMe_3 (eq 3) is strongly inhibited by the presence of oxygen. As such, essentially no reaction has occurred in aerated MeCN at 333 K.

Rates of the reduction of $AcrH^+$ by Me_3SnMMe_3 in deaerated MeCN were followed by the decay of the absorption band due to $AcrH^+$ (λ_{max} 358 nm) under conditions in which the concentrations of Me_3SnMMe_3 [$(4.8 \times 10^{-3}) - (1.4 \times 10^{-2})$ M] were maintained in large excess of $AcrH^+$ (e.g., 1.0×10^{-4} M) at 333 K. The rates obey pseudo-one-half-order kinetics, when $[AcrH^+]^{1/2}$ decreases linearly with an increase in the reaction time. The observed pseudo-one-half order rate constants ($k_{1/2}$) are proportional to $[Me_3SnMMe_3]^{3/2}$. Thus, the kinetic formulation is given by eq 4. The observed overall second-order rate constants

$$-d[AcrH^+]/dt = k_{obsd}[Me_3SnMMe_3]^{3/2}[AcrH^+]^{1/2} \quad (4)$$

(k_{obsd}) in deaerated MeCN at 333 K are listed in Table I, together with the ionization potentials of $Me_3MM'Me_3$.[7] The k_{obsd} value decreases in the order $Me_3SnSnMe_3 > Me_3SnGeMe_3 > Me_3SnSiMe_3$, when the donor ability of $Me_3MM'Me_3$ decreases as indicated by the increase in the I_D value (Table I).

The strong inhibitory effect of oxygen and the unusual kinetic formulation (eq 4) indicate that the one-electron reduction of $AcrH^+$ by Me_3SnMMe_3 proceeds via electron-transfer radical chain processes as shown in Scheme I.[12] The reaction may be initiated by electron transfer (k_i) from Me_3SnMMe_3 to $AcrH^+$ to produce $Me_3SnMMe_3^{\bullet+}$ and $AcrH^{\bullet}$.[13] The Sn-M bond of $Me_3SnMMe_3^{\bullet+}$ (M = Sn, Ge, Si) is known to be readily cleaved to give mainly $Me_3Sn^{\bullet}$ and Me_3M^+.[8,14] Then, electron transfer from $Me_3Sn^{\bullet}$ to $AcrH^+$ may occur to give acridinyl radical $AcrH^{\bullet}$, which may react with $AcrH^+$ to form the dimer radical cation $(AcrH)_2^{\bullet+}$. The electron transfer from Me_3SnMMe_3 to $(AcrH)_2^{\bullet+}$ (k_p) may be the rate-determining step to yield $(AcrH)_2$, accompanied by regeneration of $Me_3SnMMe_3^{\bullet+}$ (Scheme I). The chain carrier radical $AcrH^{\bullet}$ may be coupled in the termination step (k_t) to yield $(AcrH)_2$.[15] The steady-state approximation is applied to the reactive intermediates in Scheme I to derive the kinetic formulation which agrees with eq 4, where k_{obsd} corresponds to $k_p(k_i/k_t)^{1/2}$. The strong inhibitory effect of oxygen, which may be ascribed to the efficient trap of the chain carrier radical $AcrH^{\bullet}$ by oxygen,[2a,16] indicates a long chain length of the radical chain reactions.[15] Such a long chain length causes the highly selective formation of the dimer $(AcrH)_2$, in contrast with usual radical reactions. The unreactivity of the group 4B dimetals $Me_3MM'Me_3$ (M, M' = Ge, Si) that do not contain Sn may be ascribed to the much less reducing ability of $Me_3Ge^{\bullet}$ or $Me_3Si^{\bullet}$ in the propagation step (k_p) compared with $Me_3Sn^{\bullet}$, combined with the less reducing ability of $Me_3MM'Me_3$ compared to Me_3SnMMe_3 (M = Sn, Ge, Si) in the initiation step (k_i), as indicated by the I_D values in Table I. By the same token, BNA^+, which is a much weaker oxidant than $AcrH^+$,[11] has no ability to start or to continue the chain reactions with Me_3SnMMe_3.

Acknowledgment. This work was supported in part by a grant-in-aid (to S. Fukuzumi) for scientific research from the Ministry of Education, Science and Culture, Japan.

Supplementary Material Available: Kinetic data for the derivation of eq 4 (1 page). Ordering information is given on any current masthead page.

(9) Typically, a deaerated MeCN solution containing 19.6 mg of $Me_3SnSnMe_3$ (3.0×10^{-2} M) and 35.2 mg of $AcrH^+ClO_4^-$ (6.0×10^{-2} M) was heated at 333 K for 30 min and filtered to yield 23.3 mg (79%) of $(AcrH)_2$, when the conversion of $AcrH^+$ was 80%. Analytical and spectral data found for $(AcrH)_2$: C, 86.4; H, 6.1; N, 7.2. Calcd for $C_{28}H_{24}N_2$: C, 86.6; H, 6.2; N, 7.2. ^1H NMR (100 MHz): $(AcrH)_2$ $\delta(CDCl_3)$ 3.06 (6 H, s), 3.99 (2 H, s), 6.5-7.3 (16 H, m); Me_3SnClO_4 $\delta(CD_3CN)$ 0.63 (9 H, s); Me_3GeClO_4 δ 0.79 (9 H, s); Me_3SiClO_4 δ 0.36 (9 H, s).

(10) There has been discussion as to whether the perchlorate of Me_3SiClO_4 in MeCN is a covalent ester (or tight ion pair) or Me_3Si^+ is a free cation; see: Lambert, J. B.; McConnell, J. A.; Schilf, W.; Schulz, W. J., Jr. *J. Chem. Soc., Chem. Commun.* **1988**, 455. Lambert, J. B.; Schilf, W. *J. Am. Chem. Soc.* **1988**, *110*, 6364. Prakash, G. K. S.; Keyaniyan, S.; Aniszfeld, R.; Heiliger, L.; Olah, G. A.; Stevens, R. C.; Choi, H.-K.; Bau, R. *J. Am. Chem. Soc.* **1987**, *109*, 5123.

(11) Fukuzumi, S.; Koumitsu, S.; Hironaka, K.; Tanaka, T. *J. Am. Chem. Soc.* **1987**, *109*, 305.

(12) Radical chain mechanisms involving dimer radical cations have been well established for photoinduced isomerization and cyclodimerization of unsaturated and strained hydrocarbons, in which, however, no overall redox reactions are involved; see: Mattes, S. L.; Farid, S. *Organic Photochemistry*; Padwa, A., Ed.; Marcel Dekker: New York, 1983; Vol. 6, p 223. Julliard, M.; Chanon, M. *Chem. Rev.* **1983**, *83*, 425. Lewis, F. D. *Photoinduced Electron Transfer*; Fox, M. A., Chanon, M., Eds.; Elsevier: Amsterdam, 1988; Part C, Chapter 1. Lewis, F. D.; Kojima, M. *J. Am. Chem. Soc.* **1988**, *110*, 8664. Ledwith, A. *Acc. Chem. Res.* **1972**, *5*, 133. Mattes, S. L.; Farid, S. *Acc. Chem. Res.* **1982**, *15*, 80.

(13) Alternatively, the homolytic cleavage of the Sn-M bond of Me_3SnMMe_3, followed by electron transfer from $Me_3Sn^{\bullet}$ to $AcrH^+$, may initiate the chain reaction. However, electron transfer from Me_3SnMMe_3 to $AcrH^+$ may be much faster than the homolytic cleavage, since the activation Gibbs energy of the electron transfer from Me_3SnMMe_3 to $AcrH^+$ (e.g., $\Delta G^{\ddagger}$ = 25.8 kcal mol^{-1} for $Me_3SnSnMe_3$), which is calculated by using the Marcus theory (Marcus, R. A. *Annu. Rev. Phys. Chem.* **1964**, *15*, 155), is much smaller than the Sn-M bond dissociation energy (e.g., 39 kcal mol^{-1} for $Me_3SnSnMe_3$; cf.: Keiser, D.; Kana'an, A. S. *J. Phys. Chem.* **1969**, *73*, 4264). The parameters used for the calculation were determined by the analyses of the cyclic voltammograms according to the method reported previously.[11]

(14) (a) Walther, B. W.; Williams, F.; Lau, W.; Kochi, J. K. *Organometallics* **1983**, *2*, 688. (b) Wang, J. T.; Williams, F. *J. Chem. Soc., Chem. Commun.* **1981**, 666.

(15) No cross-coupling products such as $AcrH(SnMe_3)$[3] have been detected in the present case, probably because of the long chain length of the radical chain reactions (Scheme I).

(16) (a) Fukuzumi, S.; Ishikawa, M.; Tanaka, T. *J. Chem. Soc., Perkin Trans. 2* **1989**, 1037. (b) Fukuzumi, S.; Chiba, M.; Tanaka, T. *J. Chem. Soc., Chem. Commun.* **1989**, 941.

Site-Specific Cleavage of the Protein Calmodulin Using a Trifluoperazine-Based Affinity Reagent

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Reagents that react specifically with protein chains are extremely useful in chemistry and biology. Affinity labeling reagents react covalently with proteins and enable identification and modification of receptor sites.[1] Cleavage of the protein chain may be achieved with proteolytic enzymes or small molecules (such as cyanogen bromide).[2] Protein cleavage reagents permit sequence analysis of large or blocked proteins, functional analysis of protein domains, and structural analysis of receptors. In this communication, we describe the synthesis and evaluation of a molecule that combines the properties of both classes of protein probes: *an active site specific protein cleaving molecule.* Our target was trifluoperazine-EDTA (TFE (**1**), see Figure 1), which consists of the iron chelate ethylenediaminetetraacetic acid (EDTA) covalently tethered to the calmodulin antagonist trifluoperazine (TFP). TFE binds calmodulin under physiological conditions and, in the presence of Fe, O_2, and dithiothreitol (DTT), cleaves calmodulin to produce six major cleavage fragments. The appearance of these fragments is blocked by TFP and requires calmodulin to exist in an active conformation. *TFE is an affinity*

(1) Baker, B. R.; Lee, W. L.; Tong, E.; Rose, L. O. *J. Am. Chem. Soc.* **1961**, *83*, 3713-3714. Singh, A.; Thornton, E. R.; Westheimer, F. H. *J. Biol. Chem.* **1962**, *237*, PC3006-PC3008. Lawson, W. B.; Schramm, H. J. *J. Am. Chem. Soc.* **1962**, *84*, 2017-2018. Schoellmann, G.; Shaw, E. *Biochem. Biophys. Res. Commun.* **1962**, *7*, 36-40. Knowles, J. R. *Acc. Chem. Res.* **1972**, *5*, 155-160.

(2) Gross, E.; Witkop, B. *J. Am. Chem. Soc.* **1961**, *83*, 1510-1511.

0002-7863/90/1512-3247$02.50/0

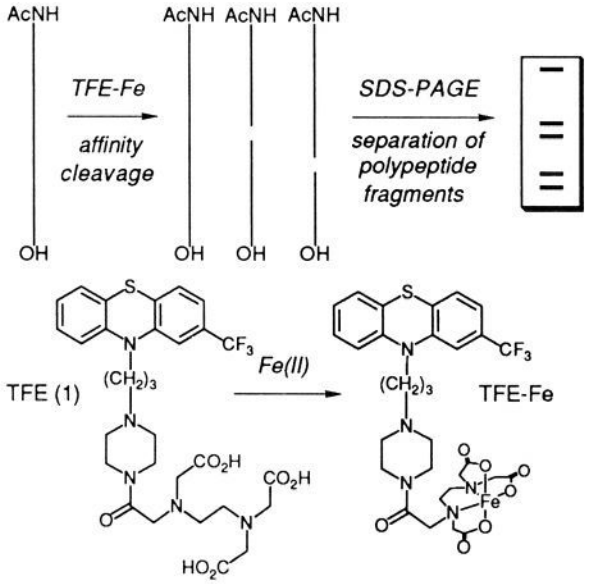

Figure 1.

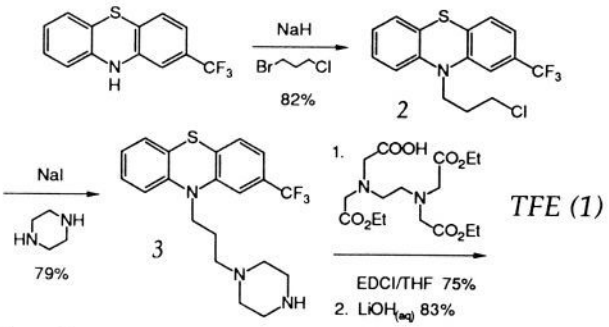

Figure 2.

cleavage reagent for calmodulin.[3]

Calmodulin[4] is a multifunctional calcium receptor protein[5] that plays a commanding role in cellular regulation.[6] Trifluoperazine binds calmodulin in a Ca^{2+}-dependent manner (K_d = 1.5 μM) and antagonizes virtually all of the known biochemical actions of this protein.[7] The three-dimensional structure of calmodulin is known,[8] yet the precise trifluoperazine binding site(s) are not.[9]

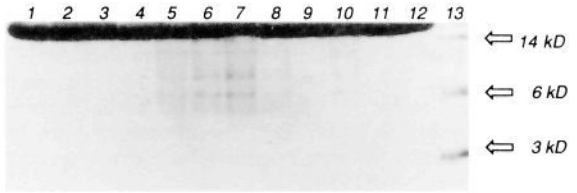

Figure 3. Coomassie blue stained polyacrylamide gel illustrating protein cleavage by TFE. Calmodulin (10 μM) was incubated at 7 °C in a 10 mM Tris pH 7.2, 1 mM Ca^{2+} buffer alone (lane 1) or with the indicated reagents (lanes 2–12) for 2 h (lanes 1–4, 7–12), 30 min (lane 6), or 15 min (lane 5). Lane 2: 30 mM DTT. Lane 3: 40 μM Fe(II), 30 mM DTT. Lane 4: 40 μM EDTA–Fe(II), 30 mM DTT. Lanes 5–7: 40 μM TFE–Fe(II), 30 mM DTT. Lane 8: 40 μM TFE–Fe(II). Lane 9: 40 μM TFE–Mg(II), 30 mM DTT. Lane 10: 40 μM TFE–Fe(II), 30 mM DTT, Ar purge. Lane 11: 40 μM TFE–Fe(II), 30 mM DTT, 400 μM TFP. Lane 12: 40 μM TFE–Fe(II), 30 m*M* DTT (buffer did not contain Ca^{2+}). Molecular weight markers (BRL) are shown in lane 13. Reactions were incubated for the indicated times and then halted by addition of EGTA to a final concentration of 3 mM. The reaction mixtures were frozen immediately, lyophilized, resuspended in loading buffer, and subjected to electrophoresis on 0.75- or 1.5-mm gels as described by Giulian.[12] Gel compositions: stacking gel, 10% acrylamide, 4.8% bis(acrylamide), 10% glycerol; separating gel, 20% acrylamide, 0.5% bis(acrylamide), 10% glycerol. All buffers contained 1 mM EDTA. Gels were electrophoresed at 16 mA for 8 h and stained.

Nevertheless, structure/function studies on TFP analogues clearly identify N-4 as appropriate for attachment of EDTA to the trifluoperazine nucleus.[10] TFE was prepared in four synthetic steps as shown in Figure 2 and converted into TFE–Fe immediately before use.[11]

Cleavage of calmodulin by TFE–Fe was monitored by SDS–PAGE.[12] Cleavage of the protein at any point along its length will result in the appearance of peptides that are shorter than calmodulin when the products are subjected to electrophoresis under denaturing conditions.[13] Six discrete calmodulin fragments are observed after a 15-min incubation with micromolar concentrations of TFE–Fe (Figure 3). These bands are clearly visible in lanes 5–7 and correspond to peptides with approximate molecular weights of 11.3, 10.2, 9.1, 7.9, 6.4, and 5.3 kD.[14] Cleavage reactions are performed at 7 °C, but specific cleavage is also observed at 25 °C.

Control experiments indicate that TFE, Fe, and DTT are all required for specific cleavage. No cleavage is observed when

(3) Two examples of *stoichiometric* protein affinity cleavage have been reported: Middlemas, M. Ph.D. Thesis, California Institute of Technology, 1987. Donadio, S.; Perks, H. M.; Tsuchiya, K.; White, E. H. *Biochemistry* **1985**, *24*, 2447–2458.

(4) Cheung, W. Y. *Biochem. Biophys. Res. Commun.* **1967**, *29*, 478–482. Cheung, W. Y. *J. Biol. Chem.* **1971**, *246*, 2859–2869. Kakiuchi, S.; Yamazaki, R. *Biochem. Biophys. Res. Commun.* **1970**, *41*, 1104–1111.

(5) *Calmodulin and Intracellular Ca^{2+} Receptors*; Kakiuchi, S., Ed.; Plenum: New York, 1981. *Calcium Regulation by Calcium Antagonists*; Rahwan, R. G., Ed.; ACS Symposium Series 201; American Chemical Society: Washington, DC, 1982. Klee, C. B.; Vanaman, T. C. *Adv. Protein Chem.* **1982**, *35*, 213–321. Cormier, M. J. In *Calcium in Biology*; Spiro, T. G., Ed.; Wiley: New York, 1983; Chapter 2. *Intracellular Calcium*; Campbell, A. K., Ed.; Wiley: New York, 1983. *Calcium and Cell Function*; Cheung, W. Y., Ed.; Academic: New York, 1984. *Calmodulin Antagonists and Cellular Physiology*; Hidaka, H., Hartshorne, D. J., Eds.; Academic: Orlando, 1985. *Calcium and the Cell*; Ciba Foundation Symposium 122; Wiley: New York, 1986. *Methods in Enzymology*; Means, A. R., Conn, P. M., Eds.; Academic: New York, 1987; Vol. 139. Swan, D. G.; Hale, R. S.; Dhillon, N.; Leadlay, P. F. *Nature (London)* **1987**, *329*, 84–85.

(6) Means, A. R.; Tash, J. S.; Chafouleas, J. G. *Physiol. Rev.* **1982**, *62*, 1–39. Cheung, W. Y. *Science* **1980**, *207*, 19–27.

(7) Weiss, B.; Fertel, R.; Figlin, R.; Uzunov, P. *Mol. Pharmacol.* **1974**, *10*, 615–625. Levin, R. M.; Weiss, B. *Biochim. Biophys. Acta* **1978**, *540*, 197–204.

(8) Babu, Y. S.; Sack, J. S.; Greenhough, T. J.; Bugg, C. E.; Means, A. R.; Cook, W. J. *Nature (London)* **1985**, *315*, 37–40. Kretsinger, R. H.; Rudnick, S. E.; Weissman, L. J. *J. Inorg. Biochem.* **1986**, *28*, 289–302. Babu, Y. S.; Bugg, C. E.; Cook, W. J. *J. Mol. Biol.* **1988**, *204*, 191–204.

(9) For NMR studies: Forsen, S.; Thulin, E.; Drakenberg, T.; Krebs, J.; Seamon, K. *FEBS Lett.* **1980**, *117*, 189–194. Klevit, R. E.; Levine, B. A.; Williams, R. J. P. *FEBS Lett.* **1981**, *123*, 25–29. Krebs, J.; Carafoli, E. *Eur. J. Biochem.* **1982**, *124*, 619–627. Dalgarno, D. C.; Klevit, R. E.; Levine, B. A.; Scott, G. M. M.; Williams, R. J. P.; Gergely, J.; Garbarek, Z.; Leavis, P. C.; Grand, R. J. A.; Draibikowski, W. *Biochim. Biophys. Acta* **1984**, *791*, 164–172. Thulin, E.; Andersson, A.; Drakenberg, T.; Forsen, S.; Vogel, H. J. *Biochemistry* **1984**, *23*, 1862–1870. For affinity labeling studies: Newton, D. L.; Burke, T. R.; Rice, K. C.; Klee, C. B. *Biochemistry* **1983**, *22*, 5462–5476. Newton, D. L.; Klee, C. B. *FEBS Lett.* **1984**, *165*, 269–272. Giedroc, D. P.; Sinha, S. K.; Brew, K.; Puett, D. *J. Biol. Chem.* **1985**, *260*, 13406–13413. Jarrett, H. W. *J. Biol. Chem.* **1986**, *261*, 4967–4972. Faust, F. M.; Slisz, M.; Jarrett, H. W. *J. Biol. Chem.* **1987**, *262*, 1938–1941. Newton, D. L.; Klee, C. B. *Biochemistry* **1989**, *28*, 3750–3757 and references cited therein. For computational studies: Holtje, H.-D.; Hense, M. *J. Comput. Aided Mol. Des.* **1989**, *3*, 101–109. Strynadka, N. C. J.; James, M. N. G. *Proteins: Struct., Funct., Genet.* **1988**, *3*, 1–17.

(10) Levin, R. M.; Weiss, B. *Mol. Pharmacol.* **1976**, *12*, 581–589.

(11) Piperazine **3** was prepared as described by Hait. Hait, W. N.; Glazer, L.; Kaiser, C.; Kennedy, K. A. *Mol. Pharmacol.* **1987**, *32*, 404–409. Condensation of **3** with EDTA triethyl ester (Hay, R. W.; Nolan, K. B. *J. Chem. Soc., Dalton Trans.* **1975**, 1348–1351) by use of 1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride gave **4** in 74% yield. Hydrolysis (9 equiv of LiOH, MeOH/CH_2Cl_2) and acidification gave TFE (**1**) in 82% yield after silica gel chromatography (50:48:2 CH_2Cl_2–MeOH–NH_{3aq}). TFE produced this way was free of contaminating transition metals, as judged by elemental analysis and mass spectroscopy. Complete spectroscopic characterization of **1**–**4** is available as supplementary material.

(12) Giulian, G. G.; Shanahan, M. F.; Graham, J. M.; Moss, R. L. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1985**, *44*, 486.

(13) Laemmli, U. K. *Nature (London)* **1970**, *277*, 680–685.

(14) Watterson, D. M.; Sharief, F.; Vanaman, T. C. *J. Biol. Chem.* **1980**, *255*, 962–975. The low cleavage yield (10%) may be due in part to the inaccessibility of the main chain in a hydrophobic, helical protein domain.

calmodulin is incubated with equivalent concentrations of DTT, Fe(II) and DTT, or Fe(II)-EDTA and DTT (lanes 2-4). Very little cleavage is generated by TFE-Fe in the absence of DTT (lane 8), and none is generated by TFE-Mg(II) in the presence of DTT (lane 9).[15] Cleavage by TFE is dependent on the presence of oxygen. If oxygen is removed from the reaction mixture with an argon purge, far less cleavage is observed (lane 10). Addition of catalase (100 μg/mL) to the reaction mixture eliminated protein cleavage, while boiled catalase had no effect (data not shown).[16] These results are consistent with oxidative cleavage of the protein backbone by TFE-Fe.[17]

Several experiments were performed to determine whether cleavage was occurring in the trifluoperazine binding pocket. At saturation, calmodulin stimulates by 3-10-fold the rate of phosphodiesterase-catalyzed hydrolysis of cyclic AMP.[18] A concentration of 75 μM TFE or TFE-Mg^{2+} was required to reduce this stimulation by 50% (IC_{50} = 75 μM).[19] TFP shows a corresponding IC_{50} of 15 μM. Although TFE is 5 times less effective than TFP, an IC_{50} of 75 μM is well within the range observed for phenothiazine ligands.[10] Therefore, TFE and TFE-Mg^{2+} are calmodulin antagonists.

A rigorous test for any affinity reagent is a competition experiment. Site-specific binding/cleaving demands that cleavage be attenuated by the original ligand. To test whether TFE would fulfill this requirement, calmodulin was incubated with 400 μM trifluoperazine, 40 μM TFE-Fe, and 30 mM DTT.[20] Comparison of lanes 7 and 11 in Figure 3 shows clearly the absence of cleavage in lane 11.

A mandatory test for affinity cleavage of calmodulin recognizes that the structure and activity of this protein are tied intimately to the concentration of Ca^{2+} in the medium. At saturation, calmodulin binds four Ca^{2+} ions in two EF-hand domains.[21] Only in this Ca^{2+}-bound form does calmodulin activate cellular enzymes or bind trifluoperazine drugs.[22] Therefore, TFE-Fe should be unable to cleave calmodulin in the absence of Ca^{2+}. Indeed, no protein cleavage is observed when Ca^{2+} is omitted from the reaction mixture (lane 12). Taken together, these experiments demonstrate that TFE-Fe is an affinity cleavage reagent for calmodulin. Studies directed toward determination of the precise cleavage sites, analysis of other cleaving reagents, and the evaluation of the power of these reagents as potent *receptor modifying/damaging reagents* are in progress.

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Supplementary Material Available: Spectroscopic characterization of compounds **1-4** and combustion analysis of **1** and **4** (1 page). Ordering information is given on any current masthead page.

(15) Fe(II) does induce nonspecific protein cleavage in the absence of TFE; however, millimolar concentrations are required. Discrete bands are not observed under these conditions; the products appear as a diffuse smear on the gel.

(16) This is an important control, since catalase itself is degraded by redox-active metal ions. Orr, C. W. M. *Biochemistry* **1967**, *6*, 3000-3006.

(17) The first example of Fe(II)-mediated protein cleavage was reported over 30 years ago: Jayko, M. E.; Garrison, W. M. *Nature (London)* **1958**, *181*, 413-414. More recently, see: Kim, K.; Rhee, S. G.; Stadtman, E. R. *J. Biol. Chem.* **1985**, *260*, 15394-15397.

(18) Weiss, B.; Lehne, R.; Strada, S. *Anal. Biochem.* **1972**, *45*, 222-235. Phosphodiesterase activity was determined at [calmodulin] = 3.8 nM. The assay mixture contained 0-100 μM trifluoperazine analogue, 2 μg of pyruvate kinase, 2 μg of myokinase, 0.32 mM cAMP, 25 mM ammonium acetate, 3 mM $MgCl_2$, 0.26 mM phosphoenolpyruvate, 25 mM DTT, 0.1 mM EDTA, 1 nM ATP, and 0.1 mM $CaCl_2$ in 125 μL of pH 8.0 glycylglycine buffer.

(19) The IC_{50} for TFE-Fe(II) could not be determined due to competitive inhibition of phosphodiesterase. Neither TFP, TFE, nor TFE-Mg^{2+} (100 μM) inhibited phosphodiesterase in the absence of calmodulin.

(20) Reactions were performed at 7 °C and incubated for 120 min.

(21) Kretsinger, R. H. *Annu. Rev. Biochem.* **1976**, *45*, 239-266.

(22) Levin, R. M.; Weiss, B. *Mol. Pharmacol.* **1977**, *13*, 690-697. Vogel, H. J.; Andersson, T.; Braunlin, W. H.; Drakenberg, T.; Forsen, S. *Biochem. Biophys. Res. Commun.* **1984**, *122*, 1350-1356.

A New Strategy for Selective Protein Cleavage

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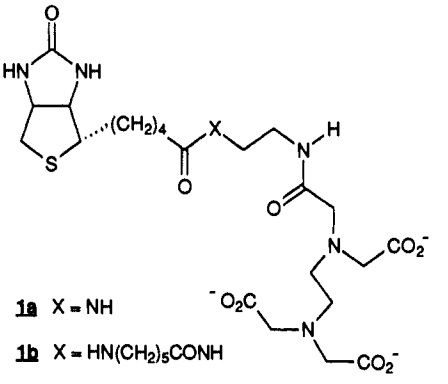
The ability of proteolytic enzymes and chemical reagents to selectively cleave peptides and proteins at defined sequences has greatly facilitated studies of protein structure and function.[1] Unfortunately, only a limited number of selective peptide cleavage agents exist, in contrast to the wide array of selective nucleases available for analyzing and manipulating nucleic acid structure. The development of strategies for generating site-specific peptidases of any defined sequence would greatly facilitate the mapping of protein structural domains, protein sequencing, the generation of semisynthetic proteins, and would likely lead to the development of new therapeutic agents. We report here a new approach to the generation of selective protein cleavage agents that is based on oxidative cleavage of the polypeptide backbone.[2] Attachment of the metal chelator ethylenediaminetetraacetic acid (EDTA) to biotin allows delivery of redox-active Cu^{2+} or Fe^{3+} to the binding site of streptavidin and selective cleavage of the polypeptide backbone. Similarly, attachment of redox-active chelates to other ligands or to antibody combining sites may lead to a new class of affinity cleavage agents,[3] to the design of catalytic drugs, or to the generation of a family of sequence-selective peptidases.

Chemical studies have demonstrated that cupric ion can catalyze the cleavage of peptide and proteins by oxidation of the polypeptide backbone.[4] In addition, the metal-dependent enzyme peptidylglycine monooxygenase catalyzes the oxidative cleavage of glycine from the C-terminus of peptides.[5] Consequently, we reasoned that attachment of a Cu^{2+} chelate to biotin should result in selective cleavage of streptavidin in close proximity to the biotin binding site (just as EDTA·Fe^{2+} conjugates of DNA binding ligands oxidatively cleave nucleic acids[6]). The 72-kDa tetrameric protein streptavidin binds one biotin per monomer with an association constant of 10^{15} M^{-1}.[7] Biotin-EDTA derivatives **1a** and **1b** were prepared by treatment of (+)-biotin *N*-hydroxysuccin-



* Author to whom correspondence should be addressed.

(1) (a) Stark, G. *Adv. Protein Chem.* **1970**, *24*, 261. (b) Spandle, T. F.; Witcop, B.; Degani, Y.; Patchornik, A. *Ibid.* **1970**, *24*, 98.

(2) A similar approach has been independently described; see: Schepartz, A.; Cuenoud, B. *J. Am. Chem. Soc.*, preceding paper in this issue.

(3) Schultz, P. G.; Dervan, P. B. *J. Am. Chem. Soc.* **1983**, *105*, 7748.

(4) (a) Bateman, R. C.; Youngblood, W. W.; Busby, W. H.; Kizer, J. S. *J. Biol. Chem.* **1985**, *260*, 9088. (b) Sigel, H. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 167. (c) Kurtz, J. L.; Burce, G. L.; Margerum, D. W. *Inorg. Chem.* **1978**, *17*, 2454. (d) Phelps, R. A.; Neet, K. E.; Lynn, L. T.; Putnam, F. W. *J. Biol. Chem.* **1961**, *236*, 96. (e) Young, S. D.; Tamburinis, P. P. *J. Am. Chem. Soc.* **1989**, *111*, 1933. (f) Ramer, S. E.; Cheng, H.; Palcic, M. M.; Vederas, J. C. *J. Am. Chem. Soc.* **1988**, *110*, 8526.

(5) (a) Bradbury, A. F.; Finnie, M. D. A.; Smyth, D. G. *Nature* **1982**, *298*, 686. (b) Kizer, J. S.; Busby, W. H.; Cottle, C.; Youngblood, W. W. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3228. (c) Eipper, B. A.; Mains, R. E.; Glembotski, C. C. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *51*, 44.

(6) Dervan, P. B. *Science* **1986**, *232*, 464.

(7) (a) Wilchek, M.; Bayer, E. A. *Trends Biochem. Sci. (Pers. Ed.)* **1989**, *14*, 408. (b) Weber, P. C.; Ohlendorf, D. H.; Wandoloski, J. J.; Salemme, F. R. *Science* **1989**, *243*, 85. (c) Hendrickson, W. A.; Pahler, A.; Smith, J. L.; Satow, Y.; Merritt, E. A.; Phizackery, R. P. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2190.

0002-7863/90/1512-3249$02.50/0