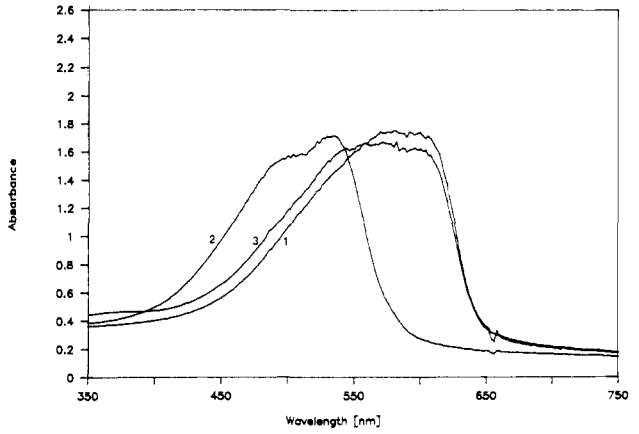

Figure 2. Absorption spectra of photopolymerized multibilayer film of **1** at 22 °C (1), at 50 °C (2), and after cooling back to 22 °C.

temperature in a thermostated cup-horn sonicator. Differential scanning calorimetry (Microcal MC-2) of membranes of **1** indicates the T_c is 14.6 °C. Hydrated bilayer membranes of **1** were not photosensitive unless they were cooled below room temperature with an ice bath. As noted previously lipid diacetylene bilayers are photopolymerizable only in the solid analogous phase ($<T_c$).

A few drops of the bilayer membranes of **1** (5 mM in lipid) were spread on a clean glass slide (75 × 25 mm) and allowed to dry slowly. After 2 days a thin transparent film was formed. The film on glass was further dried under vacuum for 2 h. It was then irradiated at room temperature by a low-pressure mercury lamp (Pen Ray) at a distance of 8 cm. The film immediately became blue, and the color intensified with continued irradiation in a manner typical of diacetylene polymerizations. After irradiation the PDA film was stripped off the glass to give a 10-μm thick, dark purple-blue, flexible free standing film. The absorption was too intense for spectrophotometric measurement; therefore, a thinner film was prepared from 0.25 mM lipid. Figure 1 shows the absorption spectra obtained by irradiation of this film with 254 nm light for various times. Visible PDA absorbance is readily detected after a few seconds. Note that photopolymerization of the film can occur at room temperature, because the T_c of lipid multilayers shifts to higher temperatures as they are dehydrated.[14] Thus the cast multilayer film of **1** has sufficient molecular order to allow the topotactic polymerization to proceed. We estimate the extent of polymerization of the diacetylenic lipids to be about 75% after 180 s exposure. At this point about 20% monomeric lipid may be extracted from the photopolymerized film. Although monomer may be extracted from the films, the free standing polymer film was not disrupted by treatment at room temperature, with any of the following organic solvents: chloroform, chlorobenzene, THF, DMSO, and DMF.

The absorption maxima of PDAs are indicative of the length of the polymer chain and/or the order of the polymer structure. Longer and/or more highly ordered PDAs, e.g., fatty acid diacetylene monolayers, exhibit absorption maxima at 650 nm or longer (blue form),[11] whereas shorter and/or less ordered PDAs, e.g., phosphatidylcholine diacetylene bilayers, show absorption maxima at 540 nm (red form).[15] The absorption maxima of poly-**1** in extended bilayers is about 640 nm and shifted to somewhat shorter wavelength (610 nm) for poly-**1** formed in cast multilayers. The film of poly-**1** shows reversible thermochromic behavior. As illustrated in Figure 2, if the film of poly-**1** was warmed from room temperature to 50 °C, the color changes to orange-red (534 nm) and then back on cooling.

Cast films were also prepared from the bilayers of poly-**1** in a manner similar to that described above for the bilayers of **1**. After drying a red-purple film was obtained. This prepolymerized film was significantly less stable to treatment with organic solvents, e.g., chloroform, than the PDA film obtained by first casting followed by polymerization of **1**.

Ordered multibilayer structures formed by lipid hydration and careful casting appear to be required for the formation of stable free standing films from **1**. As a control crystals of **1** were dissolved in chloroform and spread on a glass surface. After drying, a thin layer of lipid crystals was formed. Ultraviolet irradiation produced the typical PDA color, but films could not be obtained.

In conclusion, these data substantiate the retention of bilayer characteristics in cast multibilayer films as suggested by Kunitake and co-workers. These observations indicate that in some circumstances cast multibilayer are a possible alternate to Langmuir–Blodgett films for the formation of ordered thin film molecular assemblies. Moreover we have prepared the first insoluble, free standing PDA films. Investigations of the thermochromic, electrical conducting, and nonlinear optical properties of these PDA films will be described elsewhere.

Acknowledgment is made to the donors of The Petroleum Research Fund, administered by the American Chemical Society, for support of this research.

(14) Chapman, D. In *Form and Function of Phospholipids*, 2nd ed.; Ansell, G. B., Hawthorne, J. N., Dawson, R. M. C., Eds.; Elsevier Scientific Publishing Co.; Amsterdam, 1973; pp 117–142.
(15) Lopez, E.; O'Brien, D. F.; Whitesides, T. H. *Biochim. Biophys. Acta* **1982**, *693*, 437–443.

Design and Chemical Synthesis of a Sequence-Specific DNA-Cleaving Protein†

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We report the design and chemical synthesis of a sequence-specific DNA-cleaving protein consisting wholly of naturally occurring α-amino acids. The tripeptide, H-glyglyhis-OH (GGH), which is a consensus sequence for the copper-binding domain of serum albumin, was attached to the amino terminus of the DNA-binding domain of Hin recombinase (residues 139–190) to afford a new 55-residue protein, GGH(Hin 139–190) with two structural domains each with distinct functions, sequence-specific recognition, and cleavage of double helical DNA (Figure 1). The designed protein was synthesized by solid-phase techniques and shown, by footprinting, to be competent to bind at μM concentrations to four Hin sites, each 13 base pairs in length. In the presence of Cu(II), hydrogen peroxide, and sodium ascorbate, strong cleavage of DNA by GGH(Hin 139–190) occurs at one of the four sites by oxidative degradation of the deoxyribose backbone.

A 52-residue peptide identical with the carboxy terminal domain of Hin recombinase (190 amino acids) has been shown to bind to Hin recombination half sites (13 bp) and to inhibit Hin activity.[1] We recently described the conversion of this sequence-specific DNA-binding protein, Hin(139–190), into a sequence-specific DNA-cleaving protein by covalent attachment of an iron chelator, ethylenediaminetetraacetic acid (EDTA), to the amino-terminus.[2,3] In the presence of Fe(II) and reducing agent, EDTA-Hin(139–190) oxidatively cleaves DNA at Hin binding sites, revealing the base position and minor groove location of the amino terminus of the peptide when bound to DNA.[2] The issue arises whether the unnatural amino acid, EDTA, could be replaced by a sequence of α-amino acids that bind transition metals capable of facilitating oxidative cleavage of DNA.

The tripeptide GGH binds Cu(II) in a 1:1 complex over the pH range 6.5–11 with a dissociation constant of 1.2×10^{-16} M^{-1}.[4] A crystal structure of Cu(II)·GGH reveals square-planar complexation of the Cu(II) by an imidazole nitrogen, two deprotonated

† Dedicated to Professor E. T. Kaiser.

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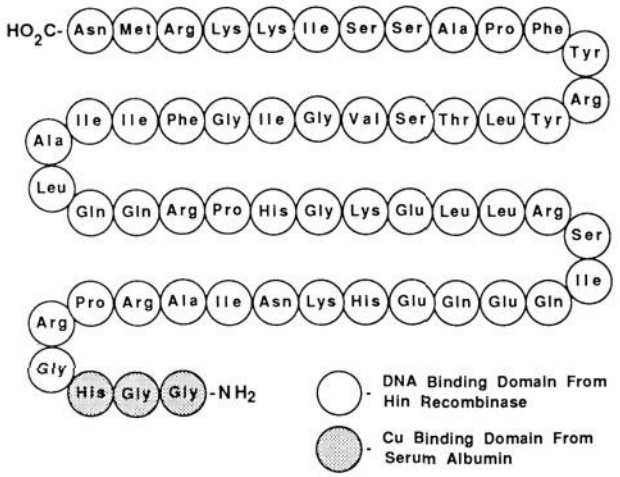

Figure 1. Sequence of designed protein combining the DNA-binding domain of Hin recombinase (residues 139–190) with the copper binding domain (GGH) from serum albumin.

peptide nitrogens, and the terminal amino group.[5] It is known that free Cu(II) at mM concentrations as well as Cu(II) in the presence of GGH[6] will degrade DNA randomly in the presence of reducing agents such as sodium ascorbate.

The 55-residue protein was synthesized by stepwise solid-phase methods (0.5 mM scale) with optimized manual protocols[7] on phenylacetamidomethyl (PAM) resin solid support substituted with *tert*-butoxycarbonyl (BOC) asparagin (0.75 g, 0.67 mmol/g) with BOC-protected amino acids to afford 2 g of peptide-resin. Each cycle was monitored by quantitative ninhydrin analysis,[8] and couplings were repeated until the yield was maximal for each step (99.7% early in the synthesis decreasing to 99.0% at the end). After deprotection of 0.1 g of peptide-resin,[9] the crude synthetic protein was purified by reverse phase preparative HPLC to afford 5 mg of synthetic protein, GGH(Hin 139–190).[11]

MPE·Fe(II) footprinting (cleavage protection)[13] reveals that the synthetic GGH(Hin 139–190) at 0.5 μM concentrations (pH 7.5, 25 °C, 20 mM NaCl) binds all four Hin half sites, each 13 base pairs in length (Figures 2 and 3). The 55-residue protein

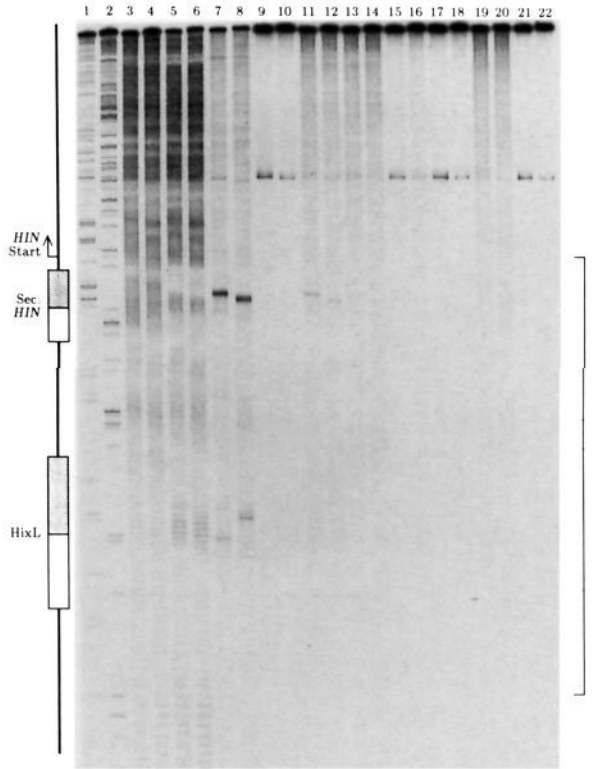

Figure 2. Autoradiogram of high resolution denaturing gel of MPE·Fe footprinting of GGH(HIN 139–190) and Cu·GGH(Hin 139–190) cleavage of a ^{32}P end-labeled fragment (XbaI/*Eco* RI) from pMFB36. Reaction conditions were 20 mM NaCl, 20 mM phosphate, pH 7.5, calf thymus DNA (100 μM base pair), and ≈ 15 000 cpm end-labeled DNA in a total volume of 20 μL. Final concentrations in control and footprinting lanes were MPE·Fe(II) at 10 μM, dithiothreitol at 5 mM, and GGH(Hin 139–190) at 0.5 μM. Final concentrations in control and cleavage lanes were GGH(nHin 139–190) and Cu·GGH(Hin 139–190) at 5 μM; sodium ascorbate and hydrogen peroxide are at 1 mM. Reactions were run at 25 °C for 90 min for Cu·GGH(Hin 139–190) and controls and 12 min for MPE·Fe footprinting and controls, respectively. Cleavage products were analyzed on an 8%, 1:20 crosslinked, 50% urea denaturing polyacrylamide gel, 0.4 mm thick. Odd-numbered lanes and even-numbered lanes are DNA labeled at the 5′ and 3′ end with ^{32}P, respectively. Lanes 1 and 2 are Maxam–Gilbert chemical sequencing G lanes. Lanes 3 and 4 are MPE·Fe controls. Lanes 5 and 6 are MPE·Fe footprints in the presence of GGH(Hin 139–190) (0.5 μM). Lanes 7 and 8 contain $CuCl_2$ (2.5 μM) and GGH(Hin 139–190) (5 μM) followed by sodium ascorbate (1 mM) and hydrogen peroxide (1 mM). Lanes 9 and 10 contain GGH(Hin 139-190) (5 μM). Lanes 11 and 12 and 13 and 14 contain GGH(Hin 139–190) (5 μM), sodium ascorbate (1 mM), and hydrogen peroxide (1 mM) without and with EDTA (1 mM), respectively. Lanes 15 and 16 contain $CuCl_2$ (2.5 μM), GGH(Hin 139–190) (5 μM), and sodium ascorbate (1 mM). Lanes 17 and 18 contain $CuCl_2$ (2.5 μM), GGH(Hin 139–190) (5 μM), and hydrogen peroxide (1 mM). Lanes 19 and 20 contain sodium ascorbate (1 mM) and hydrogen peroxide (1 mM). Lanes 21 and 22 are intact DNA.

alone or in the presence of Cu(II) does not cleave the DNA. However, GGH(Hin 139-190) at 5 μM concentrations (pH 7.5, 25 °C, 20 mM NaCl) in the presence of less than 1 equiv of Cu(II) (2.5 μM),[14] followed by addition of sodium ascorbate (1 mM) and hydrogen peroxide (1 mM), cleaves the DNA predominantly at one of the Hin half sites (Figures 2 and 3). Controls show that *both* sodium ascorbate and hydrogen peroxide are necessary for cleavage.

The major cleavage pattern at the upper half of the secondary Hin site is located toward the center of the 13 bp site and covers

(1) Bruist, M. F.; Horvath, S. J.; Hood, L. E.; Steitz, T. A.; Simon, M. I. *Science* (*Washington, D.C.*) **1987**, *235*, 777.
(2) Sluka, J. P.; Horvath, S. J.; Bruist, M. F.; Simon, M. I.; Dervan, P. B. *Science* (*Washington, D.C.*) **1987**, *238*, 1129.
(3) For attachment of 1,10-phenanthroline–copper complex to lysyl residues of DNA-binding proteins, see: Chen, C.-H.; Sigman, D. S. *Science* (*Washington, D.C.*) **1987**, *237*, 1197.
(4) Lau, J. S.; Kruck, T. P.A.; Sarkar, B. *J. Biol. Chem.* **1973**, *249*, 5878.
(5) Cameraman, N.; Camerman, A.; Sarkar, B. *Can J. Chem.* **1976**, *54*, 1309.
(6) Chiou, S.-H. *J. Biochem.* **1983**, *94*, 1259.
(7) (a) Kent, S. B. H. *Ann. Rev. Biochem.* **1987**, *57*, 957. (b) Kent, S. B. H.; Martin Selk, L.; Parker, K. F. *Int. J. Peptide Prot. Res.*, in press. We are grateful to Dr. S. B. Kent and K. Parker for generous guidance and advice with these protocols.
(8) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147.
(9) The dinitrophenyl (DNP)-protecting group on histidine was removed with 20% 2-mercaptoethanol and 10% diisopropylethylamine in dimethylformamide. The terminal BOC group was then removed as usual. Removal of the peptide from the resin as well as removal of all other side-chain protecting groups was accomplished by standard HF cleavage.[10] The residual DNP groups were removed from the lyopholized peptide by using 20% 2-mercaptoethanol in 4 M guanidine·HCl/50 mM tris pH 8.5 at 50 °C.[7a] This solution was filtered and purified by means of a C_4 reverse phase preparative HPLC column. A gradient of 0–60% acetonitrile in H_2O/0.1% trifluoroacetic acid over 240 min was used. Fractions collected were checked by analytical HPLC, and those containing the correct peak were combined and lyopholized to give the final product.[11]
(10) Stewart, J. M.; Young, J. D. *Solid-Phase Peptide Synthesis*; Pierce Chemical Co.: Rockford, IL, 1984; pp 85–89.
(11) Peptide sequence analysis by Edmann degradation confirms the identity of the purified synthetic protein.[12]
(12) We thank David Teplow and Haul Wong, Division of Biology, California Institute of Technology, for their generous assistance.
(13) Dervan, P. B. *Science* (*Washington, D.C.*) **1986**, *232*, 464.
(14) Typically the synthetic protein is combined with less than 1 equiv of Cu(II) and then added to the DNA. We find that even traces of unbound Cu(II) in the presence of ascrobate (1 mM) and hydrogen peroxide (1 mM) randomly degrades DNA.

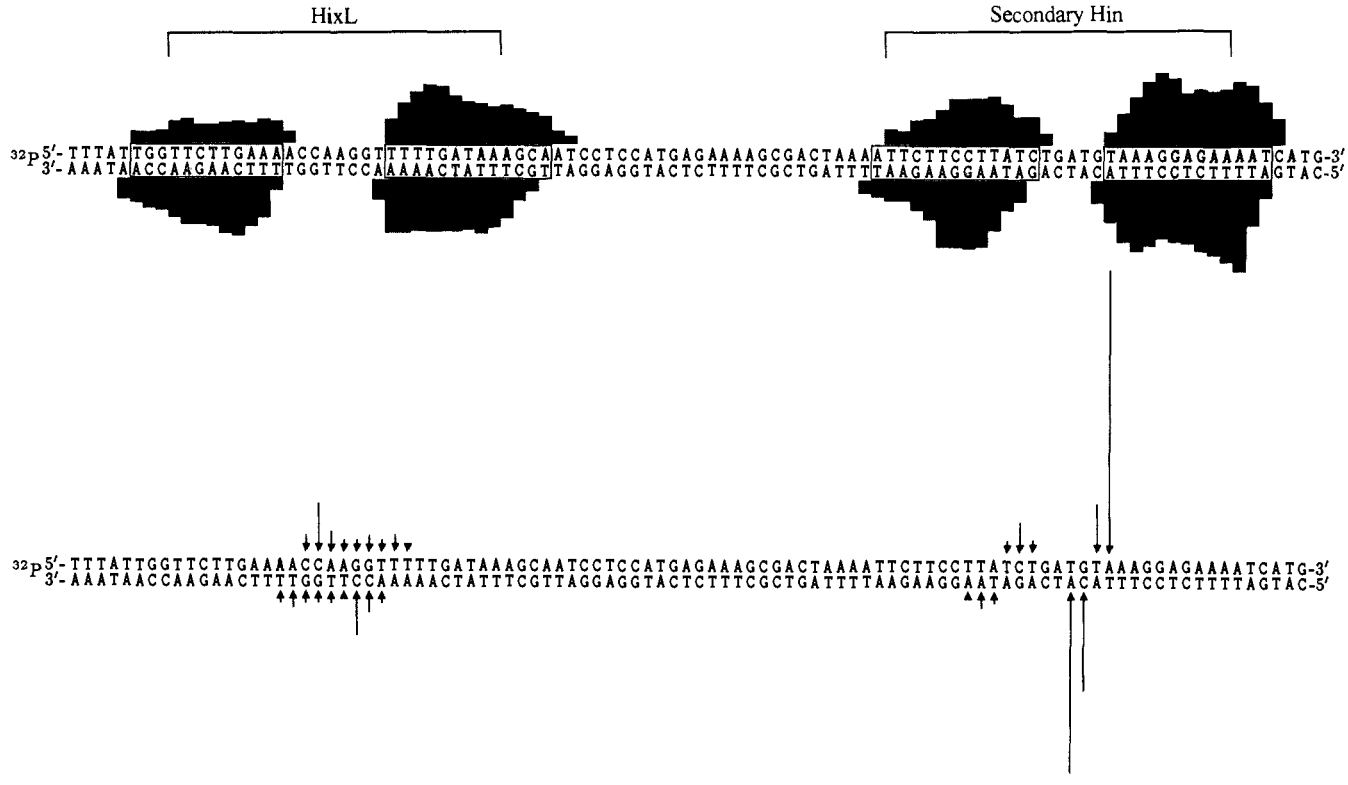

Figure 3. The sequence left to right represents the cleavage data from the bottom to the middle of the gel (bracket on the right) in Figure 2. Boxes are the half sites of the Hix L and secondary Hin binding site. Solid bars are histograms representing extent of protection from MPE·Fe cleavage in the presence of GGH(Hin 139–190) (0.5 μM) (Figure 2, lanes 5 and 6). Arrows represent extent of cleavage for Cu·GGH(Hin 139–190) at 5 μM in the presence of sodium ascorbate and hydrogen peroxide (Figure 2, lanes 7 and 8). Extent of cleavage was determined by densitometric analysis of the gel autoradiogram.

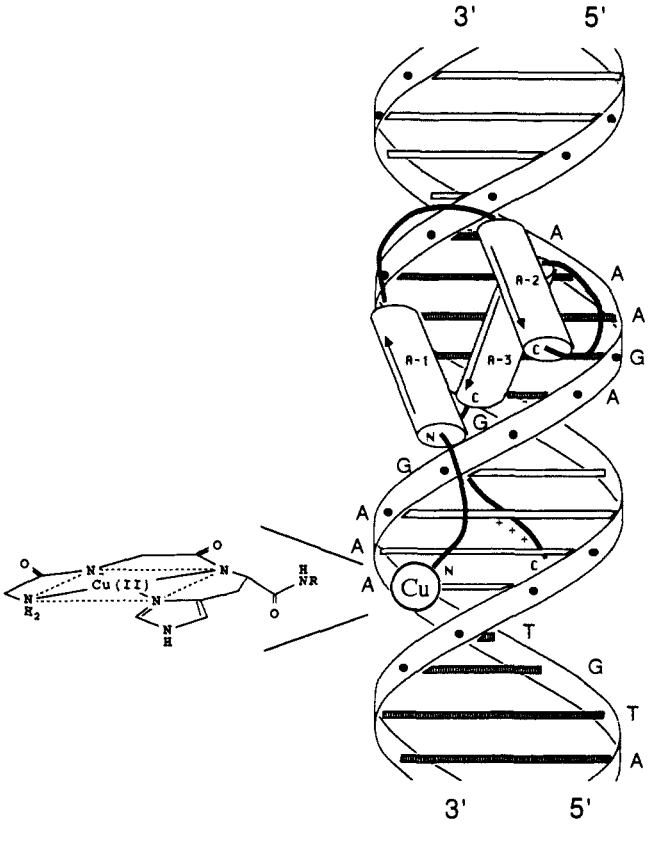

Figure 4. Schematic representation of a model for designed metalloprotein Cu·GGH(Hin 139–190) binding to one Hin half-site. Putative α helices are shown as cylinders with an arrow pointing from the NH_2 to COOH terminus. See ref 2 (Figure 6) and references cited therein for more details.

two base pairs with maximal cleavage asymmetric to the 3′ side. This is consistent with the Cu·GGH complex at the amino terminus of the protein being located toward the symmetry axis of the Hin site in the minor groove (Figure 4). The termini at the cleavage site are 3′ and 5′ phosphate indicating that the cleavage reaction is oxidative degradation. The range of the cleavage pattern (2 bp) is shorter than that usually seen with the diffusible oxidant generated by EDTA·Fe(II) (typically 5 bp).[13] Although we will defer a detailed mechanistic analysis until more data is available, one interpretation of the restricted range of oxidative cleavage at the major site is that a nondiffusible oxidant is generated by the Cu·GGH complex in the minor groove.[15]

The cleavage pattern at the upper half of the secondary Hin site is strong and specific, while those at the other half-sites are weak and diffuse. Because the footprinting data suggest that all four half-sites are occupied, one explanation for the difference in cleavage patterns is that the structure of the DNA is different at each half-site[17] and the oxidative pathway is sensitive to these differences. Sequence-dependent structures at the two Hin sites might afford different accessibility of the Cu·GGH complex for oxidation of the deoxyribose ring in the minor groove at each half site.

Acknowledgment. We are grateful for generous grant support from the DARPA University Research Initiative Program, an unrestricted research grant from Merck Sharp & Dohme Research Laboratories, and National Research Service Awards form the General Medical Sciences (D.P.M., B.L.I.).

(15) We cannot distinguish at this time whether the nondiffusible oxidant generated by the Cu·GGH complex is a metal-bound oxygen or a peptide ligand-radical species.[16]

(16) Margerum, D. W.; Chellappa, K. L.; Bossu, F. P.; Burce, G. L. *J. Am. Chem. Soc.* **1975**, *97*, 6894 and references cited therein.

(17) HixL and the secondary Hin sites have different biological roles. HixL is a recombination site while secondary Hin is a binding site whose function is less well understood.[1]