Inorg. Chem. **2006**, *45*, 490−492

Recognition of Secondary Structures in Proteins by a Diiron(III) Complex via a Hydrolytic Pathway

Qunhui Pan,[†] Wei Jiang,[†] Zhanru Liao,[‡] Tianle Zhang,[†] and Changlin Liu*[,†]

Department of Chemistry, Huazhong University of Science and Technology, Wuhan 430074, Hubei, China, and Department of Chemistry, Huazhong Normal University, Wuhan 430079, Hubei, China

Received May 4, 2005

The diiron(III) complex $Fe_2(DTPB)(\mu_2\text{-}O)(\mu_2\text{-}OAc)Cl(BF_4)_2$ [DTPB = 1,1,4,7,7-pentakis(2′-benzimidazol-2-yl-methyl)triazaheptane, OAc = acetate] exhibits a similar affinity for proteins belonging to different structural patterns. However, this diiron complex is sensitive to secondary structures in a protein when it is used to promote the protein hydrolysis, indicating that some metal complexes, such as artificial proteolytic agents, could become a new hydrolytic probe of protein structures.

The limited native proteolysis has widely been accepted as a probe for protein structures, folding and refolding.[1] However, often the hydrolytic reactions take place under denaturing conditions, so that every peptide bond is cut where the local amino acid sequence satisfies the specificity requirement of the protease in question. The denaturation steps preclude any information being obtained on the three-dimensional structures of proteins.[1] Therefore, more choices in the proteomic times are obviously desirable. Recently, there has been considerable interest in the protein hydrolysis promoted by metal complexes bound at particular sites.[2,3] Protein hydrolysis catalyzed by the metal complexes tested up to date only shows a residue specificity to a certain degree.[2,3] On the other hand, artificial proteolytic reagents, which are directed by structure and proximity rather than by residue types, represent a new approach that could be employed to obtain secondary and tertiary structural information, to assess conformational changes in a protein, and to determine membrane protein topology. These reagents could also become new and targeted therapeutic agents for diseases linked to protein folding.[4]

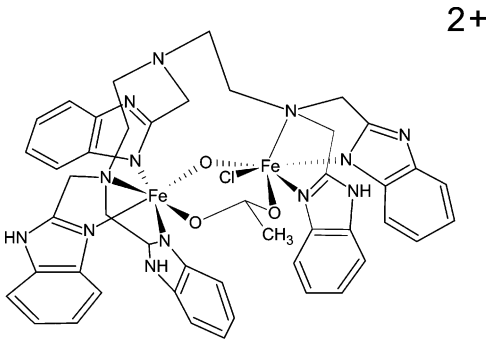

Figure 1. Diiron(III) complex $[Fe_2(DTPB)(\mu_2\text{-}O)(\mu_2\text{-}OAc)Cl]^{2+}$ (Fe_2).

We have reported that the diiron(III) complex $Fe_2(DTPB)(\mu_2\text{-}O)(\mu_2\text{-}OAc)Cl(BF_4)_2$ [Fe_2; DTPB = 1,1,4,7,7-pentakis(2′-benzimidazol-2-yl-methyl)triazaheptane, OAc = acetate; Figure 1] effectively promotes DNA and adenosine triphosphate hydrolysis.[5] It is possible that the diiron complex is also a potent promoter for protein degradation via a hydrolytic pathway because (1) the labile ligand Cl^- is easily replaced by other ligands and (2) the OH^- produced by the conversion of $Fe_2(\mu_2\text{-}O_2^-) \rightarrow Fe_2(OH^-)$ in an acidic medium is an effective nucleophile.[5] On the other hand, there might be significant differences in the rate and efficiency of the Fe_2-mediated hydrolytic reactions of proteins belonging to different structural patterns. In this work, the recognition of secondary structures in proteins by this diiron complex via a hydrolytic pathway was examined. Five proteins, hemoglobin (Hb), bovine serum albumin (BSA), lysozyme (Lyso), RNase, and Cu,Zn superoxide dismutase (SOD), were chosen with different contents of secondary structures, and the changes in their hydrolytic efficiency were observed.

Binding interactions between Fe_2 and a protein are essential to the Fe_2-mediated protein degradation and were first probed by the absorption titration of Fe_2 with each protein. The absorption spectra of Fe_2 in the presence of

* To whom correspondence should be addressed. E-mail: liuchl@mail.hust.edu.cn.
[†] Huazhong University of Science and Technology.
[‡] Huazhong Normal University.

(1) Hubbard, S. J. *Biochim. Biophys. Acta* **1998**, *1382*, 191−206.
(2) Milovic, N. M.; Badjic, J. D.; Kostic, N. M. *J. Am. Chem. Soc.* **2004**, *126*, 696−697. Milovic, N. M.; Dutca, L. M.; Kostic, N. M. *Chem.−Eur. J.* **2003**, *9*, 5097−5106. Milovic, N. M.; Kostic, N. M. *J. Am. Chem. Soc.* **2003**, *125*, 781−788. Milovic, N. M.; Kostic, N. M. *J. Am. Chem. Soc.* **2002**, *124*, 4759−4767.
(3) Hegg, E. L.; Burstyn, J. N. *J. Am. Chem. Soc.* **1995**, *117*, 7015−7016.
(4) Liu, R.; McAllister, C.; Lyubchenko, Y.; Sierks, M. R. *Biochemistry* **2004**, *43*, 9999−10007.
(5) Liu, C.; Yu, S.; Li, D.; Liao, Z.; Sun, X.; Xiu, H. *Inorg. Chem.* **2002**, *41*, 913−922. Liu, C.; Wang, M.; Zhang, T.; Sun, H. *Coord. Chem. Rev.* **2004**, *248*, 147−168.

10.1021/ic050695a CCC: $33.50
Published on Web 12/17/2005

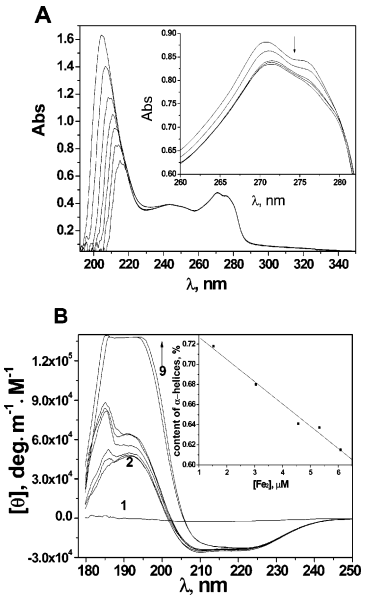

Figure 2. (A) UV−visible absorption titration of 19.4 μM Fe_2 with increasing BSA concentration (0−4.8 μM, from top to bottom, reaction for 1 min at 25 °C in a 20 mM HAc−NaAc buffer, pH 5.6, prior to measurement). The controls were the corresponding BSA solutions. (B) CD spectra of native BSA (0.25 μM) treated using Fe_2 of increasing concentration (0−9.1 μM, from bottom to top; reaction for 2 min at 25 °C prior to measurement). The curves 1 and 2−9 represent the CD spectra of Fe_2 and of BSA in the presence of Fe_2, respectively. The inset shows that the α-helical content in the BSA structure is linearly reduced with increasing Fe_2 concentration.

increasing concentrations of BSA are shown in Figure 2A. The absorption in the region of 227−272 nm, caused by benzimidazolyl groups in Fe_2,[5] was slightly reduced in acidic buffer as the concentration of BSA was raised. However, the band at 211 nm that stems from the $Cl^- \rightarrow Fe^{3+}$ charge transfer was dramatically decreased with increasing BSA concentration, indicating that the labile ligand Cl^- in Fe_2 is displaced by a protein ligand. The clear isosbestic point at 282 nm also shows that binding interactions occur between Fe_2 and BSA. The absorption in the region of 330−450 nm caused by μ_2-$O^{2-} \rightarrow Fe^{3+}$ charge transfer was not observed, suggesting that μ_2-O^{2-} might be converted into OH^- prior to the addition of BSA, which remains to be further confirmed. Similar results were obtained with other proteins. The apparent binding constants of Fe_2 to each protein obtained by fitting the absorbance values at 211 nm to the previously proposed model[6] are 3.5×10^5 M^{-1} for BSA, 5.9×10^4 M^{-1} for Hb, 1.4×10^5 M^{-1} for Lyso, 6.2×10^4 M^{-1} for RNase, and 5.1×10^4 M^{-1} for SOD. These data indicate that the affinities of Fe_2 for the proteins are almost equally strong and not parallel to their α-helical contents.

To understand the influence of Fe_2 binding on a protein structure, circular dichroism (CD) spectra of each protein and their complex with Fe_2 were measured. The CD spectra of BSA in the presence of Fe_2 (Figure 2B) show that a new positive CD band at 186 nm was progressively increased as the concentration of Fe_2 was raised, indicating that a new chiral site appears on the protein because of the interactions between Fe_2 and the protein. The quantitative analysis of the CD signal at 208 nm, which reflects the degree of α-helical character in a protein, indicates that the presence of Fe_2 at low concentrations (0−6 μM) leads to a linear reduction in the α-helical content in the BSA (see the inset in Figure 2B), suggesting that the binding of Fe_2 to a protein may break parts of the α-helical structures in the protein. In addition, the discontinuous jump in the CD bands suggests that the presence of Fe_2 at higher concentrations (>6 μM) may lead to the BSA unfolding.

The degradation of five native proteins mediated by Fe_2 was examined in a 20 mM HAc−NaAc buffer, pH 5.6, under both aerobic and anaerobic conditions. The sodium dodecyl sulfate−poly(acrylamide) gel electrophoresis (SDS−PAGE) of the degradation products shows that, for the cases of Hb, BSA, and SOD, the progressive disappearance of the intact proteins was observed as the concentration of Fe_2 was raised. The fact that large and separated polypeptide fragments were not observed indicates that the degradation reaction is not sensitive to residue types in the proteins. From the Fe_2 concentration dependences of degradation reactions of the proteins (Figure 3A), any parallel relationship was not found between their degradation yields and their contents of secondary structures (see Figure 4). Similar results were obtained under the anaerobic conditions (see Figure S1 in the Supporting Information), indicating that the degradation reactions of the proteins occur via a hydrolytic pathway.

We also observed the degradation reactions of the partly denatured form of five proteins, produced by 1.9 M guanidinium chloride and 0.54% 2-mercaptoethanol,[7] in order to examine the influence of protein unfolding on the Fe_2-mediated degradation reactions. Notably, the concentrations of proteins and Fe_2 used here are 10- and 4-fold, respectively, of those for the natural proteins to obtain a clear gel picture. Figure 3B displays the SDS−PAGE results for the degradation products of partly denatured Hb (143 μM). The partly denatured form is more easily degraded because of the formation of relaxed structures as compared to the native form. Figure 3C shows the Fe_2 concentration dependences of degradation of five partly denatured proteins under the aerobic conditions. The same is true under the anaerobic conditions. The quantitative analysis shows that their highest hydrolytic yield is parallel with the contents of secondary structures in the proteins. Alternatively, the highest hydrolytic yield is reduced in the order of Hb (780 μM) > BSA (120 μM) > Lyso (780 μM) > RNase (250 μM) > SOD (380 μM) (see Figure 4) as the content of α-helices decreases, or the content of β-sheets or coiled structures increases, if the largest concentrations of Fe_2 required for the highest yield of each protein, displayed in brackets, is left out of account. Here, it must be emphasized that the largest concentrations of Fe_2 are those that do not lead to any protein precipitate. The results reveal that the Fe_2-mediated hydrolysis of the partly denatured proteins is sensitive to the secondary

(6) Pyle, A. M.; Rehmann, J. P.; Meshoyrer, R.; Kumar, C. V.; Turro, N. J.; Barton, J. K. *J. Am. Chem. Soc.* **1989**, *111*, 3053−3058. Shrivastava, H. Y.; Kanthimathi, M.; Nair, B. U. *Biochim. Biophys. Acta* **2002**, *1573*, 149−155.

(7) Carlsson, U.; Aasa, R. L.; Henderson, E.; Jonsson, B. H.; Lindskog, S. *Eur. J. Biochem.* **1975**, *52*, 25−36. Porter, E. A.; Weisblum, B.; Gellman, S. H. *J. Am. Chem. Soc.* **2002**, *124*, 7324−7330.

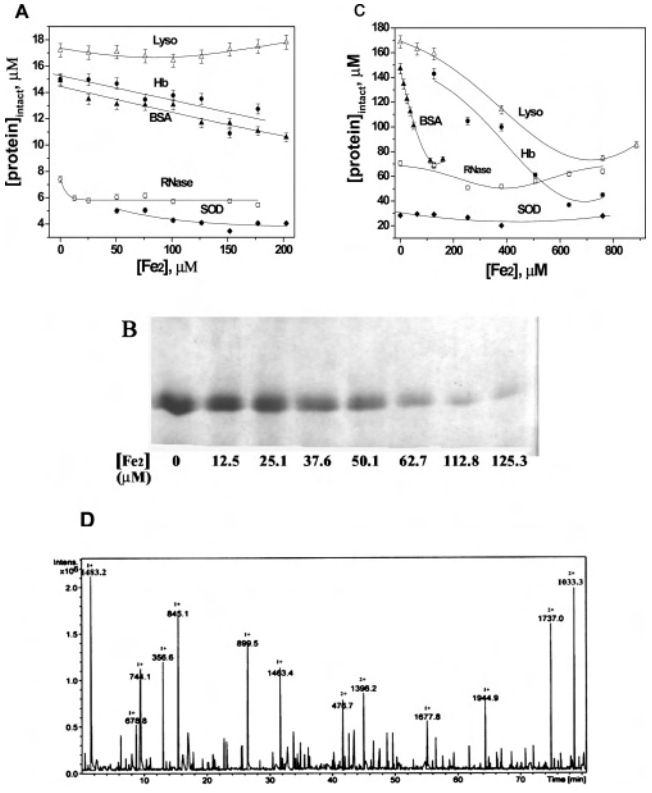

Figure 3. (A) Fe_2 concentration dependences of hydrolytic reactions of native Hb (15 μM, ●), BSA (15 μM, 7), Lyso (17 μM, △), RNase (7 μM, ○), and SOD (6 μM, ◆). Each native protein was incubated for 48 h at 50 °C with a given amount of Fe_2 in a 20 mM HAc−NaAc buffer, pH 5.6, prior to electrophoresis. (B) SDS−PAGE results of hydrolytic products of 147 μM partly denatured BSA treated by 0−125.3 μM Fe_2 under the experimental conditions. (C) Fe_2 concentration dependences of hydrolytic reactions of partly denatured Hb (143 μM, ●), BSA (147 μM, 7), Lyso (169 μM, △), RNase (71 μM, ○), and SOD (28 μM, ◆). Each partly denatured protein was incubated with Fe_2 for 24 h at 50 °C in the buffer prior to electrophoresis. Here, $[protein]_{intact}$ represents the concentration of each intact protein. (D) LC-ESIMS results for the products of 1 μM Hb treated for 36 h at 50 °C by 15 μM Fe_2 in the buffer.

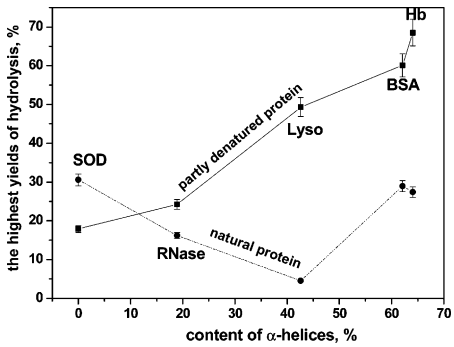

Figure 4. Plots of the highest hydrolytic yields by a given amount of Fe_2 in a given period for both the native and partly denatured forms of five proteins against their α-helical contents. The α-helical contents in the proteins were estimated by using the data in PDB (PDB ID: Hb, 1GO9; BSA, 1AO6; Lyso, 1AKI; RNase, 1RCA; SOD, 1E9Q).

structures in the proteins and the hydrolytic yields are higher than those of the native proteins. For the case of BSA, the ratio of Fe_2 to the protein required for the hydrolytic yield of 30% is 14.4 for the native form, whereas the ratio is reduced to 0.32 for the partly unfolding form. The reason may be that the presence of a denaturant leads to the conversion of proteins from the compacted state to the relaxed state, which is in favor of the nucleophilic attack of OH^-. In addition, as described for the case of native forms, the sensitivity of hydrolysis to residue types was also not observed here because the large and separated protein fragments were not found.

To verify that the Fe_2-mediated protein hydrolysis is nonspecific to residue types, the products of the hydrolytic reactions were assayed by liquid chromatography−electrospray ionization mass spectroscopy (LC−ESIMS). The data (Figure 3D) indicate that a pool of oligopeptides of different lengths generated by the degradation reaction of native Hb have molecular weights of less than 2000 Da except for a fragment of 3474 Da. This result confirms that Hb was completely broken into oligopeptides by Fe_2 and suggests that these small oligopeptides might run beyond SDS−PAGE gels and become invisible.

For the case of partly denatured proteins, the plots of the highest hydrolytic yields by a given amount of Fe_2 in a given period against the contents of their α-helical, β-sheet, and coiled structures are shown in Figures 4 and S2 in the Supporting Information. In contrast to the case of native proteins, here, an observed trend is that the highest hydrolytic yields of five partly denatured proteins are dependent on their α-helical contents; i.e., the degradation reactions are sensitive to the α-helices in the partly denatured form. Moreover, the highest yields are reduced in an exponential fashion as the content of β-sheet or coiled structures rises (Figure S2A,B in the Supporting Information). The following suggestions are proposed to explain the results. First, the hydrolytic yields may be dependent on the higher structures of a protein but not associated with both residue types and sequences because the Fe_2-mediated hydrolytic reactions are nonspecific to residue types. Then, the disruption of a few of the hydrogen bonds in an α-helix can result in the relaxation of the α-helix because the hydrogen bonds in an α-helix are a cooperative and intrachain interaction. Obviously, the extended peptide stretches facilitate the nucleophilic attack of OH^- on the scissile peptide bonds. On the other hand, the disruption of a few of the hydrogen bonds in a β-sheet motif does not affect the integrity of the hydrogen-bond network in the β-sheet motif because the hydrogen bonds in a β-sheet motif are an interchain interaction. Therefore, α-helices in a protein are hydrolyzed more easily than are β-sheets.

The affinities of Fe_2 for the five native proteins that were tested show that the binding of Fe_2 to a protein is not selective to its secondary structures, but Fe_2 preferentially hydrolyzes its α-helices. This suggests that all of the secondary structural elements in a protein may be hydrolyzed, but there are significant differences in their hydrolytic rate and efficiency by a given amount of Fe_2 in a given period. Therefore, the protein hydrolytic reactions by Fe_2 are kinetically controlled.

Acknowledgment. This work was supported by NSFC (Grant 20571028).

Supporting Information Available: Experimental procedures and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

IC050695A