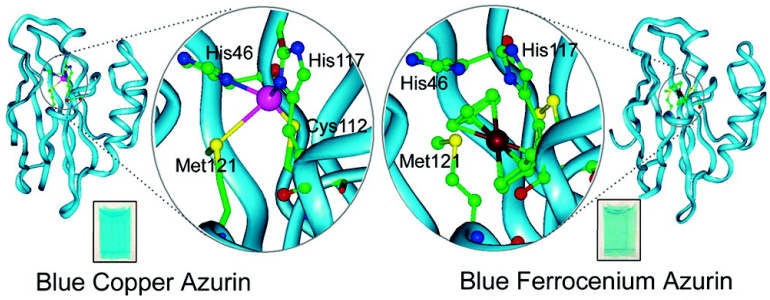
Communication

Blue Ferrocenium Azurin: An Organometalloprotein with Tunable Redox Properties

Hee Jung Hwang, James R. Carey, Evan T. Brower, Alan J. Gengenbach, Joseph A. Abramite, and Yi Lu

J. Am. Chem. Soc., **2005**, 127 (44), 15356-15357• DOI: 10.1021/ja054983h • Publication Date (Web): 19 October 2005



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 7 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 10/19/2005

Blue Ferrocenium Azurin: An Organometalloprotein with Tunable Redox Properties

Hee Jung Hwang, James R. Carey, Evan T. Brower, Alan J. Gengenbach, Joseph A. Abramite, and Yi Lu*

Department of Chemistry, University of Illinois at Urbana−Champaign, Urbana, Illinois 61801

Received July 25, 2005; E-mail: yi-lu@uiuc.edu

Organometallic compounds are known to be very efficient in redox and catalytic reactions. It is thus surprising that only a limited number of organometallic compounds are found in proteins.[1] While more organometalloproteins may be discovered in nature, specific incorporation of organometallic compounds into proteins may allow us to learn guiding principles for designing organometalloproteins and to prepare novel proteins with functional properties that combine benefits of both organometallic compounds (such as new and efficient catalytic activities) and proteins (such as water solubility and high regio- and enantioselectivity).[2] It will make an important contribution to the emerging area of bioorganometallic chemistry.[3] We report here the design and covalent attachment of ferrocene (bis(η^5-cyclopentadienyl) iron(II), Fc) to the active site of azurin (Az) free of copper (called FcAz) and control of its redox properties by pH and residues around the secondary coordination sphere. Increasing water solubility of Fc, stabilizing ferrocenium species (Fc^+), and use of such a redox active protein in oxidizing cytochrome *c* (cyt *c*) are also established.

Fc is an attractive organometallic cofactor to be designed into a protein for creating redox-active enzymes. It is one of the most widely used redox agents in chemistry,[4] such as in protein ET studies[5] and sensing applications,[6] because Fc has a very low inner-sphere reorganization energy in electron transfer (ET) (0.3 kcal/mol).[7] It therefore has been conjugated to many different biomolecules including nucleic acids and proteins.[8] Despite these advantages, application of the redox couple in aqueous solution is limited by the low solubility of most Fc and the instability of the Fc^+, as Fc^+ undergoes rapid decomposition in neutral or basic aqueous solutions.[9] Encapsulation of Fc inside proteins can overcome both limitations as proteins are generally hydrophobic inside while hydrophilic on the surface and thus can increase Fc solubility in water. It may also stabilize the Fc^+ by protecting it from the solvent. In addition, the reduction potential (E_m) of Fc can be fine-tuned by variations in the protein pocket.

Az is well-suited to provide the protein framework for the redox-active artificial metalloenzyme with non-native metal cofactors. It is one of the most common ET proteins in biology,[10] and its electrochemical properties and ET kinetics have been thoroughly studied.[11] Apo-Az is well-folded without copper, as shown in the X-ray crystal structure.[12a] Therefore, it is an ideal host for Fc.

We choose to covalently attach Fc through the highly conserved cysteine in apo-Az because anchoring the redox center can minimize fluxional movement inside the protein. Cysteine is not only a well-defined bioconjugation point,[2b,13] but Cys112 is also a key entry or exit point for ET in Az.[11c] Figure 1 shows an X-ray crystal structure of blue copper Az[12b] and a computer model structure of Fc covalently attached to the apo-Az through Cys112.[14] The model suggests that Fc can be incorporated into the blue copper site with minimal structural perturbation.

2-[(Methylsulfonyl)thio]ethylferrocene (**1**) was designed and synthesized[15] for selective attachment to a Cys residue. It is minimally soluble in water, and no discernible absorption band could be observed in saturated aqueous solution (Figure 2A). In addition, the **1**/**1**$^+$ redox couple has very limited stability in aqueous buffers. Cyclic voltammograms (CV) of this compound were obtained in aqueous buffer of various pH (Figure S1). The cathodic-to-anodic peak current ratio (i_{pc}/i_{pa}) is 0.41 at pH 4 and further decreases to 0.22 at pH 7, indicating rapid decomposition of oxidized species (Figure 2B). In addition, the E_m of **1** showed little dependence on pH (Figure S1). In contrast, addition of this compound into Az in aqueous buffer resulted in a new species with a stronger absorption band around 400−500 nm (Figure 2A). This spectrum in water is similar to that of Fc in an organic solvent, suggesting that incorporation of Fc inside Az can help increase the compound's solubility in water.[16] Covalent attachment of Fc inside Az was confirmed by electrospray mass spectrometry; a single peak corresponding to FcAz was observed (calculated 14187.72 Da, observed 14188.28 ± 1.19 Da) (Figure S2).[17] The FcAz was electrochemically oxidized to Fc^+Az and displayed a blue color with an absorption band at 632 nm (Figure 2A).[15] This spectrum is similar to that of Fc^+ in aqueous solution.[18]

After demonstrating the increased solubility of the Fc, we investigated the use of the protein environment to stabilize the Fc^+ in aqueous solution and fine-tune its E_m. The E_m of FcAz increased significantly when covalently attached to the protein; **1** has an E_m value of 402 mV (versus NHE) in pH 4 aqueous solution, while FcAz has an E_m of 579 mV at the same pH (Figure 2C).[15] This 177 mV increase is consistent with the Fc being encapsulated inside the hydrophobic protein matrix. Similar increases, although to a lesser extent, in the E_m of Fc were reported in β-cyclodextrin encapsulated Fc (∼80 mV)[19] and dendrimers containing Fc in the core (∼100 mV).[20] In addition, increasing the pH from 4 to 9 resulted in more than 80 mV decrease in E_m (from 579 to 495 mV) (Figure 2C). This decrease is probably due to deprotonation of amino acid residues around the Fc at high pH, which creates a more negatively charged environment that can stabilize the Fc^+. In contrast to **1**, FcAz exhibits reversible CV with i_{pc}/i_{pa} close to unity at all pH values (Figures 2B and S3), indicating increased stability of the ferrocenium ion in the protein pocket. Furthermore, replacing Met121, an amino acid residue in close proximity to Fc (Figure 1), with a positively charged Arg or negatively charged Glu resulted in the predicted increase and decrease in E_m at all pH values (wt 520 ± 5, Arg 543 ± 4, Glu 508 ± 4 mV at pH 7, Figure 2C). Finally, substitution of Met121 with a more hydrophobic Leu raised the Fc potential (537 ± 3 mV at pH 7, Figure 2C), consistent with destabilization of a positively charged Fc^+ by a hydrophobic environment. Compared to the Cu(I)/Cu(II) azurin, where large variation in the E_m was observed (e.g., wt 304, Met121Glu 220, Met121Leu 390 mV),[21] smaller changes observed in FcAz reflect

 10.1021/ja054983h CCC: $30.25

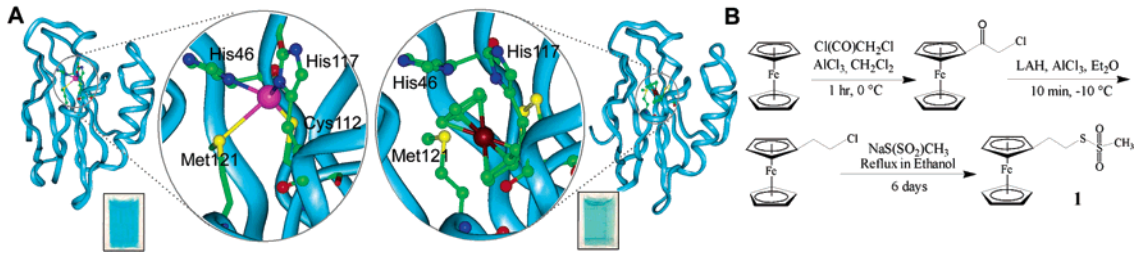


Figure 1. (A) Crystal structure of blue copper azurin (4AZU, left panel) and computer model structure of azurin with covalently attached **1** (FcAz, right panel). Apo-azurin structure (1E65) was used as the template for the FcAz model. The figure was generated using INSIGHT II (Accelrys, San Diego). The insets show photographs of blue copper azurin and blue ferrocenium azurin solutions. (B) Synthetic scheme of **1**.

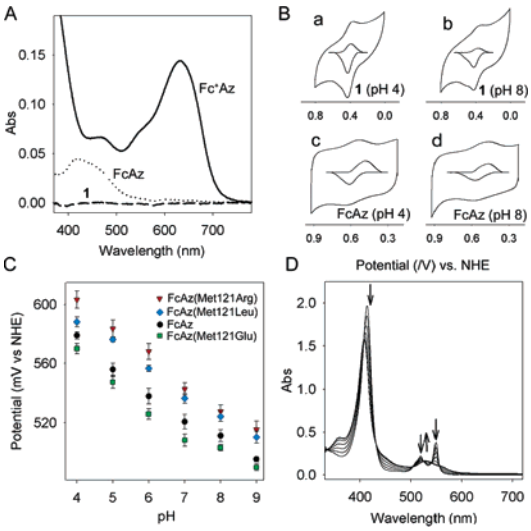


Figure 2. (A) UV−vis spectra of oxidized Fc^+Az (solid line), reduced FcAz (dotted line), and **1** (dashed line) in 50 mM ammonium acetate at pH 4. (B) CV of saturated aqueous solution of **1** at pH 4 (a) and 8 (b), and of FcAz at pH 4 (c) and 8 (d). Shown inside the CV is background-corrected peaks.[22] (C) Reduction potential of FcAz, FcAz (Met121Glu), FcAz (Met121Arg), and FcAz (Met121Leu) at various pH. (D) Oxidation of ferro-cyt *c* by Fc^+Az: 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 equiv of Fc^+Az was added to 14 μM of ferro-cyt *c* in 50 mM ammonium acetate, pH 4.

the weaker interaction of metal ion and the amino acid residue in the second coordination sphere.

With the exception of a few redox couples, such as ferri/ferro-cyanides, few metal-containing redox couples are stable in both oxidation states in water, and therefore, the choice of redox agents in biology is limited. We therefore sought to demonstrate the use of FcAz as a redox agent for biomolecules. Figure 2D displays the oxidation of horse heart ferro-cyt *c* using Fc^+Az as the oxidant. The UV−vis spectrum of ferro-cyt *c* has characteristic absorption bands at 414, 520, and 550 nm. Upon addition of up to 1 equiv of Fc^+Az, the peak intensities at 520 and 550 nm decreased and the Soret band shifted to 409 nm, which are typical of ferri-cyt *c*. This result suggests that the Fc^+Az can be used as a redox active agent in biology. With the tune-ability accomplished by substitution of either the cyclopentadienyl ring or second coordination sphere of Fc provided by protein cavity, and the remarkable efficiency in ET due to small reorganization energy, the artificial organometalloprotein FcAz could find potential applications in many biological ET studies and in the design of new biosensors.

Acknowledgment. We thank Drs. T. B. Rauchfuss and T. D. P. Stack for helpful discussions, Drs. F. A. Armstrong, H. A. Heering, and C. Léger for providing a CV baseline correction program, and Ms. M. Lee for help with protein preparations. This work is supported by NSF (CHE-0139203) and NIH (GM62211).

Supporting Information Available: Detailed experimental procedures, ESI-MS of FcAz, CV of **1** and FcAz (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Lippard, S. J.; Berg, J. M. *Principles of Bioinorganic Chemistry*; University Science Books: Mill Valley, CA, 1994. (b) Bertini, I.; Gray, H. B.; Lippard, S. J.; Valentine, J. S. *Bioinorganic Chemistry*; University Science Books: Sausalito, CA, 1994. (c) Holm, R. H.; Kennepohl, P.; Solomon, E. I. *Chem. Rev.* **1996**, *96*, 2239−2314 and references therein.
(2) (a) Lu, Y.; Berry, S. M.; Pfister, T. D. *Chem. Rev.* **2001**, *101*, 3047−3080. (b) Lu, Y. *Curr. Opin. Chem. Biol.* **2005**, *9*, 118−126.
(3) (a) Fish, R. H.; Jaouen, G. *Organometallics* **2003**, *22*, 2166−2177. (b) Collot, J.; Humbert, N.; Skander, M.; Klein, G.; Ward, T. R. *J. Organomet. Chem.* **2004**, *689*, 4868−4871.
(4) Togni, A.; Hayashi, T. *Ferrocenes: Homogeneous Catalysis, Organic Synthesis, Materials Science*; Wiley-VCH: Weinheim, Germany, 1995.
(5) Carney, M. J.; Lesniak, J. S.; Likar, M. D.; Pladziewicz, J. R. *J. Am. Chem. Soc.* **1984**, *106*, 2565−2569.
(6) (a) Badia, A.; Carlini, R.; Fernandez, A.; Battaglini, F.; Mikkelsen, S. R.; English, A. M. *J. Am. Chem. Soc.* **1993**, *115*, 7053−7060. (b) Riklin, A.; Katz, E.; Willner, I.; Stocker, A.; Bueckmann, A. F. *Nature (London)* **1995**, *376*, 672−675.
(7) Nielson, R. M.; Golovin, M. N.; McManis, G. E.; Weaver, M. J. *J. Am. Chem. Soc.* **1988**, *110*, 1745−1749.
(8) (a) van Staveren, D. R.; Metzler-Nolte, N. *Chem. Rev.* **2004**, *104*, 5931−5985. (b) Di Gleria, K.; Nickerson, D. P.; Hill, H. A. O.; Wong, L.-L.; Fueloep, V. *J. Am. Chem. Soc.* **1998**, *120*, 46−52.
(9) Holecek, J.; Handlir, K.; Pavlik, I. *Collect. Czech. Chem. Commun.* **1972**, *37*, 1805−1815.
(10) (a) Gray, H. B.; Malmström, B. G.; Williams, R. J. P. *J. Biol. Inorg. Chem.* **2000**, *5*, 551−559. (b) Vila, A. J.; Fernández, C. O. In *Handbook on Metalloproteins*; Bertini, I., Sigel, A., Sigel, H., Eds.; Marcel Dekker: New York, 2001; pp 813−856. (c) Solomon, E. I.; Randall, D. W.; Glaser, T. *Coord. Chem. Rev.* **2000**, *200*−*202*, 595−632.
(11) (a) Goldberg, M.; Pecht, I. *Biochemistry* **1976**, *15*, 4197−4208. (b) Wittung-Stafshede, P.; Hill, M. G.; Gomez, E.; Di Bilio, A. J.; Karlsson, B. G.; Leckner, J.; Winkler, J. R.; Gray, H. B.; Malmström, B. G. *J. Biol. Inorg. Chem.* **1998**, *3*, 367−370. (c) Farver, O.; Jeuken, L. J. C.; Canters, G. W.; Pecht, I. *Eur. J. Biochem.* **2000**, *267*, 3123−3129. (d) Jeuken, L. J. C.; Armstrong, F. A. *J. Phys. Chem. B* **2001**, *105*, 5271−5282. (e) Fujita, K.; Nakamura, N.; Ohno, H.; Leigh, B. S.; Niki, K.; Gray, H. B.; Richards, J. H. *J. Am. Chem. Soc.* **2004**, *126*, 13954−13961.
(12) (a) Nar, H.; Messerschmidt, A.; Huber, R.; Van de Kamp, M.; Canters, G. W. *FEBS Lett.* **1992**, *306*, 119−124. (b) Nar, H.; Messerschmidt, A.; Huber, R.; van der Kamp, M.; Canters, G. W. *J. Mol. Biol.* **1991**, *221*, 765−772.
(13) Carey, J. R.; Ma, S. K.; Pfister, T. D.; Garner, D. K.; Kim, H. K.; Abramite, J. A.; Wang, Z.; Guo, Z.; Lu, Y. *J. Am. Chem. Soc.* **2004**, *126*, 10812−10813.
(14) **1** is positioned to minimize steric conflict with the apoprotein structure.
(15) Detailed experimental procedures are given in Supporting Information.
(16) Scott, D. R.; Becker, R. S. *J. Chem. Phys.* **1961**, *35*, 516−531.
(17) The yield of FcAz adduct was higher than 90%.
(18) Sohn, Y. S.; Hendrickson, D. N.; Gray, H. B. *J. Am. Chem. Soc.* **1970**, *92*, 3233−3234.
(19) Matsue, T.; Evans, D. H.; Osa, T.; Kobayashi, N. *J. Am. Chem. Soc.* **1985**, *107*, 3411−3417.
(20) Cardona, C. M.; Kaifer, A. E. *J. Am. Chem. Soc.* **1998**, *120*, 4023−4024.
(21) Di Bilio, A. J.; Chang, T. K.; Malmström, B. G.; Gray, H. B.; Karlsson, B. G.; Nordling, M.; Pascher, T.; Lundberg, L. G. *Inorg. Chim. Acta* **1992**, *198*−*200*, 145−148.
(22) Background current subtraction was performed with a program developed by Dr. H. A. Heering and Dr. C. Léger.

JA054983H