Noncovalent insertion of ferrocenes into the protein shell of apo-ferritin†

Jochen Niemeyer,[a] Satoshi Abe,‡[b] Tatsuo Hikage,§[c] Takafumi Ueno,‡*[bd] Gerhard Erker[a] and Yoshihito Watanabe*[e]

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Apo-ferritin was used for the incorporation of functionalized ferrocene derivatives and the resulting composites were studied by means of single-crystal X-ray diffraction and electrochemical analysis, revealing a different incorporation behaviour of the ferrocenes governed by the functional group present.

The combination of naturally occuring proteins with artificial guest molecules has been investigated using a variety of approaches.[1] These include the covalent binding of an artificial cofactor to the protein backbone,[2] the anchoring using a natural host molecule[3] or the noncovalent insertion of guests into a suitable protein cavity.[4] The resulting composites allow the combination of features previously assigned to either low-molecular weight organic molecules or supramolecular protein moieties, thus creating systems helping to understand the interaction between proteins and organic molecules or potentially finding new applications in catalytic reactions.[5]

Ferritin is the universal iron storage protein in nature. It consists of 24 monomeric subunits, creating a spherical protein shell having a 432 symmetry (see Fig. 1). This protein shell, having an outer diameter of 12 nm and enclosing an inner cavity with a diameter of 8 nm, allows the storage of up to 4500 Fe-atoms as a ferric oxyhydroxide cluster.[6] The iron uptake and release mechanisms have been studied in detail, showing that specific nucleation and ferrioxidase sites are responsible for the mineralization process.[7] In the case of bacterial ferritin, up to 24 heme cofactors can be bound, which have been shown to facilitate the initial Fe^{3+} reduction during iron-release.[8]

Removal of the ferritin iron cluster leads to apo-ferritin, which was initially discovered by Papanicolaou.[9] Since then, many attempts have been made to make use of the empty inner cavity of apo-ferritin, *e.g.* for the generation of non-iron clusters[10] or for the non-covalent incorporation of organic guest molecules.[11] Yet, in only one case such an apo-ferritin/guest composite has been structurally characterized,[12] being an important prerequisite for a deeper understanding of the exact interactions of apo-ferritin with incorporated guest molecules.

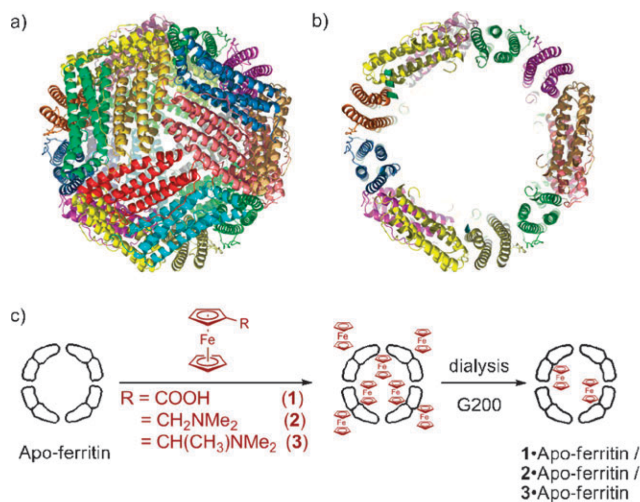

Fig. 1 (a) Whole structure of apo-ferritin (PDB ID: 1 DAT), (b) interior view of the protein shell, (c) synthesis of the composites of apo-ferritin with the ferrocenes **1**, **2** and **3**.

For this reason, we were interested in the possibility to noncovalently incorporate simple organometallic compounds, namely functionalized ferrocene derivatives, into the apo-ferritin shell. In addition to a better structural understanding of the interactions of apo-ferritin with organometallic moieties, we were interested in the electrochemical behaviour of the generated hybrid species.

Although several reports about the covalent modification of proteins with ferrocene derivatives have been published,[13] the noncovalent interaction of a protein with a ferrocene moiety has not yet been investigated to our knowledge.

We have successfully incorporated three differently functionalized monosubstituted ferrocene derivatives into recombinant horse L-chain apo-ferritin. These ferrocene derivatives, ferrocene carboxylic acid (**1**), dimethylaminomethylferrocene (**2**) and 1-dimethylaminoethylferrocene (**3**) were chosen in order to investigate the influence of the type of substitution on the incorporation behaviour.

The composites were generated by mixing a solution of apo-ferritin at pH = 8 with a 10 000-fold excess of the corresponding ferrocene derivative, giving clear yellow solutions in all cases. This was followed by dialysis and size-exclusion chromatography in order to ensure the removal of any non-incorporated ferrocene molecules (Fig. 1(c)).

[a] *Organisch-Chemisches Institut, Universität Münster, Corrensstraße, 40, D-41849 Münster, Germany. E-mail: erker@uni-muenster.de; Fax: +49-251-8336503*
[b] *Department of Chemistry, Graduate School of Science, Nagoya University, Furo-cho, Nagoya, 464-8602, Japan. E-mail: taka@mbox.chem.nagoya-u.ac.jp; Fax: +81-52-789-2953*
[c] *High Intensity X-ray Diffraction Laboratory, Nagoya University, Furo-cho, Nagoya, 464-8603, Japan*
[d] *PRESTO, Japan Science and Technology Agency (JST), Honcho, Kawaguchi, Saitama, 332-0012, Japan*
[e] *Research Center for Materials Science, Nagoya University, Furo-cho, Nagoya, 464-8602, Japan*

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‡ Present address: Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Katsura, Nishikyou-ku, Kyoto 615-8510, Japan.

§ X-Ray crystal structure analyses.

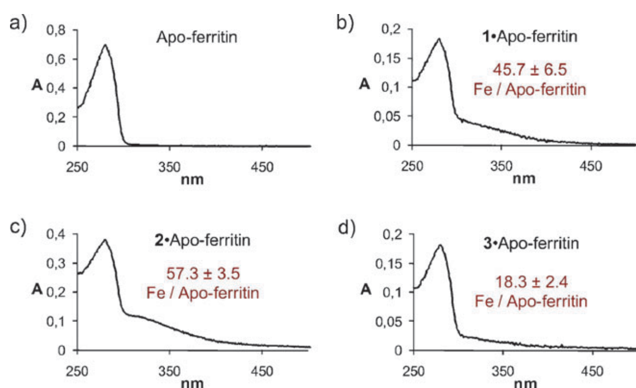

Fig. 2 UV-vis spectra and exact composition of the composites **1**/**2**/**3**·apo-ferritin (b–d) in comparison to apo-ferritin (a).

The UV-vis spectra of the resulting composites show a broad shoulder between 300 and 500 nm, indicating the successful incorporation of the ferrocene derivatives into apo-ferritin (see Fig. 2). The exact numbers of incorporated ferrocenes per apo-ferritin unit were determined by ICP-OES spectroscopy and protein content analysis. In order to prove the reproducibility of the composite synthesis, at least three independent preparations were performed in each case, showing that 45.7 ± 6.5 (for **1**·apo-ferritin), 57.3 ± 3.5 (for **2**·apo-ferritin) or 18.3 ± 2.4 (for **3**·apo-ferritin) ferrocene moieties were incorporated, respectively (see Fig. 2). Prolonged dialysis times did not result in a diminished number of incorporated ferrocenes, so that we assume a tight binding of the ferrocene moieties to apo-ferritin. Comparing the numbers of incorporated ferrocenes per ferritin, it seems that the exact substituent at the ferrocene backbone has a strong influence on the total number of available binding sites. This is especially striking when comparing the composites **2**·apo-ferritin and **3**·apo-ferritin, which show that the addition of one additional methyl group leads to a significant drop in the number of incorporated ferrocenes.

Crystal structures of all three ferrocene/apo-ferritin composites were determined with resolutions of 2.0 Å (for **1**·apo-ferritin and **3**·apo-ferritin) and 1.6 Å (for **2**·apo-ferritin). The anomalous fourier difference map calculation[14] for **2**·apo-ferritin and **3**·apo-ferritin shows the existence of a new anomalous peak at the twofold symmetry axis of the apo-ferritin molecule, located between the monomeric subunits of the apo-ferritin shell (see ESI†).

Although the structures of the ferrocene derivatives within the protein shell could not be determined, the X-ray analysis indicates the incorporation of the ferrocene moieties in this position. This results in anomalous peak density arising from the central Fe(II)-ion and suggests a specific binding of the ferrocenyl amines **2** and **3** in this position.

This binding site has been reported by Eckenhoff *et al.* for the incorporation of molecules such as halothane and isoflurane[12] in apo-ferritin, indicating that this binding site is able to incorporate different non-protic molecules of suitable size. Yet, the position identified for the incorporation of the ferrocenes **2** and **3** would only allow a total number of twelve ferrocenes being incorporated into the 24-meric structure of apo-ferritin. Compared with numbers obtained from ICP-analysis, this suggests the existence of further binding sites which are not observable by X-ray analysis.

Similarly, no anomalous peak could be detected for the composite **1**·apo-ferritin, neither at the position observed for the ferrocenes **2** and **3** nor at any other position. Thus, the ferrocene carboxylic acid molecule cannot be incorporated into the binding site found for **2**·apo-ferritin and **3**·apo-ferritin, nor could the actual binding site be determined by X-ray crystallographic analysis.

The electrochemical analysis was performed using a Au working electrode, as used earlier for direct electrode/protein electron transfer reactions.[15] The observed redox activity of the composites confirmed the incorporation of the electrochemically active ferrocene moieties into the apo-ferritin shell, proven by the observation of a reversible oxidation/reduction process in the cyclovoltammetric analyis (for details see ESI†). In addition to this, the different incorporation environment of the ferrocenylamines **2** and **3** in contrast to ferrocene carboxylic acid **1** leads to a different oxidation potential change, when investigated at different pH values (see Fig. 3).

The composites **2**·apo-ferritin and **3**·apo-ferritin exhibit a higher $E_{1/2}$ (*ca.* +8 mV) compared to that of the free ferrocenylamines **2** and **3** over the whole pH-range investigated, indicating the encapsulation in a hydrophobic environment.[16] Yet, the oxidation potential increases with lower pH values, which can be attributed to a protonation of the ferrocenylamine moiety.[17]

The same trend can be observed for the free ferrocene carboxylic acid **1**, although a significant increase in oxidation potential can only be observed for pH-values <5. In contrast to this, the oxidation potential of the composite **1**·apo-ferritin shows a strong increase in the oxidation potential, starting from a value of +252 mV (*vs.* SCE) at pH = 9, rising up to +335 mV at pH = 6 (no clear analysis of the oxidation potential was possible for pH values <6).

As previously found by Lu *et al.* for a ferrocene moiety covalently attached to Azurin,[18] this increase of the oxidation potential suggests the incorporation of the ferrocene carboxylic acid **1** in a highly protic protein environment. Protonation of the amino-acid backbone due to lowering the pH value leads to an increase in the oxidation potential of the nearby ferrocene species.

In summary we have reported the successful noncovalent incorporation of differently functionalized ferrocene derivatives into apo-ferritin. These organometallic moieties show a

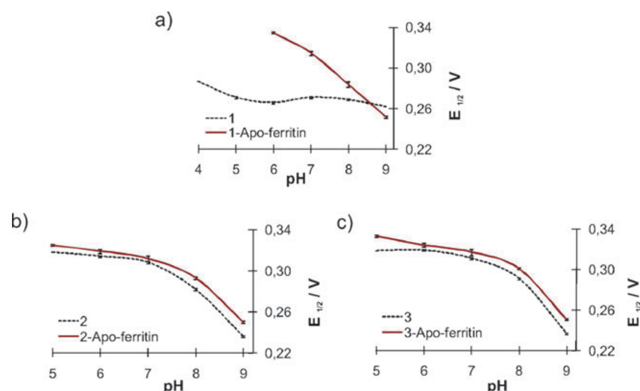

Fig. 3 pH-dependence of the oxidation potentials for (a) **1**·apo-ferritin/**1**, (b) **2**·apo-ferritin/**2** and (c) **3**·apo-ferritin/**3**.

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highly reproducible incorporation behaviour, with the number of incorporated molecules being strongly dependent on the ferrocene substitution pattern. X-Ray crystal structure analysis and electrochemical analysis of the composites revealed a different incorporation behaviour for the ferrocenyl amines **2** and **3** as compared to the ferrocene carboxylic acid **1**.

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