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Artificial [FeFe]-Hydrogenase: On Resin Modification of an Amino Acid to Anchor a Hexacarbonyldiiron Cluster in a Peptide Framework

Souvik Roy,[a,b] Sandip Shinde,[a,b] G. Alexander Hamilton,[a] Hilairy E. Hartnett,[a,b,c] and Anne K. Jones*[a,b]

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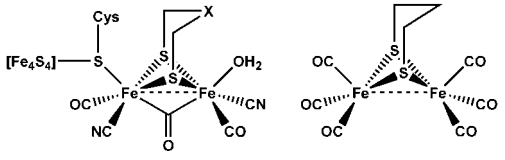
A general method for immobilization of synthetic analogues of the [FeFe]-hydrogenase in designed peptides via on resin modification of an amino acid side chain with a dithiol functional group is described. Utilizing a unique amine side chain as anchor, the dithiol unit is coupled to the peptide via formation of an amide. This dithiol unit precisely positions the two required sulfur atoms for the formation of a [(μ-SRS){Fe(CO)$_3$}$_2$] cluster on reaction with [Fe$_3$(CO)$_{12}$]. UV/Vis and FTIR spectroscopy demonstrate formation of the desired complex.

Introduction

Hydrogen is an energy currency in both biological and industrial settings, and hydrogenases are the biological catalysts for the interconversion of protons and hydrogen. This reaction is at the heart of hydrogen's usage as a fuel.[1] Unlike industrial catalysts that rely on precious metals to catalyze hydrogen activation, biology takes advantage of the earth-abundant metals nickel and iron. FTIR and X-ray crystallographic studies have shown that both [NiFe]- and [FeFe]-hydrogenases contain unusual, organometallic active sites utilizing biologically unprecedented CO and CN as intrinsic ligands.[2–9]

The structure of the active site of [FeFe]-hydrogenases (Scheme 1) consists of an unusual [Fe$_2$S$_2$] subunit bridged to an ordinary [4Fe4S] cubane via a single cysteinyl sulfur atom. The diiron subsite contains two features that are remarkable from a biological perspective: (1) the aforementioned diatomic ligands and (2) a dithiol organic bridging molecule that has been alternatively proposed to contain carbon, nitrogen or oxygen at its central position.[10–13] From a synthetic chemistry perspective, the active site of [FeFe]-hydrogenases has remarkable structural similarities to many known iron carbonyl compounds, and considerable progress in understanding the mechanism of this enzyme has been achieved via synthesis of relevant model compounds.[14–20]



Scheme 1. [FeFe]-hydrogenase active site and first-generation model compound utilizing a propanedithiol ligand.

Despite the wealth of biomimetic chemistry that has been reported, there still exist several important functional differences between synthetic model compounds and [FeFe]-hydrogenases. Model compounds cycle through the more reduced Fe^{I}-Fe^{0} redox state during electrocatalytic reduction of protons whereas [FeFe]-hydrogenases are believed to utilize the Fe^{II}-Fe^{I} state.[14] Similarly, model compounds require substantial overpotentials to catalyze the reduction of protons at slower rates than can be achieved by the enzymes. Finally, none of the [FeFe]-hydrogenase models is able to oxidize hydrogen while the enzymes are known to perform reversible catalysis.

Increasingly, attention is being focused on generation of functional [FeFe]-hydrogenase model complexes that can perform electrocatalysis or photocatalysis. In these investigations, a number of synthetic strategies have been developed for coupling relevant diiron models to electrode surfaces, redox cofactors, or photoactive centers.[21–25] In parallel, strategies for investigating the impact of second coordination sphere interactions and burial of the active site in superstructures isolated from bulk solvent are starting to emerge.[22,26–32] On the other hand, relatively little attention has been paid to developing strategies for attachment of

[a] Department of Chemistry and Biochemistry, Arizona State University,
Tempe, AZ 85287-1604, USA
Fax: +1-4809652747
E-mail: jonesak@asu.edu
[b] Center for Bioenergy and Photosynthesis, Arizona State University,
Tempe, AZ 85287-1604, USA
[c] School of Earth and Space Exploration, Arizona State University,
Tempe, AZ 85287-1404, USA
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diiron models to amino acids[28,33–35] or peptidic scaffolds[36,37] – this, despite the fact that evidence continues to build indicating that the enzyme tertiary structure plays a crucial role in tuning the properties of the active site to achieve near diffusion-limited, bidirectional catalysis.

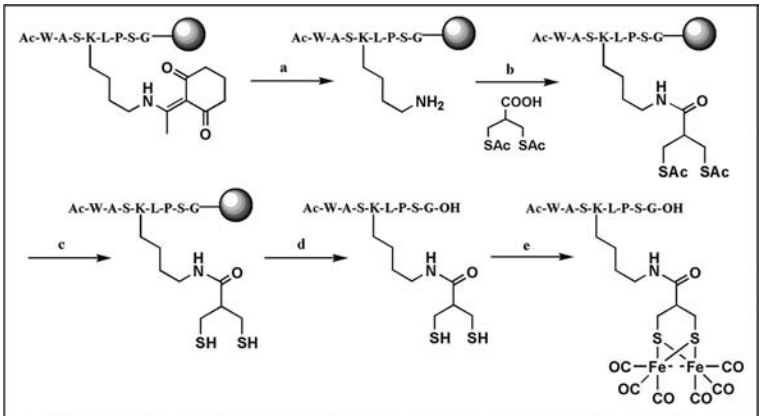
De novo designed proteins have proven an invaluable tool in the study of complex oxidoreductases and have been designed to bind a number of both naturally occurring and artificial metallocofactors.[38–43] The construction of miniaturized, peptidic systems coordinating hydrogenase-related metallocenters presents unique synthetic challenges. One approach has been utilization of the naturally occurring amino acid cysteine as an anchor for construction of a $[(\mu\text{-SRS})\{Fe(CO)_3\}_2]$ cluster.[36] This strategy has the disadvantage of relying on the correct placement of two distinct cysteines to form the desired complex. A second synthetic route involves modification of an amino acid to create an artificial amino acid ligand for the desired metal complex. In this report, we present a method to modify a peptide still attached to its resin support in order to create an artificial derivative of a lysine bearing a propanedithiol unit. The appended sulfur side chain posesses two sulfurs poised to anchor a covalently attached $[Fe_2(SRS)(CO)_6]$ unit. This approach can serve as a general method for modification of a wide range of designer synthetic peptides to probe interactions between amino acids and [FeFe]-hydrogenase small models.

Results and Discussion

Scheme 2 shows the general strategy for synthesis of an $[Fe_2(SRS)(CO)_6]$ unit appended to a dithiol-modified lysine residue. First, the desired peptide is constructed via standard Fmoc/*t*Bu solid-phase peptide synthesis. During synthesis, an orthogonally protected amino acid residue, in this case lysine, is introduced that serves as the site for dithiol incorporation. The dithiol unit is provided by 3-(acetylthio)-2-(acetylthiomethyl)propanoic acid.[44] This propanoic acid derivative can be coupled to a primary amine to form a stable amide. After modification of the lysine, the rest of the peptide can be simultaneously deprotected and cleaved from the resin via standard trifluoroacetic acid methods and purified before incorporation of the diiron unit via reaction with $[Fe_3(CO)_{12}]$.

Reactions with peptides can present unique challenges relative to reactions with small, organic ligands. First, peptides are not usually soluble in the organic solvents traditionally utilized for organometallic synthesis. Second, the number and diverse composition of functional groups present in a peptide require carefully orchestrated protection/deprotection to avoid side products. To demonstrate the efficacy of this synthetic approach, a simple variant of the N-terminal sequence of the nickel-superoxide dismutase (SODA) from *Streptomyces coelicolor* was utilized. The SODA peptide, ACDLPCG, binds nickel, is soluble and monomeric in aqueous solution, is spectroscopically well characterized, and retains some of the catalytic activity of the complete enzyme.[45] Three variations in this sequence were introduced. First, the two cysteine residues were exchanged for serines to eliminate competition from these thiols for interaction with iron while maintaining the approximate length and polarity of the side chain. Second, the aspartic acid was exchanged for a lysine as an attachment point for the iron cluster. Finally, a tryptophan residue was introduced at the N-terminus to facilitate quantification of the peptide, yielding the final sequnce, WASKLPSG.

Figure 1 shows the analytical HPLC and Figure 2 the NMR spectra from (A) deprotected, unmodified peptide, (B) peptide modified with the dithiol acid, and (C) peptide modified with the diiron cluster. The HPLC traces demonstrate that with each additional modification the peptide became more hydrophobic, eluting at a higher percentage of acetonitrile. These changes in hydrophobicity facilitated efficient purification to yield high quality products as shown in the NMR spectra. The peaks of peptide modified with the dithiol acid can clearly be observed after coupling to the lysine at 1.10 (t, 2 H), 2.4–2.55 (1 H) and 3.0–3.15 (4 H). These peaks are then shifted after incorporation of the



a. 2 % NH_2NH_2, DMF, yield 100 %; b. HATU, DIEA, DMF, yield 50%; c. 2 % NH_2NH_2, DMF, yield 100 %; d. (i) cleavage: 95 % TFA, 2.5 % water, 2.5 % TIPS (ii) HPLC purification; e. $Fe_3(CO)_{12}$, aq. methanol, reflux, yield 80 %

Scheme 2. Synthetic strategy for modification of a unique lysine with a dithiol functional unit and incorporation of an $\{Fe_2(CO)_6\}$ unit. The sphere represents the resin bead utilized for solid phase peptide synthesis.

diiron unit to overlap with the resonances of the ε-H of the lysine residue. Meanwhile, the peaks for all other protons in the peptide can be accounted for and are virtually unaffected by the modifications of the peptide (complete assignment can be found in the Supporting Information). ESI mass spectrometry has also been utilized to demonstrate that the products have the expected composition and the iron cluster is coordinated via a single peptide monomer.

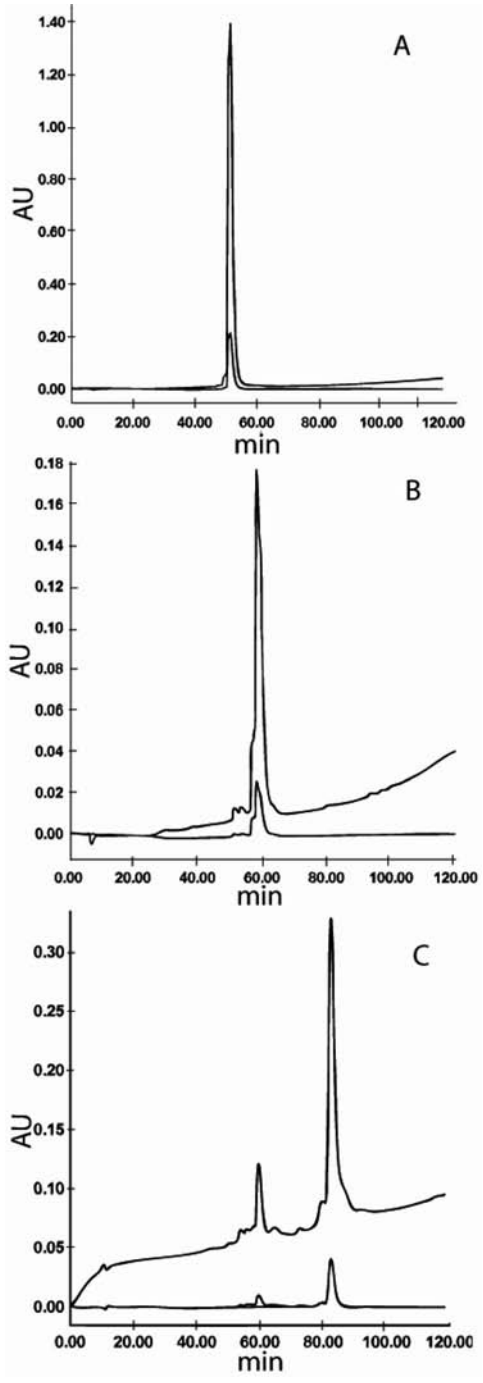


Figure 1. Analytical HPLC traces from (A) WASKLPSG, (B) peptide with dithiol side-chain modification WAS(Dt)LPSG and (C) peptide modified with both a dithiol side-chain and the $\{Fe_2(CO)_6\}$ cluster. HPLC gradient 1% acetonitrile/min; flow rate 0.25 mL/min. The higher amplitude signal in each case is absorbance at 220 nm (peptide bond) and the lower at 280 nm (tryptophan).

The UV/Vis and FTIR spectra of $Fe_2(CO)_6$-modified peptide are shown in Figure 3. The UV/Vis spectrum consists of three notable features: an absorption at 334 and a shoulder at 465 nm both associated with the Fe-S core, and the characteristic absorbance of the tryptophan residue at 280 nm. The ratio of the extinction coefficients of these features is similar to that observed previously for incorporation of cluster into peptide via cysteine, providing additional evidence for nearly stoichiometric incorporation of cluster in the purified product.[36] The FTIR spectrum of the $Fe_2(CO)_6$-modified peptide has three bands at 2075, 2035 and 1999 cm^{-1} arising from the coupled CO vibrational modes of the complex. These confirm the presence of the carbonyls on each iron. Furthermore, the close agreement between these values and those observed for $[\{\mu\text{-}S(CH_2)_3S\}\{Fe(CO)_3\}_2]$ suggests that, as expected, the iron complex is completely exposed to solution when attached to the peptide. Finally, ESI-MS data of $Fe_2(CO)_6$-modified peptide also implies the presence of the CO ligands since ions formed by loss of CO ligands [complex-CO, complex-$(CO)_3$ and complex-$(CO)_6$] could clearly be identified (Supporting Information).

The complex $[\{\mu\text{-}S(CH_2)_3S\}Fe_2(CO)_6]$ is an unremarkable catalyst, but it can undergo substitution of the carbonyls by a number of different types of ligands such as cyanide or phosphanes to form more active species.[14] Similarly, $Fe_2(CO)_6$-modified peptide is also able to undergo substitution reactions. As shown in Figure 3, addition of excess PMe_3 to an acetonitrile solution of the peptide, results in a uniform shifting of the frequencies of the ν(CO) bands to approximately 100 cm^{-1} lower wavenumbers (1974, 1939, and 1894). The compound $[\{\mu\text{-}S(CH_2)_3S\}\{Fe(CO)_2(PMe_3)\}_2]$ is reported to have ν(CO) bands at 1979, 1942, and 1898 cm^{-1}.[46] Thus, the observed data is consistent with formation of a disubstituted phosphane derivative and indicates that the cluster is accessible for ligand exchange.

Conclusions

We have demonstrated the ability to incorporate a $[(\mu\text{-}SRS)Fe_2(CO)_6]$ model of [FeFe]-hydrogenases into a peptide via on resin construction of a modified amino acid. This synthetic scheme is general and allows covalent connection of the diiron unit to any unique primary amine. Importantly, the synthesis can be performed in water/methanol mixtures yielding a water-soluble product in high yield.

This approach provides a convenient method for constructing peptide-based models of [FeFe]-hydrogenases. Through variation of the peptide scaffold, it will be possible to construct complexes in carefully tailored cavities designed to incorporate selected aspects of the enzyme active site. The 3-(acetylthio)-2(acetylthiomethyl)propanoic acid unit may also be utilized as a handle to construct other sulfur-ligated metallocenters.

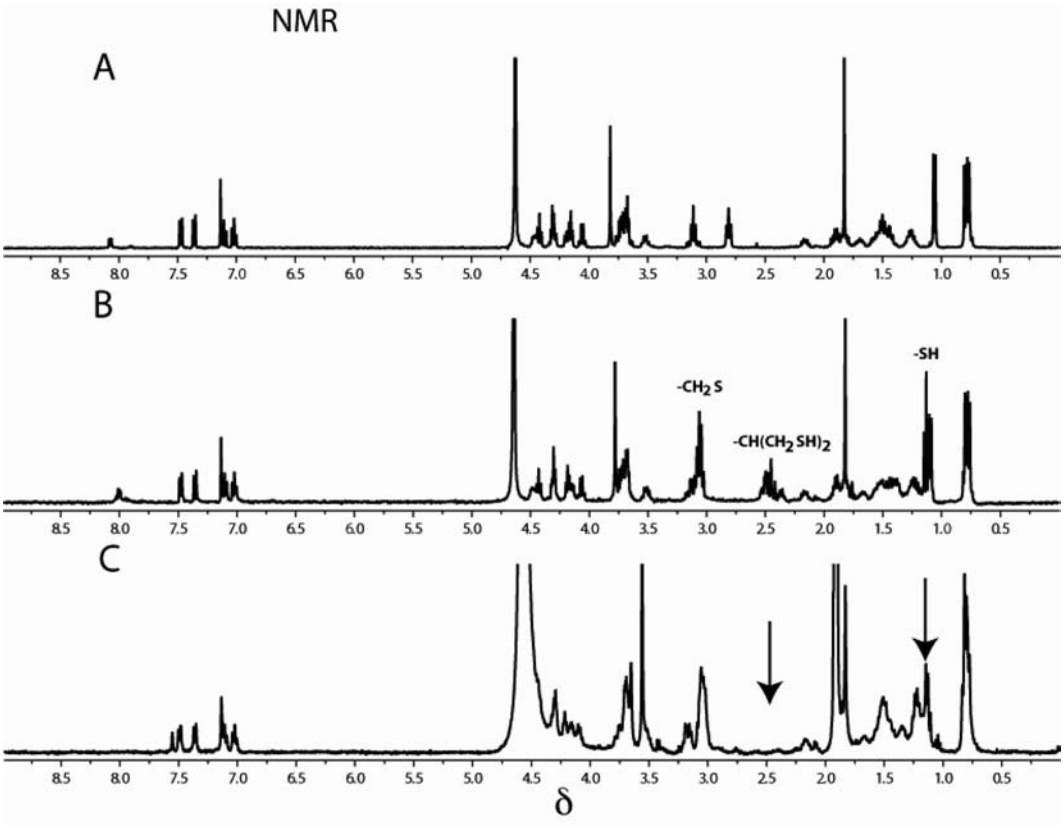


Figure 2. ^{1}H NMR spectra from (A) WASKLPSG, (B) peptide with dithiol side-chain modification WAS(Dt)LPSG and (C) both a dithiol side-chain and the $\{Fe_2(CO)_6\}$ cluster. NMR spectra were obtained in D_2O.

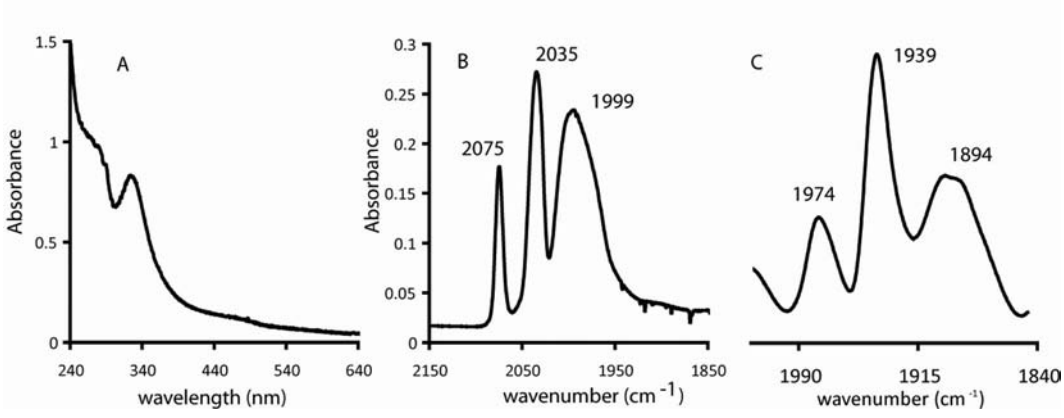


Figure 3. Characterization of $[Fe_2(CO)_6]$ complex coordinated to dithiol-modified peptide via (A) UV/Vis and (B) FTIR spectroscopy and (C) FTIR spectrum of [peptide-$Fe_2(CO)_4(PMe_3)_2$]. UV/Vis spectrum was obtained in a 1:1 mixture of water/acetonitrile. For FTIR experiments, sample was applied to a single CaF_2 window as a methanolic solution and dried under a vacuum to form a thin film. Experiments were then performed with the film.

Experimental Section

General: All reactions were performed under an atmosphere of nitrogen. Unless otherwise specified, all chemicals were of the highest purity available from Sigma–Aldrich. Fmoc protected amino acids and peptide coupling reagents were obtained from Protein Technologies (Tucson, AZ, USA). Solvents including DMF, acetonitrile, methanol, ethyl acetate, hexanes, ether $CDCl_3$ and D_2O were obtained from Sigma Aldrich.

Instrumentation: MALDI-MS (matrix assisted laser desorption/ionization mass spectrometry) characterization of peptides was performed on a Voyager DE STR in the Proteomics and Protein Chemistry Laboratory at Arizona State University. ESI-MS (electrospray ionization mass spectrometry) was performed using a Thermo Quantum Discovery Max triple-quadrupole mass spectrometer in the Environmental Biogeochemistry Laboratory at Arizona State University. Measurements were conducted in positive

(+) and negative (–) ionization modes using a methanol/water (50:50 by volume) mobile phase at a flow rate of 10 mL min^{-1} and the following ionization conditions: spray voltage, 4000 (+, –); capillary temperature, 270 °C; sheath gas pressures, 25 (+) and 15 (–); auxiliary gas pressure, 2 (+, –). NMR spectra were recorded at 400 or 500 MHz (^1H) on Varian Liquid-State NMR instruments in $CDCl_3$ solutions containing 0.1% TMS (tetramethylsilane) unless otherwise mentioned. UV/Vis measurements were performed on a Hewlett–Packard 8453 spectrophotometer using quartz cuvettes with a 1 cm pathlength. Peptide concentrations were determined via absorbance of the tryptophan residue (ε_{280} = 5600 M^{-1} cm^{-1}). FTIR spectra were recorded on a Thermo Nicolet Avatar-360 spectrometer as a dry film on a CaF_2 window. Each spectrum is an average of 1024 scans collected under nitrogen at 1 cm^{-1} resolution. The clean CaF_2 window was used as a reference.

2-(Bromomethyl)propenoic Acid: Diethyl bis(hydroxymethyl)malonate (13 g, 0.058 mmol) was dissolved in HBr (25 mL of 48% aqueous solution, ca. 0.2 mol) and was heated at 120–125 °C in an oil bath for 45 min. On cooling the solution to –80 °C, white crystals appeared. The crystals were removed by filtration and the filtrate was heated again to 120–125 °C for 45 min. This solution was again cooled to –80 °C and additional crystals were filtered off. This process was repeated a third time. All the white crystals were collected and dissolved in 30 mL of dichloromethane, washed with 2 × 10 mL of 1 N HCl, dried with anhydrous $MgSO_4$ and concentrated under reduced pressure to yield a white crystalline solid (2.8 g, 29.4%). ^1H NMR (400 MHz): δ = 6.48 (s, 1 H), 6.09 (s, 1 H), 4.17 (s, 2 H) ppm.

3-(Acetylthio)-2-(acetylthiomethyl)propanoic Acid: To a stirred suspension of 2-(bromomethyl)propenoic acid (2.8 g, 0.017 mol) in 50 mL of water at 0 °C was added an aqueous solution of sodium carbonate (3.1 g, 0.029 mol in 15 mL) in small portions. To the resulting mixture, thiolacetic acid (1.25 mL, 0.017 mol) was added dropwise, and the solution was stirred at 0 °C for 2 hours. The solution was acidified with 6 M HCl, and the white precipitate was extracted with ethyl acetate (3 × 25 mL). The EtOAc layer was dried with anhydrous $MgSO_4$ and concentrated under reduced pressure to yield a colorless oil. With this colorless oil, the reaction was repeated (as ^1H NMR of the oil indicated the presence of about 33% unreacted starting material, 2-(bromomethyl)propenoic acid) to produce pure 2-(acetylthiomethyl)acrylic acid as a colorless oil (3 g). ^1H NMR (400 MHz): δ = 6.38 (s, 1 H), 6.02 (s, 1 H), 3.75 (s, 2 H), 2.32 (s, 3 H) ppm. To this 2-(acetylthiomethyl)acrylic acid was added thiolacetic acid (2 mL, 0.027 mol) and stirred at room temperature. After 36 hours, ^1H NMR spectra of the reaction mixture showed no olefinic peaks. The unreacted thiolacetic acid was removed from the reaction mixture under reduced pressure and the yellow oil like residue was purified by column chromatography on silica gel with hexane/ethyl acetate (3:1) as eluent followed by hexane/ethyl acetate (2:1) giving 3-(acetylthio)-2-(acetylthiomethyl)-propanoic acid as a white solid (2.8 g, 70%). R_f = 0.2 (3:1 hexane/ethyl acetate). ^1H NMR (400 MHz): δ = 3.19 (m, 4 H), 2.91 (p, 1 H), 2.35 (s, 6 H) ppm.

Peptide Synthesis: The eight amino acid containing peptide (WASKLPSG) was synthesized on a Protein Technologies PS3 automated peptide synthesizer using the standard Fmoc/*t*Bu (Fmoc, 9-fluorenylmethoxycarbonyl) protection strategy and HBTU (*o*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate) as coupling reagent on Fmoc-Gly wang resin (Aapptec 0.54 mmol/g, 100–200 mesh) at 0.1 mmol scale.[25] Lysine with an orthogonally protected side chain, Fmoc-Lys(ivDde)-OH [ivDde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl], was used for solid-phase peptide synthesis so that the side-chain amino group of lysine can be selectively deprotected and modified. Following synthesis, the peptide was acetylated at the N-terminus [1:1 (v/v) acetic anhydride: *N*-methylmorpholine for 20 min, two times].

Coupling of 3-(Acetylthio)-2-(acetylthiomethyl)propanoic Acid to Peptide and Purification: Resin-bound peptide (64 mg, 0.033 mmol) was treated with 2% hydrazine in DMF (10 mL) for 45 min at room temperature under nitrogen to remove the protecting group from lysine. Following deprotection, 3-(acetylthio)-2-(acetylthiomethyl)-propanoic acid (35 mg, 0.15 mmol), HATU [2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium] (55 mg, 0.14 mmol) and DIEA [*N*,*N*-diisopropylethylamine] (30 μL, 0.16 mmol) dissolved in 5 mL of DMF were added to the resin under nitrogen and stirred at room temperature for 30 min. This coupling reaction was repeated. The resin was then washed with DMF (5 × 2 mL). The thioacetal groups were reduced to thiol by treatment with 2–3% hydrazine in DMF (10 mL) under nitrogen for 40 min. Then the peptide was simultaneously deprotected and cleaved from the resin using 95:2.5:2.5 trifluoroacetic acid (TFA)/water/triisopropylsilane (TIPS) for 2 h and 15 min. The crude cleavage solution was filtered and the residual resin was washed with neat TFA (3 × 1 mL). The TFA solution of peptide was concentrated under reduced pressure to a light yellow colored liquid (ca. 0.5–1 mL). The crude peptide was precipitated by adding cold diethyl ether (–20 °C). Followed by incubation with four equivalents TCEP, the crude peptide was purified by reverse-phase HPLC on a Waters 600E HPLC system with a photodiode array detector (3 × 250 mm C-18 column for analytical and PrepLC 25 mm module C-18 column for semi-preparative HPLC) using aqueous acetonitrile gradients containing 0.1% TFA (v/v). Molecular weight of the peptide was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) mass spectrometry. $[M]^+$ calcd. 1020.99; found 1043.3 $[(M + Na - H)^+]$.

Cluster Incorporation Into Peptide: Dodecacarbonyltriiron (5 mg, 0.01 mmol) in methanol (1.5 mL) and 0.16 mM aqueous solution of peptide (1 mL) were combined and refluxed for 1.5 hours resulting in a color change from green to pale red. The reaction mixture was concentrated under reduced pressure and was extracted with 3 mL of water. The complex was purified by reverse phase HPLC using aqueous-acetonitrile gradients.

Substitution of Carbon Monoxide Ligands with Trimethylphosphane: Trimethylphosphane (4 μL, 0.038 mmol) was added to a 0.02 mM acetonitrile solution of [peptide-{μ-$S_2Fe_2(CO)_6$}] complex (1 mL) under N_2 in the dark and stirred at room temperature for 1.5 h resulting in color change to red. The FTIR of the crude reaction mixture showed ν(CO) bands at 1974, 1939, 1894 cm^{-1}.

Supporting Information (see also the footnote on the first page of this article): Assignments of peptide ^1H NMR spectra, ESI-MS for the $Fe_2(CO)_6$-modified peptide.

Acknowledgments

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