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Oxidized Rubredoxin Models. II. Iron(III) Complexes of Z-Cys-Pro-Leu-Cys-OMe and Z-Cys-Thr-Val-Cys-OMe[1)]

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Fe(III) complexes of cysteine-containing peptides such as Z–Cys–Pro–Leu–Cys–OMe and Z–Cys–Thr–Val–Cys–OMe corresponding to the Fe binding amino acid sequences in *Cl. pasteurianum* rubredoxin were prepared in solution and characterized by the absorption, circular dichroism (CD), and magnetic circular dichroism (MCD) spectra and were compared with those of the Fe(III) complexes of Z–Cys–Ala–Ala–Cys–OMe, Z–Ala–Cys–OMe, and Z–Cys–Ala–Cys–OMe. These model compelxes exhibited the existence of an electronic configuration and a core structure similar to that of native rubredoxin. The thermal stabilities of the Fe(III) peptide complexes decrease in the following order, Z–Cys–Thr–Val–Cys–OMe$>$Z–Cys–Ala–Ala–Cys–OMe$>$Z–Cys–Pro–Leu–Cys–OMe $\simeq$Z–Ala–Cys–OMe$\gg$Z–Cys–Ala–Cys–OMe.

Rubredoxin playing significant roles in biological electron transfer and oxygenation reactions has one $[Fe(S\text{–}Cys)_4]^{-,2-}$ (S–Cys denotes cysteinyl thiolate, –NH–CH(CH_2S^-)–CO–) as an active site.[2,3)] The X-ray analysis of oxidized rubredoxin from *Clostridium pasteurianum* has revealed that the Fe(III) is coordinated by two thiolates of –Cys(6)–Pro–Leu–Cys(9)–sequence and other two thiolates of –Cys(39)–Thr–Val–Cys(42)–sequence with a distorted tetrahedral geometry.[4)]

Importance of –Cys–A–B–Cys– has been discussed on the contribution to the stabilities of the chelate rings, the determination of the geometries around the metal ions, and the origins of catalytic activities. Christou *et al.* reported the Fe(III) complexes of Ac{Gly–Cys–Gly}$_n$$NH_2$ (n=1—4) in which two Gly residues were interposed between Cys residues. The Gly residues have the least sterical restriction on the peptide conformations.[5)]

Any substituent on the peptide chain will restrict the conformations and will result in preference of one of them.

Thus, our previous paper reported the absorption, CD, MCD, and electron paramagnetic resonance (EPR) spectral analyses of Fe(III) complexes of Z–Cys–Ala–Ala–Cys–OMe and Z–Ala–Cys–OMe.[1)] The tetrapeptide complex exhibited a relatively stable structure with the hairpin turn conformation of the tetrapeptide. It may be indispensable to the function of rubredoxin that the –Cys–A–B–Cys– sequences consist of amino acid residues with bulky side chains such as Pro, Leu, Val, and Thr residues.

In this paper, we describe the preparation and the characterization of Fe(III) complexes of Z–Cys–Thr–Val–Cys–OMe corresponding to the Fe(III) binding sequences in *Cl. pasteurianum* rubredoxin. For comparison, thermal stabilities of an artificial sequence, Z–Cys–Ala–Cys–OMe, in addition to those of Z–Cys–Ala–Ala–Cys–OMe and Z–Ala–Cys–OMe mentioned previously[1)] were investigated with visible spectra. Characteristic properties dependent on the identity of A and B residues in these Cys–A–B–Cys sequences will be discussed.

Experimental

All operations were carried out under argon atmosphere. Dimethyl sulfoxide and triethylamine were degassed and distilled before use. Iron(III) chloride hexahydrate was of commercial grade. Syntheses of the cysteine-containing peptides are described elsewhere.[6)]

Preparations of Fe(III) Complexes. The Fe(III) complexes of the peptides were prepared by the same manner reported by Christou *et al.*[7)] A Me_2SO solution of $FeCl_3\cdot 6H_2O$ and 4 molar equivalents of the cysteine-containing peptide based on its SH-content was treated with triethylamine to give a deep red-violet solution of the Fe(III) complex of the corresponding peptide, which was used for physical measurements without further purifications. $[Et_4N][Fe(S_2\text{-}o\text{-}xyl)_2]$ (S_2-*o*-xyl=*o*-xylene-α,α'-dithiolate) was prepared according to the literature.[8)]

Physical Measurements. Absorption spectra were recorded on a JASCO UVIDEC-5A spectrophotometer. CD and MCD spectra were measured using a JASCO J-40 spectropolarimeter equipped with an electromagnet. The magnetic field was calibrated with an aqueous $K_3[Fe(CN)_6]$ solution.[9)] Calibration of the spectropolarimeter was performed with *epi*-androsterone in dioxane.[10)] The values of ε and $\Delta\varepsilon$ were given in the unit of $M^{-1}\,cm^{-1}$ and $\Delta\varepsilon_M$ in $M^{-1}\,cm^{-1}\,T^{-1}$ where M expresses molar concentration of Fe(III) g-atom. The MCD spectra of the peptide complexes were corrected for the zero-field circular dichrosim.

EPR spectra were recorded at 77 K on a JEOL JESFE 1X with 100 KHz magnetic field modulation using Mn(II) as g-marker (g=1.981).

Results

Spectral Properties of Fe(III) Complexes of Cysteine-containing Peptides. In order to elucidate core structures of the Fe(III) complexes of a series of cysteine-containing peptides, absorption, MCD, and CD spectra of the Fe(III) peptide complexes were investigated in Me_2SO. Table 1 lists the near UV-visible absorption, MCD, and CD spectra of the Fe(III) complexes of Z–Cys–Thr–Val–Cys–OMe and Z–Cys–Pro–Leu–Cys–OMe. For the sake of comparison, those of $[Et_4N][Fe(S_2\text{-}o\text{-}xyl)_2]$ and oxidized rubredoxin from *Cl. pasteurianum*[11–13)] are also represented in Table 1.

The Fe(III) complexes of the peptides exhibited absorption maxima at 330—350 nm and $\approx$500 nm, which were assigned to the ligand-to-metal charge-transfer transitions.[14,15)] The absorption spectra of $[Fe(S_2\text{-}o\text{-}xyl)_2]^-$ and oxidized rubredoxin showed similar spectral features to those of the Fe(III) peptide com-

TABLE 1. SPECTRAL DATA OF Fe(III) COMPLEXES OF CYSTEINE-CONTAINING PEPTIDES

Ligand		$S^-\rightarrow Fe(III)$ charge-transfer									
Z-Cys-Thr-Val-Cys-OMe[a]	Absorption[b]			350 (4700)		495 (3100)					
	MCD[c]	305 (?, 1.1)		385 (A)		495 (A)		570 (B, 2.3)			
	CD[d]		320 (−0.2)	353 (3.0)	393 (−0.7)	438 (3.2)	494 (−1.5)	555 (2.3)		640 (−1.2)	
Z-Cys-Pro-Leu-Cys-OMe[a]	Absorption[b]			355 (6600)			490 (4700)				
	MCD[c]	310 (?, 0.8)	350 (B, −1.0)	385 (A)			485 (A)		580sh (B, 1.3)		
	CD[d]		350 (5.5)		385 (−0.6)	428 (3.2)	494 (−5.0)	562 (1.4)		640 (−0.5)	
Z-Cys-Ala-Ala-Cys-OMe[a,g]	Absorption[b]			353 (5500)			495 (3400)				
	MCD[c]	310 (?, 1.0)	355 (B, −0.3)	390 (A)			495 (A)		585sh (B, 1.2)		
	CD[d]		350 (4.0)		395 (−0.6)	437 (1.2)	500 (−1.6)	560 (1.3)		650 (−0.7)	
Z-Cys-Ala-Cys-OMe[a,g]	Absorption[b]			330 (2600)			500 (1500)				
	MCD[c]		340 (?, 0.4)	389 (A)			495 (A)	550 (B, 0.4)			
	CD[d]	315 (−0.7)	340 (0.7)	370 (−0.8)	415sh (0.4)	459 (0.9)	530sh (−0.9)	570 (−0.4)			
Z-Ala-Cys-OMe[a,g]	Absorption[b]			348 (4400)			495 (2700)				
	MCD[c]	305 (?, 1.1)		383 (A)			495 (A)	560 (B, 1.8)			
	CD[d]		355 (0.1)		400 (−0.1)		500 (0.3)		615 (−0.1)		
$[Et_4N]$ $[Fe(S_2\text{-}o\text{-}xyl)_2]$[e]	Absorption[b]			354 (8200)		430sh (4400)	486 (6400)			635 (1700)	685 (1700)
	MCD[c]	341 (?, 1.1)	355 (?. 0)	365 (?, −0.6)	422 (?, −3.4)		499 (A)			632 (B, 1.8)	
Oxidized rubredoxin[f]	Absorption[b,h]			353 (9600)	380 (10800)		493 (9200)	560 (4400)			
	MCD[c,i]	309 (?, 4.4)	347 (?, −0.8)	382 (A)			495 (A)	565 (B, 5.1)			
	CD[d,j]		344 (−6.2)		412 (10.8)	440 (14.3)	500 (−20.7)	564 (20.8)		637 (−6.9)	

a) In Me_2SO. b) nm(ϵ, $M^{-1}\,cm^{-1}$). c) nm(origin of the Farady effect, $\Delta\epsilon_M$, $M^{-1}\,cm^{-1}\,T^{-1}$). d) nm($\Delta\epsilon$, $M^{-1}\,cm^{-1}$). e) In *N*, *N*-dimethyl formamide. f) In aqueous solution. g) From Ref. 1. h) From Ref. 11. i) From Ref. 12. j) From Ref. 12.

plexes although several additional bands were observed (Table 1).

The MCD spectra of the Fe(III) peptide complexes were highly structured and much more informative than the absorption spectra. The MCD spectrum of oxidized rubredoxin was characterized by Ulmer *et al.* and they pointed out that two dispersion type bands with cross-over points at 382 and 495 nm are assignable to the Faraday A-term, and a negative and a positive bell-shaped bands at 347 and 565 nm to the Faraday B-term corresponding to the absorption maxima at 353, 380, 493, and 560 nm (Table 1).[12] A positive band at 309 nm was not assigned. According to the assignments for rubredoxin[11] and its model complexes with alkanedithiols,[16] tentative assignments for the peptide complexes and $[Fe(S_2\text{-}o\text{-}xyl)_2]^-$ were carried out and are summarized in Table 1. All of the Fe(III) peptide complexes had dispersion type bands with cross-over points at 380—390 nm and positive MCD shoulders at 550—585 nm corresponding to those of the native protein at 382 and 565 nm whereas the peptide complexes had no definite absorption maxima at these regions. On the other hand, $[Fe(S_2\text{-}o\text{-}xyl)_2]^-$ had no cross-over point at 380—390 nm. Among the peptide Fe(III) complexes examined, Z–Cys–Pro–Leu–Cys–OMe and Z–Cys–Ala–Ala–Cys–OMe complexes exhibited negative Faraday effects at 350 and 355 nm, respectively, similar to rubredoxin. The complexes of other peptides did not show such negative ones.

The CD spectra of the Fe(III) peptide complexes

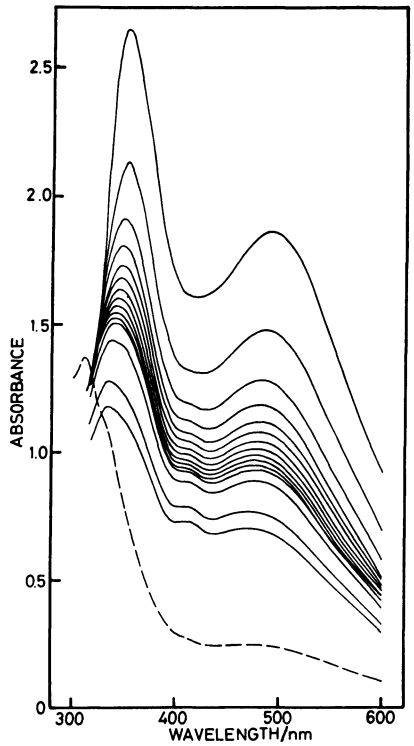

Fig. 1. Spectral change of Fe(III) complex of Z–Cys–Pro–Leu–Cys–OMe. Each spectrum from upper to lower indicates that measured after 10, 71, 130, 187, 254, 314, 383, 453, and 530 s, 10, 11, 13, 24, and 30 min from the complex formation. The broken-line indicat esthe spectrum after 13 h.

also revealed many transitions involved in the charge-transfer region as shown in Table 1. The tetrapeptide Fe(III) complexes especially Z–Cys–Ala–Ala–Cys–OMe complex previously reported,[1] exhibited spectral pattern similar to that of rubredoxin at wavelength longer than 400 nm although the magnitude of the Cotton effects was *ca.* 1/10. On the other hand, tri- and dipeptide Fe(III) complexes exhibited the CD spectra entirely different from those of the tetrapeptide complexes or rubredoxin.

Thermal Stabilities of Fe(III) Complexes of Cysteine-containing Peptides. Under anaerobic conditions, the Fe(III) complexes of the cysteine-containing peptides were unstable as reported by Christou *et al.*[6] Intense red-violet color of the Fe(III) complexes observed immediately after the addition of triethylamine faded gradually due to oxidations of the ligands by the Fe(III) ion. The spectral changes were followed in the time course. A typical example is shown in Fig. 1 for the Z–Cys–Pro–Leu–Cys–OMe complex. After 13 h, the spectrum is broken line of Fig. 1 and further change was observed. The spectrum was similar to that of the Fe(II) complex of Z–Cys–Pro–Leu–Cys–OMe which will be described elsewhere. The following reaction must be taking place:

$$4[Fe^{III}(Z\text{-cys-Pro-Leu-cys-OMe})_2]^{2-}$$
$$\longrightarrow 3[Fe^{II}(Z\text{-cys-Pro-Leu-cys-OMe})_2]^{2-}$$
$$+ 1/2n\,(Z\text{-Cys-Pro-Leu-Cys-OMe})_n + 1/4\,Fe^{II}.$$

Half-life time for each peptide complexes was obtained by monitoring the absorbance maximum at around 500 nm, which is listed in Table 2. The results indicate that the Fe(III) complexes of Z–Cys–Thr–Val–Cys–OMe and Z–Cys–Ala–Ala–Cys–OMe are less unstable among the complexes examined by us. In contrast, the Fe(III) state of rubredoxin is stable at ambient temperature in an aqueous solution.

TABLE 2. HALF-LIFE TIMES OF Fe(III) COMPLEXES OF CYSTEINE-CONTAINING PEPTIDES

Ligand	Half-life times/min	λ_{max}/nm
Z-Cys-Thr-Val-Cys-OMe	25	495
Z-Cys-Pro-Leu-Cys-OMe	7	490
Z-Cys-Ala-Ala-Cys-OMe	16	495
Z-Cys-Ala-Cys-OMe	1	500
Z-Ala-Cys-OMe	5	495

Conditions: in Me_2SO at 33 °C.

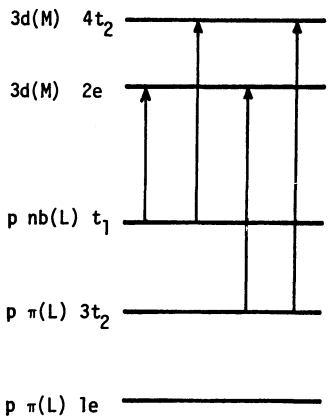

Fig. 2. Energy level diagram of tetrahedral complexes including the highest ligand levels (L) and the metal d levels (M).

Discussion

Structures of $[Fe^{III}S_4]$ *Cores.* The absorption, MCD, and EPR spectra of the Fe(III) peptide complexes led the conclusion that the peptide complexes have mononuclear tetrahedral $[Fe^{III}S_4]$ cores. A formation of binuclear core, $[Fe^{III}{}_2S_8]$, involved in $[Fe^{III}(SCH_2\text{-}CH_2S)_2]_2{}^{2-}$ [17] was ruled out by the MCD spectra. The Fe(III) complex of 1,2-ethanedithiolate exhibited no dispersion type band at around 490 nm but a small positive band at 427 nm and a negative one at 590 nm which were ascribed to the Faraday B-term.[16] The previous paper[1] also indicated that the ESR signals of the Fe(III) complexes of Z–Cys–Ala–Ala–Cys–OMe and Z–Ala–Cys–OMe arise from $S=5/2$ ground state (Fe(III)d^5 high spin) under approximately tetrahedral environments coordinated with four cysteinyl thiolates.

Although the peptide complexes had the similar MCD spectral features from 400 to 700 nm, they exhibited different spectral properties from 300 to 400 nm. They could be divided into two groups. One is the Fe(III) complexes of Z–Cys–Pro–Leu–Cys–OMe and Z–Cys–Ala–Ala–Cys–OMe which have a negative Faraday B-term at around 350 nm and the other has

TABLE 3. CHARGE-TRANSFER BANDS OF Fe(III) COMPLEXES OF CYSTEINE-CONTAINING PEPTIDES[a)]

Ligand	$t_1 \rightarrow 2e$	$t_1 \rightarrow 4t_2$	$3t_2 \rightarrow 2e$	$3t_2 \rightarrow 4t_2$
Z-Cys-Thr-Val-Cys-OMe	17500	20200	26000	—[b)]
Z-Cys-Pro-Leu-Cys-OMe	17200	20600	26000	28600
Z-Cys-Ala-Ala-Cys-OMe	17100	20200	25600	28200
Z-Cys-Ala-Cys-OMe	18200	20200	25700	—[b)]
Z-Ala-Cys-OMe	17400	20200	26100	—[b)]
$[Et_4N]$ $[Fe(S_2\text{-}o\text{-xyl})_2]$	15800	20000	—[c)]	—[b)]
Oxidized rubredoxin	17700	20200	26200	28800[d)]

a) Transition energies were estimated from MCD spectra represented in the unit of cm^{-1}. b) No Faraday B-term was observed. c) No Faraday A-term was observed. d) From Ref. 11.

no such a band at 300—400 nm.

A simple molecular orbital consideration gives a clue to interpret the charge-transfer spectra. A qualitative molecular orbital diagram for tetrahedral Fe(III) complexes is illustrated in Fig. 2, which shows the highest ligand level (L), the Fe(III) d levels, and allowed charge-transfer transitions. The molecular orbitals are constructed by a basis set consisting of 3d, 4s, and 4p of Fe(III) and 3s and 3p of S.[18)] The transition energies estimated from the MCD spectra of the peptide complexes are listed in Table 3. The transitions were assigned to those from the highest occupied ligand π or nonbonding orbitals to the half occupied metal d orbitals according to the literature.[15)] The orientations of the lone pairs on the cysteinyl S atom are shown schematically in Fig. 3. There are two lone pairs; one is perpendicular to the Fe–S–C_β plane (π-type) and the other in the plane (nonbonding type). As shown in Fig. 2 and Table 3, the transitions at around 350 nm (28600 cm^{-1}) were assignable to those from the occupied ligand orbitals ($3t_2$) to the half-occupied metal t_2 orbitals ($4t_2$). The MCD spectral characteristics sensitively reflect differences of the orientation of the π-orbitals on S atom toward Fe(III) ion (Fig. 3b). The orientation of the π-type S lone pair toward the Fe(III) ion change the energies of the Fe d_{xy} and d_{yz} as described by Bair and Goddard III.[19)] The nonbonding orbital on the S atom does not affect the Fe d orbitals in a manner observed for the π-type orbital. Thus, the MCD spectral differences in 300—400 nm region are related to the differences in the electronic states of the Fe(III) t_2 orbitals affected by the lone pairs of the S atom, mainly π-orbitals, whose orientation is determined by steric disposition of the S–C_β bond relative to the Fe–S bond as shown in Fig. 3. Conformational restriction of the peptide ligands determines these orientations and, hence, electronic structures of the $[Fe^{III}S_4]$ cores.

Chemical environments surrounding the $[Fe^{III}S_4]$ core are also important in determining properties of the core. For example, the MCD spectrum of $[Fe(S_2\text{-}o\text{-xyl})_2]^-$ exhibited entirely different feature within 300—400 nm from those of the peptide complexes and rubredoxin (Table 1) in spite of the similarities of the $[Fe^{III}S_4]$ core structures as confirmed by the X-ray structural analyses.[3,8)]

Although the absorption and MCD spectra of the peptide Fe(III) complexes did not show remarkable differences among a series of the peptide ligands examined, their CD spectra exhibited different spectral profiles as shown in Table 1. The Cotton effects in the charge-transfer region are originated with chiral charge-transfer from thiolate to Fe(III) influenced by asymmetric α-carbons of the peptide ligands. Spatial distribution of the asymmetric carbons is crucial in determining the CD spectral features. Restricted conformation of the peptide ligands around the metal ion will result in large Cotton effects. Therefore, chelate ring formation is related to the large Cotton effects.

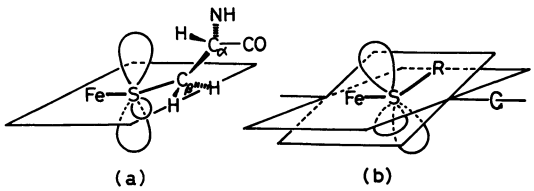

Fig. 3. (a) Schematic representation of lone pairs on S atom in $[Fe(S\text{–}Cys)_4]$ core. (b) Rotation about Fe–S bond in (a).

According to the above criteria, the Fe(III) complex of Z–Cys–Thr–Val–Cys–OMe, Z–Cys–Pro–Leu–Cys–OMe, and Z–Cys–Ala–Ala–Cys–OMe have *S,S*-chelate structures when comparisons were made with the CD spectrum of the Fe(III) complex of Z–Ala–Cys–OMe. The tetrapeptide complexes had the stronger Cotton effects; 10—50 times greater than the dipeptide complex (Table 1). The tripeptide complex shows about half CD strength of the tetrapeptide (Table 1). Further studies are required to determine the *S,S*-chelation of the tripeptide to Fe(III) ion.

Comparisons between Fe(III) Complexes of Cysteine-containing Peptides and Oxidized Rubredoxin. Relationships of the properties of the $[Fe^{III}S_4]$ cores with the spectral arrangement of the cysteine-containing peptides among the peptide Fe(III) complexes and oxidized rubredoxin will be discussed in terms of the spectral characteristics as diagnostic tools. A given orientation of the S–C_β bond around the Fe–S bond fixes location of the lone pairs on the S atom and, consequently, determines the electronic state of the $[Fe^{III}S_4]$ core, which is reflected sensitively by the MCD spectra as discussed above. Therefore, similarity of the MCD spectra between the Fe(III) complexes of Z–Cys–Pro–Leu–Cys–OMe or Z–Cys–Ala–Ala–Cys–OMe and rubredoxin (Table 1) allows us to conclude that they have similar $[Fe^{III}S_4]$ core electronic structures

controlled by the orientations of the S–C_β bonds around the Fe–S bonds. The other peptide complexes presumably have somewhat different orientations of the S lone pairs, especially π-type orbitals, which influence their MCD spectra in the $3t_2$–$4t_2$ transitions (Fig. 2).

The CD spectral resemblances between the peptide Fe(III) complexes and the native protein mean the similar arrangements of the asymmetric α-carbons between them as well as the similar orientations of the S–C_β bonds (Fig. 3a). From such a point of view, the Fe(III) complex of Z–Cys–Ala–Ala–Cys–OMe has spatial arrangement of the peptide ligands closely similar to rubredoxin around the [$Fe^{III}S_4$] core. The other tetrapeptide, Z–Cys–Thr–Val–Cys–OMe or Z–Cys–Pro–Leu–Cys–OMe, could not reproduce the CD spectral characteristic of rubredoxin completely. Cooperative effects of the both tetrapeptide units or whole protein environments seem to be important for subtle adjustments of the active site properties of native rubredoxin.

Thermal Stabilities of Fe(III) Complexes of Cysteine-containing Peptides. The thermal stabilities of the Fe(III) peptide complexes followed in the order (Table 2): Z–Cys–Thr–Val–Cys–OMe>Z–Cys–Ala–Ala–Cys–OMe>Z–Cys–Pro–Leu–Cys–OMe>Z–Ala–Cys–OMe>Z–Cys–Ala–Cys–OMe. It is interesting that the Fe(III) complex of Z–Cys–Pro–Leu–Cys–OMe, although having one of the Fe binding peptide units of rubredoxin, is somewhat less stable under these conditions. On the other hand, the Fe(III) complex of Z–Cys–Thr–Val–Cys–OMe had fairly good stability toward the oxidation of the thiolate ligand. Steric effects caused by different conformations of the two tetrapeptides in the Fe(III) complexes presumably resulted in such differences in their thermal stabilities, which were supported by their CD spectra. A recent synthesis of thermally stable $[Fe^{III}(SC_{10}H_{13})_4]^-$ ($C_{10}H_{13}S^-$= 2,3,5,6-tetramethylbenzenethiolate) revealed that the steric and conformational properties of the thiolate ligands are crucial in accounting for the stabilities of Fe(III) thiolate complexes because the synthesis of the corresponding benzenethiolate derivative has not been successful.[20] Importance of the steric and conformational effects on the cysteine-containing peptide ligands is supported by the fact that reconstituted adrenodoxin containing one Fe(III) ion at its active site, which exhibits oxidized rubredoxin-like spectra, is unstable in the Fe(III) state.[21] Adrenodoxin, which is one of the iron–sulfur proteins, has a [$Fe_2S^*_2$] core (S* denotes inorganic sulfide) chelated by two tetrapeptide units as Cys(52)–Ser–Thr–Cys(55) and Cys(92)–Glu–Ile–Cys(95) with the cysteinyl thiolates in its native state.[21] On the reconstitution, one Fe(III) ion will be accommodated by these cysteinyl thiolate ligands, but the [$Fe^{III}S_4$] core is not stabilized by the tetrapeptide units in the active site of adrenodoxin.

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