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A Poly(oxyethylene)-Supported Cys–Pro–Leu–Cys/Fe(II) Complex as a Rubredoxin Model: Protection of the Fe–Cys Coordination from Hydrolysis in Aqueous Solution

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ABSTRACT: Hg(II) and Fe(II) complexes of PEG–CO–Cys–Pro–Leu–Cys–OMe (PEG = poly(oxyethylene)) each having an invariant sequence of rubredoxin were synthesized. The complexes were characterized by ^{13}C NMR and CD spectral methods. In aqueous solution, the Fe(II) complex is considerably resistant to hydrolysis compared to the Fe(II) complex of PEG–CO–Cys–OMe because of protection of the Fe(II) core by hydrophobic side chains of Pro and Leu residues.

KEY WORDS Fe(II) / Peptide Complex / Polymer-Supported Cys-Containing Peptide / Rubredoxin Model /

X-Ray structural studies of metalloproteins showed their metal ion to be surrounded by a hydrophobic hydrocarbon layer of a certain thickness.[1,2] Hydrophilic amino acid residues are located outside the proteins and enable the whole protein to dissolve in water. Hydrophobic amino acid residues, *e.g.*, Pro and Leu, are closest to the coordinating amino acid residues. In the case of rubredoxin, an iron-containing protein functioning as an electron transfer agent, a characteristic sequence, Cys–Pro–Leu–Cys, was found to chelate the iron ion by two cysteine thiolate groups.

The Fe(II) and Fe(III) complexes of various Cys-containing peptides were synthesized as a model of rubredoxin having one Fe(II)/(III) ion surrounded by four Cys–thiolato ligands.[3,4] An Fe(II) complex of Z–Cys–Pro–Leu–Cys–OMe (Z = benzyloxycarbonyl) was found stable in an aqueous micellar solution.[5] We attempted to synthesize some water-soluble Cys-containing peptides chemically bound to poly(oxyethylene) as the hydrophilic part. Mutter *et al.* employed poly(oxyethylene) for peptide synthesis.[6,7] Bayer *et al.* succeeded in the synthesis of heme-containing poly(oxyethylene) as a water-soluble model of hemoglobin.[8] $MoO_2(Cys–O–PEG)_2$ was synthesized by us as a model of molybdo-oxidase.[9]

This paper presents the synthesis of the Hg(II) and Fe(II) complexes of polymer-supported Cys-containing peptides prepared by condensation at the peptide amino groups, using poly(oxyethylene) with carboxylic acid groups at both ends. The amide condensation was carried out for the following reasons. 1) The amide condensation can be performed more efficiently than the ester condensation in the presence of a considerable amount of water in commercial PEG samples. 2) The deblocking of protection groups in the side chains of peptides must be done under strongly acidic or basic conditions. For example, the removal of Acm group from Cys(Acm)-containing peptides must be carried out with

$HgCl_2$ and H_2S with the peptide exposed to hydrogen chloride formed by the deblocking process. 3) Most heavy metal complexes possess weak catalytic activity for the hydrolysis of ester group.[10]

Leibfritz *et al.* analyzed the structure of Boc–(Aib–L-Ala)$_5$–O–PEG (Boc = *t*-butyloxycarbonyl, Aib = 2-methylalaninyl) by the ^{13}C NMR technique so as to distinguish between random coil and α-helical conformations.[11] In this paper, we detected a hydrophobic interaction between the bulky side chains of the Cys–Pro–Leu–Cys sequence in an aqueous solution of its Hg(II) complex. The remarkable stabilization of Fe–S(Cys) bonding by such a hydrophobic effect in water was found.

EXPERIMENTAL

Materials

All solvents were purified by distillation before use. Dicyclohexylcarbodiimide (DCC) was purchased from Protein Research Institute. Potassium permanganate and poly(oxyethylene) (PEG) were obtained from Wako Chemical Co. The synthesis of Z–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe (Acm = acetamidomethyl) and Hg_2Cl_2 (Z–Cys–Pro–Leu–Cys–OMe)[12] was carried out by the same method in the previous paper.[3]

Synthesis

$HOOCCH_2(OCH_2CH_2)_nOCH_2COOH$ (PEG–COOH). About 150 g (0.15 mol) of PEG (MW, 2000) were dissolved in 500 ml of water. To this solution was added an aqueous solution (100 ml) of potassium permanganate (25 g, 0.16 mol) at room temperature. The mixture was stirred at 40°C for 4 h. After black precipitates were filtered off, the filtrate was acidified with 2 *N* HCl aq and extracted with 300 ml of dichloromethane. The organic layer was washed twice with water. The solution was concentrated to about one-fifth the original volume under reduced pressure. The addition of 500 ml of ether to the solution resulted in the precipitation of a white material. The content of the carboxyl group was titrated with 0.1 *N* NaOH aq, using phenolphthalein as an indicator. The results indicated a conversion of about 20% of OH into COOH.

PEG–CO–Cys(Acm)–OMe (**1**). To a mixture of HCl·Cys(Acm)–OMe (0.73 g, 3 mmol) and triethylamine (0.42 ml, 3 mmol) in 50 ml of dichloromethane was added a solution of PEG–COOH (15 g; COOH content, 3 mmol) in 50 ml of dichloromethane. 0.62 g (3 mmol) of DCC was then added with vigorous stirring at 0°C. The mixture was allowed to stand for 48 h at room temperature and filtered to remove dicyclohexylurea. The filtrate was washed with water twice, dried over sodium sulfate, and concentrated to 20 ml under reduced pressure. The addition of 200 ml of ether resulted in the precipitation of a white solid, yield, 82%. Elemental analysis for nitrogen (N, 1.37%) indicated that about 16% of Cys(Acm)–OMe had been introduced into PEG.

PEG–CO–Cys(HgCl)–OMe (**2**). To a solution of PEG–CO–Cys(Acm)–OMe (4 g, 0.3 mmol) in DMF (2 ml), 0.24 g (0.9 mmol) of $HgCl_2$ was added at room temperature. After 100 ml of dichloromethane were added to the solution, the mixture was washed twice with water. The organic layer was dried over sodium sulfate and filtered. A white solid was precipitated with the addition of 200 ml of ether to the filtrate. Yield, 95%. Two Raman bands assignable to (S–Hg–Cl) were observed at 315 and 283 cm^{-1}.

PEG–CO–Cys–OMe (**3**). Hydrogen sulfide was passed through a solution of **2** in 50 ml of methanol. The resulting black precipitates were filtered off and the filtrate was concentrated under reduced pressure. The white residue obtained was washed with 100 ml of degassed ether and dried over NaOH under an argon atmosphere. Estimation of the SH group of **3** was carried out by spectroscopic titration with 2,2′-dithiobis(5-nitropyridine).[13]

The SH content of **3** per 1 g of the polymer was 0.75×10^{-4} mol g^{-1}. The polymer was air-sensitive, soluble in water, chloroform, or DMF, and insoluble in ether.

PEG–CO–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe (**4**). Z–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe (0.3 g, 0.4 mmol) was dissolved in 5 ml of HBr/acetic acid at room temperature. After 2 h, the addition of 100 ml of ether to the mixture gave HBr salts of the peptide, which were collected by filtration and dried over NaOH. To a solution of the HBr salts (0.2 g, 0.3 mmol) and triethylamine (0.04 ml, 0.3 mmol) in 100 ml of chloroform was added PEG–COOH (3 g; COOH, 0.3 mmol), DCC (0.06 g, 0.3 mmol), and N-hydroxysuccinimide (0.04 g, 0.3 mmol) at 0°C. The solution was allowed to stand at room temperature for 48 h. The solution was filtered, washed twice with water, dried over sodium sulfate, and concentrated to 10 ml under reduced pressure. The addition of 100 ml of ether to the solution caused precipitation of a white solid. Yield, 85%.

PEG–CO–Cys(HgCl)–Pro–Leu–Cys(HgCl)–OMe (**5**). The complex was synthesized by the same procedure described for **2**. Yield, 93%. Raman bands were found at 314 and 281 cm^{-1}.

PEG–CO–Cys–Pro–Leu–Cys–OMe (**6**). The polymer was synthesized by the same method described for **3**. Estimation of the SH group was carried out by the same method for **3**. SH content in the polymer (**6**) was 1.2×10^{-4} mol g^{-1}. Yield, 80%. The polymer was air sensitive, soluble in water, chloroform, or DMF, and insoluble in ether.

Solution of $[Et_4N]_2[Fe(PEG–CO–Cys–OMe)_4]$ (**7**) *and* $[Et_4N]_2[Fe(PEG–CO–Cys–Pro–Leu–Cys–OMe)_2]$ (**8**). The solution was prepared from $[Et_4N]_2[FeCl_4]$ and **3** or **6** in DMF by the same method reported previously without further purification.[5] An aqueous solution of **8** was prepared by the addition of water to the residue obtained at the concentration of the above DMF solution. The solution was used for the physical measurements described in this paper. The synthetic scheme is shown in Figure 1.

Physical Measurements

^{13}C NMR spectra were recorded on a JEOL FX-90Q spectrometer at 31°C. Circular dichroism (CD) spectra were obtained on a JASCO J-40A at room temperature. The value of $\Delta\varepsilon$ was expressed in units of $M^{-1}\,cm^{-1}$. Raman spectra were obtained so as to detect Hg–S and Hg–Cl bonds, using a JASCO R-

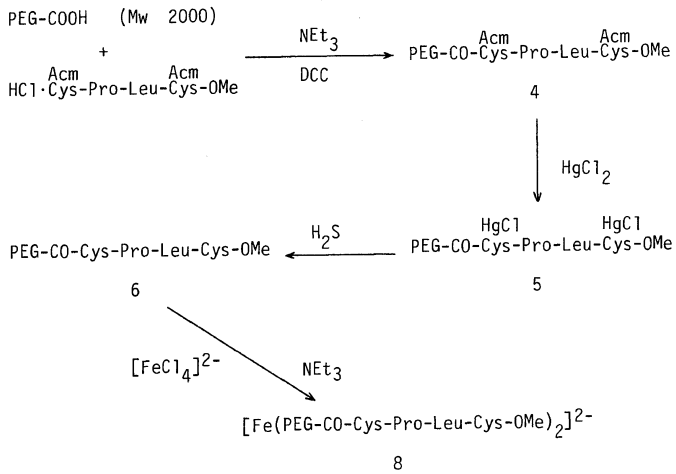

Figure 1. Synthetic processes of polymer-supported peptides and their Hg(II) and Fe(II) complexes.

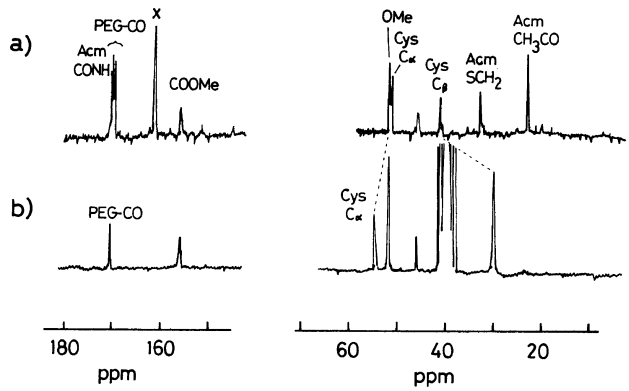

Figure 2. ^{13}C NMR spectra of a) PEG–CO–Cys(Acm)–OMe in $CDCl_3$ and b) PEG–CO–Cys(HgCl)–OMe in Me_2SO-d_6.

800 spectrometer at an exciting line of 488.0 nm.

RESULTS AND DISCUSSION

Structure in Solution of S-Protected Peptides

The ^{13}C NMR spectral technique is useful for analyzing oligopeptides/metal complexes attached to PEG parts, since the methylene carbons of the PEG parts give sharp signals centered at 73 ppm in $CDCl_3$ or Me_2SO-d_6. Figure 2 shows the ^{13}C NMR spectra of PEG–CO–Cys(Acm)–OMe in $CDCl_3$ and PEG–CO–Cys(HgCl)–OMe in Me_2SO-d_6. The assignments of Cys and PEG–CO parts were readily performed and shown in Figure 2. The Cys C_β carbon signal of Cys(Acm) was observed at 40.1 ppm in $CDCl_3$, while that of Cys(HgCl) shifted to 31.0 ppm in Me_2SO-d_6. Such a shift has also been observed for a ^{13}C NMR signal of the C_β carbon adjacent to a thiolato ligand coordinated to a Pd(II) ion.[14] A lowfield shift (12 ppm) of C_β signal was induced by Hg–S bonds (Table II) but other carbon signals, such as PEG–CO and COOMe, were not affected by such bonds. PEG–CO–Cys(HgCl)–OMe exhibited almost the same chemical shifts of ^{13}C NMR signals in Me_2SO-d_6 and in D_2O, as evident from the slight, steady shifts by solvation of Me_2SO-d_6 to all residues.

The ^{13}C NMR spectra of the PEG-supported tetrapeptide and its Hg(II) complex in Me_2SO-d_6 and D_2O are shown in Figures 3, 4 and Tables I and II along with the spectrum of Z–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe. The ^{13}C NMR signals of the polymer-

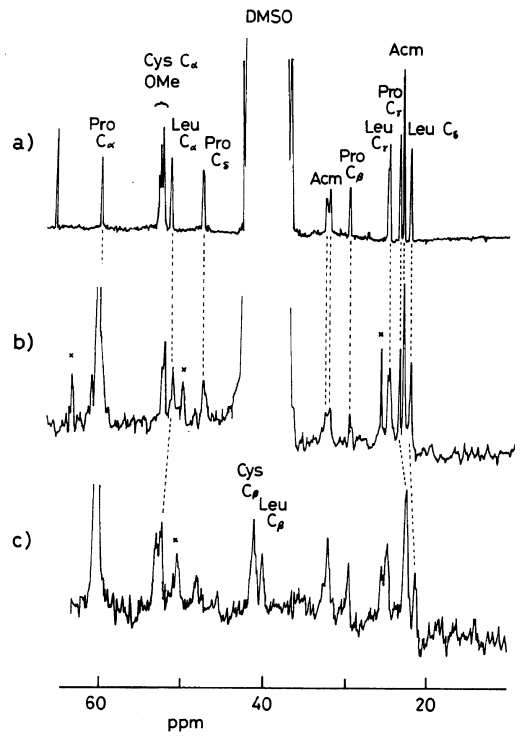

Figure 3. ^{13}C NMR spectra of a) Z–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe in Me_2SO-d_6, b) PEG–CO–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe (**4**) in Me_2SO-d_6 and c) **4** in D_2O.

Table I. ^{13}C NMR chemical shifts of Z–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe and PEG–CO–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe in Me_2SO or D_2O

Compounds	Z–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe in Me_2SO-d_6	PEG–CO–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe **4**	
		in Me_2SO-d_6	in D_2O
Cys C_α	52.3	52.2	—
	52.7		
C_β	40.0—43.0	40.0—43.0	42.0
Acm S-CH_2	31.9	31.9	31.2
CH_3CO	22.5	22.5	21.7
Pro C_α	59.5	59.5	59.5—60.5
C_β	29.4	29.4	29.0
C_γ	23.1	23.0	21.7
C_δ	46.8	46.9	47.0
Leu C_α	50.8	51.0	53.3
C_β	—	39.0—41.0	39.2
C_γ	24.3	24.3	24.2
C_δ	21.7	21.7	20.4
$COOCH_3$	52.0	52.1	51.8
CH_2Ph	65.5		

Table II. ^{13}C NMR chemical shifts of Z–Cys(HgCl)–Pro–Leu–Cys(HgCl)–OMe and PEG–CO–Cys(HgCl)–Pro–Leu–Cys(HgCl)–OMe in Me_2SO or D_2O

Compounds	Z–Cys(HgCl)–Pro–Leu–Cys(HgCl)–OMe in Me_2SO-d_6	PEG–CO–Cys(HgCl)–Pro–Leu–Cys(HgCl)–OMe **5**	
		in Me_2SO-d_6	in D_2O
Cys C_α	55.1	55.8	Undetected
C_β	29.8	29.8	32.0
Pro C_α	60.4	60.3	60.3
C_β	29.1	29.1	Undetected
C_γ	22.8	22.8	21.7
C_δ	47.1	47.1	47.0
Leu C_α	51.1	51.1	50.8
C_β	40.0—41.0	40.0—41.0	39.1
C_γ	24.3	24.3	24.5
C_δ	21.4	21.8	20.7
$COOCH_3$	51.9	51.9	52.0
CH_2Ph	65.5		

supported peptide were observed to have almost the same chemical shifts as those of Z–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe. The PEG part of PEG–CO–Cys(Acm)–Pro–Leu–Cys(Acm)–OMe did not influence any of the

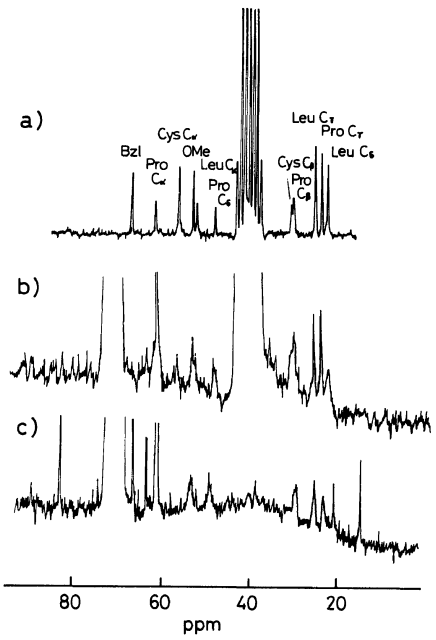

Figure 4. ^{13}C NMR spectra of a) Z–Cys(HgCl)–Pro–Leu–Cys(HgCl)–OMe in Me_2SO-d_6, b) PEG–CO–Cys(HgCl)–Pro–Leu–Cys(HgCl)–OMe (**5**) in Me_2SO-d_6 and c) **5** in D_2O.

chemical shifts of the carbons listed in Table I. The ^{13}C chemical shifts of the Leu C_α ($\Delta\delta$= 2.3 ppm) ($\Delta\delta$ is the difference between the chemical shifts in Me_2SO-d_6 and in D_2O), Leu C_δ ($\Delta\delta = 1.3$ ppm), and Pro C_γ ($\Delta\delta = 1.3$ ppm) carbons in D_2O suggest the occurrence of hydrophobic interactions by Pro and Leu side chains. Only a small difference in the CD spectra of methanol ($\Delta\varepsilon_{198}$: -0.72) and water ($\Delta\varepsilon_{198}$: -0.70) solutions was recognized. Therefore, the difference in the chemical shifts of ^{13}C NMR signals and the absence of any distinction between the CD spectra in MeOH and water are ascribed to the interactions of the side chains, but not to the change in conformational probability about amide chromophores. Thus, the slight conformational change about the chiral C_α of the main chain with a hydrophobic interaction, though local interactions in the case of chains, may leads to considerable change in the chemical shifts assigned to Pro and Leu residues.

Figure 4 and Table II show the ^{13}C NMR spectra and chemical shifts of Hg(II) complexes (**5**). The peptide part of **5** gave the same ^{13}C signals in Me_2SO-d_6 as those of Hg_2Cl_2 (Z–Cys–Pro–Leu–Cys–OMe). Assignments of the ^{13}C signals of **5** in D_2O were carried out by monitoring the ^{13}C signals in mixed solvents with various ratios of Me_2SO-d_6/D_2O. Solvent-dependent shifts of the ^{13}C signals of Cys C_β ($\Delta\delta = 2.2$ ppm), Pro C_γ ($\Delta\delta = 1.1$ ppm), Leu C_α ($\Delta\delta = 0.3$ ppm), Pro C_δ ($\Delta\delta = 1.1$ ppm) were observed in Me_2SO-d_6 and D_2O and are listed in Table II. The NMR results suggest the effect of a hydrophobic interaction by the side chain of the Cys–Pro–Leu–Cys sequence and **4** as well.

Fe(II) Complexes of Polymer-Supported Cys-Containing Peptides

In our previous paper, Fe(II) complexes of Cys-containing peptides, as models of rubredoxin, were characterized by visible, CD, and MCD spectra.[4,5] Figure 5 shows the CD spectra of Fe(II)/PEG–CO–Cys–Pro–Leu–Cys–OMe (1 : 2), **8**, in DMF or H_2O and Fe(II)/PEG–CO–Cys–OMe (1 : 4), **7**, in DMF. No CD extremum was observed for the Fe(II)/PEG–CO–Cys–OMe (1 : 4) in H_2O since the complex was apparently hydrolyzed completely under these conditions. **7** shows small extrema at 370 and 520 nm in DMF. Such a non-chelating Cys–peptide is considered not to provide a stable Fe(II) complex.[3] **8** exhibits a strong CD maximum at 310 nm ($\Delta\varepsilon$: +7.2) in DMF, as was also the case for a Fe(II) complex of Z–Cys–Pro–Leu–Cys–OMe. The strong CD extrema of **8** indicate the chelating coordination of the Cys–Pro–Leu–Cys sequence to a Fe(II) ion. A small shoulder at 380 nm ($\Delta\varepsilon$: +0.4) and a trough at 465 nm ($\Delta\varepsilon$: −0.3) are assignable to the CD extrema of a Fe(III) complex of PEG–CO–Cys–Pro–Leu–Cys–OMe formed by oxidation of **8** with contaminating dioxygen.

8 in water exhibits CD extremum at 318 nm ($\Delta\varepsilon$: +1.2) due to ligand-metal charge transfer

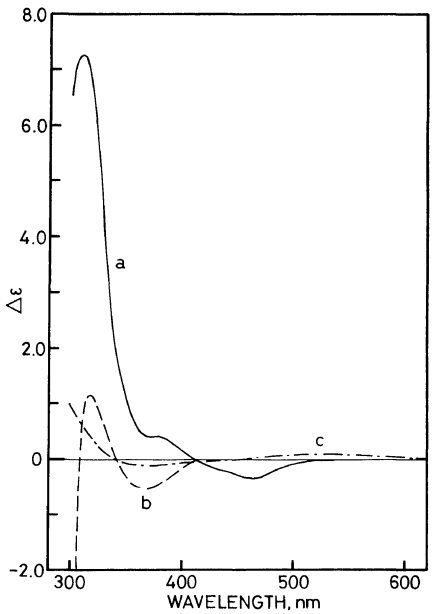

Figure 5. CD spectra of a) Fe(II)/PEG–CO–Cys–Pro–Leu–Cys–OMe (1 : 2) (**8**) in DMF, b) **8** in D_2O, and c) Fe(II)/PEG–CO–Cys–OMe (1 : 4).

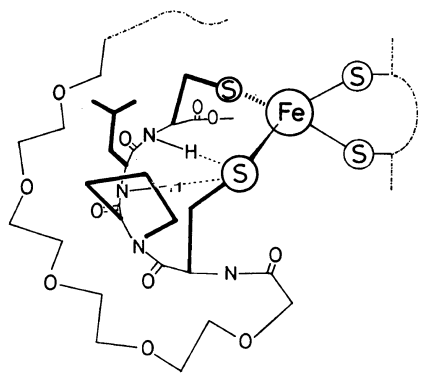

Figure 6. Proposed structure of Fe(II)/PEG–CO–Cys–Pro–Leu–Cys–OMe complex in aqueous solution.

of S→Fe(II) and an unusual CD trough at 368 nm ($\Delta\varepsilon$: −0.5). The intensity of the CD extremum at 318 nm indicates that 20% of **8** remains in an aqueous solution without hydrolysis. Thus, contact of **8** with large amounts of water on preparation of an aqueous solution of **8** may result in partial decomposition. However, after **8** was completely formed in an aqueous solution, the Fe(II)

complex was stabilized by the influence of the hydrophobic side chains of Pro and Leu residues.

An Fe(II) complex of Z–Cys–Pro–Leu–Cys–OMe has been reported to be more resistant to hydrolysis than that of Z–Cys–Thr–Val–Cys–OMe or Z–Cys–Ala–Ala–Cys–OMe in an aqueous micellar solution.[5] The Pro and Leu side chains of the Cys–Pro–Leu–Cys sequence are thus considered to protect an Fe(II) core by a hydrophobic interaction not only in an aqueous micellar solution, but in an aqueous solution of the PEG-supported peptide complex, as shown in Figure 6. The entire molecule of **8** is solubilized in an aqueous solution by the PEG part. From the present results, stability of Fe–thiolato bonding in native rubredoxin against hydrolysis is thus demonstrated.

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