Oxidized Rubredoxin Models. Iron(III) Complexes of Z-Cys-Ala-Ala-Cys-OMe and Z-Ala-Cys-OMe

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Oxidized rubredoxin models using Fe(III) ion and cysteine-containing peptide as Z-Cys-Ala-Ala-Cys-OMe were synthesized in solution and characterized by the absorption, CD, MCD, and EPR spectra. The Fe(III)/Z-Cys-Ala-Ala-Cys-OMe complex exhibits similar CD spectra as well as absorption, MCD, EPR spectra to native oxidized rubredoxin. Thus, the model complex has a similar electronic configuration and core structure to that of native protein. From the comparison with the result of Fe(III)/Z-Ala-Cys-OMe complex, the tetrapeptide complex probably has a relatively stable chelate structure with the hairpin turn conformation of the tetrapeptide.

Iron-sulfur proteins play important roles in biological electron-transfer and oxygenation reactions.[1,2] These proteins have two major classes. One is a rubredoxin type, $[Fe(SR)_4]^{-,2-}$ (SR denotes cysteine thiolate), which has one or two independent active sites per molecule. Its active site is constructed by one iron atom with four cysteine thiolate groups but without inorganic sulfide. The X-ray crystallographic studies on oxidized rubredoxin from *Clostridium pasteurianum* revealed that the iron atom is coordinated by the four cysteine thiolates with distorted tetrahedral geometry.[3] The other is ferredoxin type, which has also one or two active sites per molecule. Each active site consists of two or four iron atoms with the same number of inorganic sulfide (S^{2-}) to form $[Fe_2S^*_2(SR)_4]^{n-}$(n=2 or 3) or $[Fe_4S^*_4(SR)_4]^{n-}$(n=1, 2, or 3) cluster, respectively (S* denotes inorganic sulfide), and each cluster is coordinated by four cysteine thiolates. Very recently the existence of new type ferredoxin, [3Fe3S*], was suggested.[4]

It is interesting that amino acid sequence, –Cys–A–B–Cys–, exists at the metal binding site of many iron-sulfur proteins. In such cases, it is supposed that conformational restriction due to the steric effects caused by side chains of the two amino acid residues, A and B, interposed between the two cysteine residues may contribute to the stabilities of chelate rings, the determination of the geometries around the metal ion, and the revelation of the enzymatic activities. Although many model studies for iron-sulfur proteins have been reported,[5–10] the roles of the protein or chelating units as ligands in controlling metal ion activities still remain ambiguous. It is presumed that the model studies by the use of peptide complexes possessing partial amino acid sequences of native metalloenzymes give significant informations about the roles of proteins. The preparation of cysteine-containing peptide-iron complexes so far investigated,[6,8,10] however, has employed sequencial oligopeptides such as $\{Gly-Cys-Gly\}_n$ which possess no sterical restriction by the side chains of two amino acids interposed between two terminal cysteine residues. For example, Rydon *et al.*[8] reported the rubredoxin-like complex formation between Fe(III) and $Ac\{Gly-Cys-Gly\}_nNH_2$ (n=1—4) but these complexes were very unstable to result in the oxidation of thiols by Fe(III) ion under strictly unaerobic conditions whereas native rubredoxin is stable under such conditions. Denatured rubredoxin[11,12] in 80 or 90% aqueous dimethyl sulfoxide (DMSO) exhibits a similar absorption spectra to that of native protein and it is stable under unaerobic conditions. These informations indicate that the stability of $[Fe(SR)_4]^-$ chromophore is dependent on the amino acid sequence of the active site as well as the whole protein environment. In native rubredoxin,[1] two well-separated tetrapeptide units at the binding site, Cys(6)–Thr–Val–Cys(9) and Cys(39)–Pro–Leu–Cys(42) for *C. pasteurianum* rubredoxin, should take specific conformations by interactions of side chains of the two amino acid residues interposed between two terminal cysteine residues, of course with the aid of the other peptide sequence, to form a stable chelate complex and to prevent the oxidation of thiols by Fe(III) ion. In view of importance of identity of amino acid residues in native metalloenzymes, these simple models such as Rydon's models are not sufficient in discussion of the role of amino acids in the structures and functions of iron-sulfur proteins. Thus we took the tetrapeptide such as Z–Cys–Ala–Ala–Cys–OMe in order to establish basic informations about the points mentioned above.

In this report, we describe the formation of $[Fe(SR)_4]^-$ moiety with cysteine-containing peptides. In particular, spectroscopic properties of Fe(III)/Z–Cys–Ala–Ala–Cys–OMe complex are discussed on the basis of its relevance to rubredoxin.

Experimental

Peptide Syntheses. The syntheses of peptides were carried out by mixed anhydride method using isobutyl chloroformate as a coupling reagent by stepwise elongation from C-terminus and cysteine thiolate group was protected by acetamidomethyl group. The detail will be reported separately. The SH-free peptides, Z–Ala–Cys–OMe and Z–Cys–Ala–Ala–Cys–OMe, were prepared by the reaction of the corresponding S-blocked peptides with Hg(II) and H_2S.

Complex Formation. 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.

The metal salt and 4 molar equivalents of SH-groups based on the cysteine contents of cysteine-containing peptide were dissolved in DMSO. The addition of an equimolar amount of triethylamine for one SH-group to the solution gave a deep red-violet solution. The color faded gradually in the absence of air, especially for Fe(III)/Z-Ala–Cys–OMe complex.

Physical Measurements. Absorption and circular dichroism (CD) spectra were measured on a JASCO UVIDEC-5A and a JASCO J-40 spectrometer, respectively. Magnetic circular dichroism (MCD) spectra were measured on a JASCO J-20 spectrometer equipped with an electromagnet in field strength of 1.5 T and corrected for the zero-field circular dichroism. Electron paramagnetic resonance (EPR) spectra were recorded on a JEOL JESFE 1X with 100 kHz magnetic field modulation using Mn(II) as *g*-marker ($g=1.981$). Absorption, CD, and MCD spectra were recorded at room temperature whereas EPR spectra at 77 K. The values of ε, $\Delta\varepsilon$, and $\Delta\varepsilon_M$ were based on the molar concentration of Fe(III).

Results and Discussion

Addition of triethylamine to a DMSO solution of cysteine-containing peptide and iron(III) chloride gave a deep red-violet solution. The resulting Fe(III) complex of cysteine-containing peptide were characterized with absorption, CD, MCD, and EPR spectra in solution. Figures 1 and 2 show the absorption and CD spectra of Fe(III)/peptide complex, respectively, together with those of native oxidized rubredoxin from *C. pasteurianum*[13,14] for comparison. The CD spectrum of native rubredoxin[14] was converted from wave-number-linear scale in the original paper to wavelength-linear scale and was reproduced in Fig. 2. The CD spectra of other rubredoxins from different organisms show similar spectral pattern to *C. pasteurianum* rubredoxin.[15]

The Fe(III)/Cys-containing peptide complexes showed absorption maxima at 355 and 495 nm, which are typical for the oxidized rubredoxin. These spectral

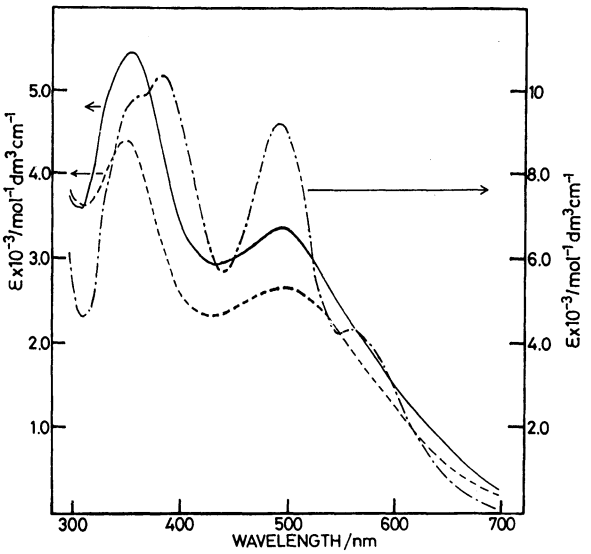

Fig. 1. Absorption spectra of Fe(III)/Cys-containing peptide complexes.
——: Fe(III)/Z-Cys-Ala-Ala-Cys-OMe complex in DMSO, ------: Fe(III)/Z-Ala-Cys-OMe complex in DMSO, -·-·-: oxidized rubredoxin (*C. pasterurianum*) in Tris buffer. The spectrum of the native protein (W. A. Lovenberg and B. E. Sobel, *Proc. Natl. Acad. Sci. U.S.A.*, **54**, 193 (1965)) was inserted together with our spectra for comparison.

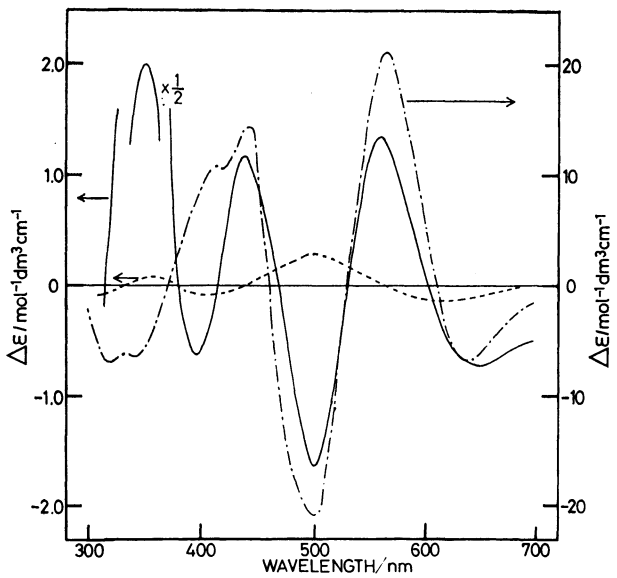

Fig. 2. CD spectra of Fe(III)/Cys-containing peptide complexes.
——: Fe(III)/Z-Cys-Ala-Ala-Cys-OMe complex in DMSO, ------: Fe(III)/Z-Ala-Cys-OMe complex in DMSO, -·-·-: oxidized rubredoxin (*C. pasteurianum*) in Tris buffer. The spectrum of the native protein (W. A. Eaton and W. Lovenberg, "Iron-Sulfur Proteins," ed by W. Lovenberg, Academic Press, New York and London (1973), Vol. II) was converted to wavelength-linear scale and was inserted together with our spectra for comparison.

patterns disappeared gradually due to the oxidation of thiols by the Fe(III) ion. Rydon *et al.*[8] reported that the rubredoxin-like spectra of Fe(III)/Ac{Gly-Cys-Gly}$_n$NH$_2$ ($n=1$—4) complex disappeared rapidly and after 5 min no typical spectra were observed in visible region under strictly unaerobic conditions. In our study, Fe(III)/Z-Ala-Cys-OMe complex was more unstable toward the autoxidation of thiols than Fe(III)/Z-Cys-Ala-Ala-Cys-OMe. The half-life times of absorbance at 495 nm of Fe(III)/peptide complexes were about 22 and 10 min for the Z-Cys-Ala-Ala-Cys-OMe complex and the Z-Ala-Cys-OMe complex, respectively.

The EPR spectra at 77 K showed signals at $g=4.0$ and 4.2 for Fe(III)/Z-Cys-Ala-Ala-Cys-OMe complex and Fe(III)/Z-Ala-Cys-OMe complex, respectively, whereas oxidized rubredoxin at $g=4.3$.[1] It is clear that these peptide complexes have high-spin d^5 Fe(III) structure under an approximately tetrahedral environment surrounded by four cysteine thiolates identical with the core structure of the active site of oxidized rubredoxin.

The MCD spectra also supported the conclusion but there was a little difference between dipeptide and tetrapeptide complex. The MCD spectrum of oxidized rubredoxin in the visible region has two dispersion type bands with crossover points at 382 and 495 nm, and a positive and negative bell-shaped bands at 565 and 357 nm, respectively.[14] Both dispersion type bands were assigned to the Faraday *A* terms, while the bell-shaped bands were assigned to the Faraday *B* terms.[14]

Hatano *et al.*[9] investigated the MCD spectra of Fe(III) complexes of various simple dithiols such as 1,2-ethanedithiol, 1,4-butanedithiol, 1,6-hexanedithiol, and *o*-xylene-α,α'-dithiol. They reported that Fe(III)/1,6-hexanedithiol complex has very similar MCD spectral pattern to that of oxidized rubredoxin and, consequently, a similar core structure, whereas Fe(III)/1,2-ethanedithiol complex has very different spectral pattern from that of oxidized rubredoxin. The complex of 1,2-ethanedithiol was reported to exhibit no dispersion type band at around 490 nm but a positive bell-shaped band at 590 nm ascribed to the Faraday *B* terms, and this indicates that this complex had a binuclear form, $[Fe(SCH_2CH_2S)_2]_2^{2-}$, with five sulfur atoms coordinated to each Fe(III) ion, and its geometry was distorted trigonal bipyramidal around the high-spin Fe(III) ion with coupling interaction between iron atoms confirmed with X-ray crystallographic study.[16]

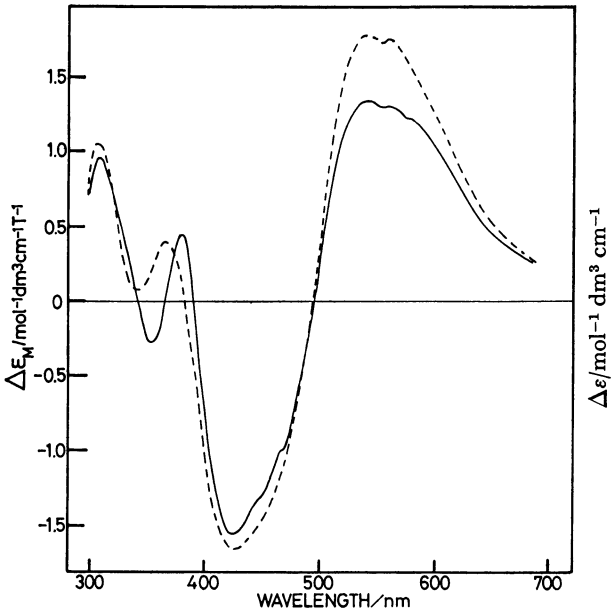

Fig. 3. MCD spectra of Fe(III)/Cys-containing peptide complexes.
——: Fe(III)/Z-Cys-Ala-Ala-Cys-OMe complex in DMSO, ------: Fe(III)/Z-Ala-Cys-OMe complex in DMSO.

Figure 3 shows the MCD spectra of the Fe(III)/peptide complexes. The MCD spectra of the di- and the tetrapeptide complexes in 400—700 nm region have similar spectral pattern. The dispersion type bands with crossover points at around 380 and 490 nm were assigned to the Faraday *A* terms and the positive bell-shaped band at around 540 nm to the Faraday *B* term tentatively. In the shorter wavelength below 400 nm, however, the complex of Fe(III)/Z-Cys-Ala-Ala-Cys-OMe had a negative bell-shaped band at 355 nm which was assignable to the Faraday *B* term. Similar MCD was observed for oxidized rubredoxin. On the other hand, Fe(III)/Z-Ala-Cys-OMe had a positive MCD band. Such difference has also been reported between Fe(III)/1,6-hexanedithiol and Fe(III)/1,4-butanedithiol or *o*-xylene-α,α'-dithiol complexes by Hatano *et al.*[9] They suggested that the MCD spectra of the dithiol Fe(III) complexes at around 350 nm reflect the chelate ring size.

In our case, the potential difference between Z-Cys-Ala-Ala-Cys-OMe and Z-Ala-Cys-OMe, affects the geometry of $[FeS_4]$ core (S denotes cysteinyl sulfur atom). The fact that Z-Cys-Ala-Ala-Cys-OMe can coordinate to Fe(III) ion as a chelating dithiolate ligand should be considered. The Fe(III)/peptide complexes have monomeric $[FeS_4]$ core structure as inferred from the MCD measurements and the core structure of Fe(III)/Z-Cys-Ala-Ala-Cys-OMe complex thus resembles to that of oxidized rubredoxin more closely than Fe(III)/Z-Ala-Cys-OMe complex.

Although the absorption, MCD, and EPR spectra of Fe(III)/peptide complexes did not show so large differences between the dipeptide and the tetrapeptide complexes, the CD spectra exhibited very different spectral pattern between them (Fig. 2). The CD spectrum of Fe(III)/Z-Cys-Ala-Ala-Cys-OMe complex was very similar to that of oxidized rubredoxin in visible region while the intensity was *ca.* 1/10. For oxidized rubredoxin the transitions at 565 and 494 nm have been assigned by MO treatments to the charge transfer transitions from occupied ligand π-orbitals to Fe d-orbitals.[17] The similarity in the CD spectra between oxidized rubredoxin and Fe(III)/Z-Cys-Ala-Ala-Cys-OMe complex in these transitions suggests that not only geometry of the $[FeS_4]$ core but also the major parts of the orientations of Fe–S–C_β (C_β denotes cysteine β carbon) groups resembles each other. The differences in the CD patterns and the CD strength between Fe(III)/Z-Cys-Ala-Ala-Cys-OMe and Fe(III)/Z-Ala-Cys-OMe complexes suggest that the $[FeS_4]$ core is formed for the dipeptide complex similar to the tetrapeptide complex but the orientations of the Fe–S–C_β groups largely differ from the tetrapeptide complex because the CD spectra are generally very sensitive to both the identity of asymmetric ligands and their orientations relative to the metal.

Bair *et al.*[18] studied the electronic properties of the active site of rubredoxin by *ab initio* methods. They used $[Fe(SH)_4]^{-/2-}$ as a model complex for the rubredoxin active site and examined several different sets of S–Fe–S–H dihedral angles. They found that energy separation of the d-d transitions is sensitive to the dihedral angle of the S–H bonds, and that the orientations of the S lone pairs pointing toward Fe ion, which are strongly influenced by the positions of the S–H bonds, directly affect the splitting energies of the Fe d-orbitals and thus play important roles in determining the redox properties of the Fe site. So it is expected that the tetrapeptide complex has different redox properties and also catalytic activities from the dipeptide complex.

We have studied Pd(II)/Z-Cys-Ala-Ala-Cys-OMe complex and revealed that the tetrapeptide coordinates to Pd(II) at two cysteine thiolate groups as a *cis*-chelate ligand from the conformational analyses of the peptide using ^{1}H-NMR spectra.[19] Other related di-cysteine peptides that we have also examined, Z-Cys-Ala-Cys-OMe, Z-Cys-Val-Val-Cys-OMe, and Z-Cys-Gly-Pro-Cys-OMe, were not able to take such stable chelate structure. These results allow us to conclude

that the tetrapeptide, Z–Cys–Ala–Ala–Cys–OMe, prefers a folded structure like a hairpin turn to form a stable *S,S-cis*-chelate Pd(II) structure which has rigid square planar geometry. Stability of such chelate structures should be influenced by the identity and sequence of amino acids intervening the two terminal cysteine residues. It is reasonable to think that the conformational restriction toward chelate ligand caused by tetrahedral metal ion as Fe(III) is looser than by square planar metal ion as Pd(II). Thus, a macro ring chelate structure probably exists for Fe(III)/Z–Cys–Ala–Ala–Cys–OMe system although direct evidences such as X-ray structure are lacking.

In conclusion, the CD spectrum as well as the absorption, MCD, and EPR spectra of Fe(III)/Z–Cys–Ala–Ala–Cys–OMe complex is similar to those of oxidized rubredoxin. The tetrapeptide complex has the mononuclear $[Fe(S\text{–}C_\beta)_4]^-$ structure similar to the rubredoxin active site and probably has the chelate structure although the amino acid sequence is different from the native protein except for two terminal cysteines. The tetrapeptide complex was more stable than the dipeptide complex. This is interesting in connection with the fact that mononuclear Fe(III) complex with monodentate thiolate ligand such as benzenethiolate anion has not been isolated whereas corresponding bidentate chelate complex, $[Fe(o\text{-xylene-}\alpha,\alpha'\text{-dithiolate})_2]^-$, is isolated.[7] Conceivably, Z–Cys–Ala–Ala–Cys–OMe chelated to Fe(III) ion takes a relatively stable conformation by the interaction between side chains of the alanyl residues to resist the oxidation of thiols. It is presumed that a tetrapeptide unit such as Cys–Gly–Gly–Cys lacking such interaction takes more flexible conformation and consequently the stability of the complex will decrease even if a chelate structure exists.

The roles of amino acid residues intervening the two terminal cysteine residues on the geometry and stability of active site of native enzyme is now being investigated using oligopeptides having the same sequence as the native enzyme active site.

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