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A Novel Method for Determining the Chelation Ability of the Cysteine-Containing Peptides with 3,4-Toluenedithiol. Application to [2Fe-2S]-Ferredoxin Model Systems

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The chelating ability of a bidentate ligand, Cys-X-Y-Cys (X, Y=amino acid residue), in 2Fe-2S peptide model complexes was examined by a ligand-exchange method using 3,4-toluenedithiol (tdt-H_2) or *o*-xylene-α,α'-dithiol. The addition of tdt-H_2 to an *N,N*-dimethylformamide solution of $[Fe_2S_2(Z\text{-cys-Ala-Ala-cys-OMe})_2]^{2-}$ results in the cleavage pf Fe-S* (S*=inorganic sulfur) bond and in the formation of hydrogen sulfide and mononuclear Fe(III) peptide thiolate complex, $[Fe(Z\text{-cys-Ala-Ala-cys-OMe})(tdt)]^{1-}$. In the case of $[Fe_2S_2(Z\text{-Ala-cys-OMe})_4]^{2-}$, $[Fe_2S_2(tdt)^2]^{2-}$ was obtained by the substitution of Z-Ala-Cys-OMe with the chelating reagent. On the other hand, *o*-xylene-α,α'-dithiol expels both Z-Cys-Ala-Ala-Cys-OMe and Z-Ala-Cys-OMe ligands from these complexes.

An inherent lability of teterahedrally coordinated Fe(II) or Fe(III) species in solution is manifest in the observed ease in the core extrusion from iron-sulfur proteins or in the thiolate ligand exchange of their synthetic model complexes, e.g. $[Fe_2S_2(SR)_4]^{2-}$.[1] Bridging μ-sulfido ligands are also labile in these iron complexes and interconversion among [4Fe-4S], [3Fe-4S], and [2Fe-2S] clusters has been recently found in many iron-sulfur proteins.[2] Similar changes in cluster structures are studied for some model complexes.[3] Thus, we have reported a smooth conversion of $[Fe_2S_2(peptide)_4]^{2-}$ to $[Fe_4S_4(peptide)_4]^{2-}$ upon one-electron reduction.[4] All these reactions depend on the lability of the Fe-S bonds which is important for their functions in biological systems.

The high lability of monodentate thiolate ligands in Fe(II) complexes or the instability in Fe(III) complexes has been found to decrease by effective chelation.[5] Thus, remarkable thermal stability of oxidized rubredoxin (Fe(III) state) is ascribed to the multiple chelation of the protein. In the related model complex, $[Fe(Z\text{-cys-Pro-Leu-cys-OMe})_2]^{1-,2-}$ (Z=benzyloxycarbonyl), we have found a remarkable stabilizing effect due to chelation.[6,7] In native [2Fe-2S]-ferredoxins, the oxidized state is also stable by chelation where an invariant peptide sequence, Cys-A-B-C-D-Cys-X-Y-Cys chelates in tridentate fashion.[8] Here, the Cys-X-Y-Cys part is bridging the two Fe(III) ions and Cys-A-B-C-D-Cys part is chelating.

In the previous paper we reported the chelation of a tetrapeptide Cys-X-Y-Cys to $[Fe_2S_2]^{2+}$ ion.[9] However, we could not determine whether the Cys-X-Y-Cys bridges between the two Fe(III) ions of $[Fe_2S_2]^{2+}$ or chelates to one Fe(III) ion of $[Fe_2S_2]^{2+}$.

In this paper, we describe a novel method for determination of the mode of chelation of Cys-X-Y-Cys to $[Fe_2S_2]^{2+}$ core utilizing the ligand-exchange reaction with 3,4-toluenedithiol. This reagent has been employed for attempt to extrude some inorganic clusters from iron-sulfur proteins.[10]

Experimental

All manipulations involving solvent purifications, air-sensitive iron thiolate, and peptide complexes were carried out under argon atmosphere.

Materials. 3,4-Toluenedithiol (tdt-H_2) was obtained from Nakarai Chemical Co. and used without purification. Tetrahydrofuran (THF) was purified by distillation after sufficient refluxing over sodium metal. *N,N*-Dimethylformamide (DMF) was purified by distillation under reduced pressure before use. *o*-Xylene-α,α'-dithiol (*o*-xyl-$(SH)_2$) was prepared by the literature method.[11] $[NEt_4]_2[Fe_2S_2(S\text{-}t\text{-}Bu)_4]$ (**1**), $[NEt_4]_2[Fe_2S_2(Z\text{-Ala-cys-OMe})_4]$ (**2**),[12] and $[NEt_4]_2[Fe_2S_2(Z\text{-cys-Ala-Ala-cys-OMe})_2]$ (**3**) were synthesized by the method reported in the previous paper.[9] $[NEt_4]_2[Fe_2S_2(S_2\text{-}o\text{-xyl})_2]$ (**4**) was prepared according to the reported procedure.[13]

$[NEt_4]_2[Fe_2S_2(tdt)_2]$ (tdt=3,4-toluenedithiolate). To a solution of $[NEt_4]_2[Fe_2S_2(S\text{-}t\text{-}Bu)_4]$ (200 mg, 2.5×10^{-4} mol) in acetonitrile (20 cm^3) was added 3,4-toluenedithiol (36 mg, 2.3×10^{-4} mol) with stirring at room temperature. The solution was concentrated under reduced pressure and the residue was washed with fresh THF. The powder obtained was recrystallized with acetonitrile and ether and gave black microcrystals. Found: C, 46.73; H, 7.39; N, 3.33. Calcd for $C_{30}H_{62}N_2S_6Fe_2$: C, 48.38; H, 7.04; N, 3.76. Absorption maxima (DMF); 550 nm (ε 4000), 496 nm (ε 5260), 475 nm (sh, ε 4800), 383 nm (ε 9100), and 320 nm (ε 9700). ^1H NMR spectrum; *o*-CH_3, δ=4.5; Ph-H, δ=10.2 and 10.5 (Me_2SO-d_6).

Detection of H_2S. Argon gas was bubbled into a DMF solution (2 cm^3) of $[NEt_4]_2[Fe_2S_2(Z\text{-cys-Ala-Ala-cys-OMe})_2]$ (30.3 mg, 2.1×10^{-5} mol) and the gas was vented through an 8 mM silver nitrate aqueous solution. After addition of tdt-H_2 (6.4 mg, 4×10^{-5} mol) into the DMF solution, the silver nitrate solution gave a black precipitate upon bubbling argon gas at room temperature. The black precipitate was collected with filtration. Yield 7 mg of silver sulfide (68%).

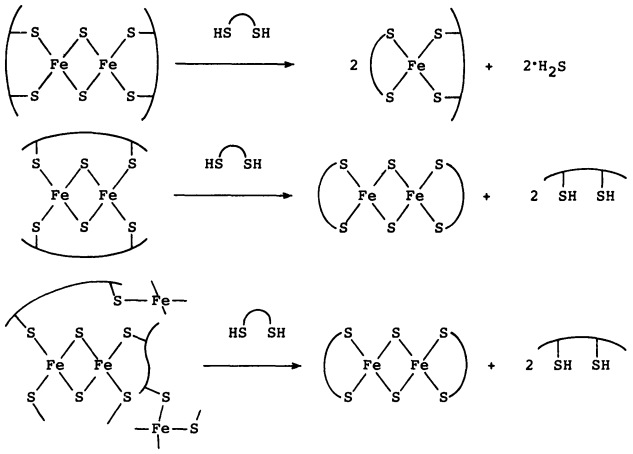
Methods. The visible spectrum (a) was obtained with a DMF solution of [2Fe-2S] complex (3×10^{-7} mol) in a

specially designed quartz 1 mm-cell equipped with stopcocks for the measurements of visible and circular dichroism (CD) spectra. The ligand-exchange reaction (b) was carried out by the addition of a DMF solution of tdt-H_2 (3×10^{-7} mol) at room temperature. The difference visible spectra of the above systems were obtained by subtracting (a) from (b) to give a spectrum corresponding to the spectrum of a DMF solution of the authentic $[NEt_4]_2[Fe_2S_2(tdt)_2]$. The addition of *o*-xyl-$(SH)_2$ to [2Fe-2S] complexes was also carried out by the above method.

Physical Measurements. The visible spectra of the [2Fe-2S] complexes were obtained on a Jasco UVIDEC-5A spectrophotometer. CD spectra were recorded using a Jasco J-40 spectropolarimeter. The calibration of the spectropolarimeter was carried out with *epi*-androsterone in dioxane.[14] The values of ε and $\Delta\varepsilon$ were given in the unit of $M^{-1}cm^{-1}$.

Results and Discussion

Ligand-Exchange Reaction. A ligand-exchange method using 3,4-toluenedithiol (tdt-H_2) or *o*-xylene-α,α'-dithiol (*o*-xyl-$(SH)_2$) was employed to determine a chelation ability of Z-Cys-X-Y-Cys-OMe to $[Fe_2S_2]^{2+}$ core in DMF at room temperature. The proposed ligand-exchange reactions for the 2Fe-2S complexes with the dithiols are shown in the following scheme.



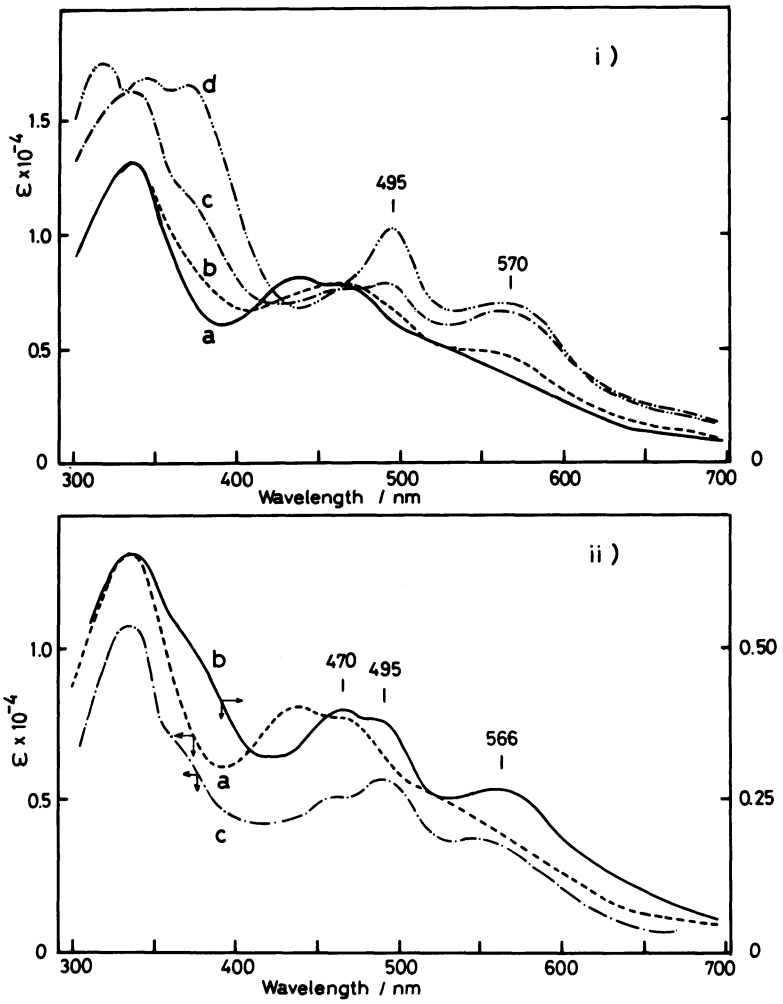


Fig. 1. Visible spectra of i) (a) a DMF solution of $[NEt_4]_2[Fe_2S_2(S\text{-}t\text{-}Bu)_4]$ at room temperature, (b) solution-(a)+tdt-H_2(1 equiv), (c) solution-(a)+tdt-H_2(2 equiv,) and (d) the solution-(a)+tdt-H_2(4 equiv) and ii) (a) a DMF solution of $[NEt_4]_2[Fe_2S_2(S\text{-}t\text{-}Bu)_4]$, (b) the difference visible spectrum of the reaction mixture of $[NEt_4]_2[Fe_2S_2(S\text{-}t\text{-}Bu)_4]$ and tdt-H_2 (1 : 1), and (c) the visible spectrum of a DMF solution of $[NEt_4]_2[Fe_2S_2(tdt)_2]$ at room temperature.

Monodentate ligand, *t*-Bu-S$^-$, in $[Fe_2S_2(S\text{-}t\text{-}Bu)_4]^{2-}$ can be easily expelled by Cys-containing peptides such as Z–Ala–Cys–OMe and Z–Cys–Ala–Ala–Cys–OMe. This ligand-exchange reaction in the following equation was utilized for the synthesis of [2Fe–2S] Cys-containing peptide complexes[9] as well as the synthesis of [4Fe–4S] peptide complexes.[15–17] The authentic $[NEt_4]_2[Fe_2S_2(tdt)_2]$ was synthesized by the same method.

$$[Fe_2S_2(S\text{-}t\text{-}Bu)_4]^{2-} \xrightarrow{RS^-} [Fe_2S_2(SR)_4]^{2-} + t\text{-BuSH} \quad (1)$$

($SR^- = tdt^{2-}/2$, Cys-containing peptides$^-$)

Examination of Chelation of Peptide Ligands on $[Fe_2S_2]^{2+}$ Core by Ligand-Exchange Reaction with 3,4-Toluenedithiol. The dithiol readily exchanges with the monodentate ligand of [2Fe–2S] complexes as shown in $[NEt_4]_2[Fe_2S_2(S\text{-}t\text{-}Bu)_4]$ (**1**) and $[NEt_4]_2[Fe_2S_2$-(Z–Ala–cys–OMe)$_4$] (**2**).

Figure 1-i or 2 shows the visible spectra of **1** or **2** in DMF and the spectral change by the addition of various molar equivs of tdt-H_2. The addition of less than 2 equiv of tdt-H_2 to $[NEt_4]_2[Fe_2S_2(S\text{-}t\text{-}Bu)_4]$ resulted in formation of a new species giving an isosbestic point at 465 nm. The visible spectrum of the new species obtained by the addition of 1 equiv of tdt-H_2 is shown in Fig. 1-b. One of the figures shows a difference spectrum between **1** and the reaction mixture. The new species exhibits absorption maxima at 566, 495, 470, 375 (sh), and 336 nm for **1** or 566, 493, 470, 373 (sh), and 335 nm for **2** in DMF. The absorption maximum at 566 nm is assignable to a characteristic ligand-metal charge-transfer (LMCT) band for $[Fe_2S_2(SR)_4]^{2-}$ complex. The species having these absorption maxima (550, 496, 470, 370, 320 nm) was thus identified as the authentic $[Fe_2S_2(tdt)_2]^{2-}$ complex. The formation of $[Fe_2S_2(tdt)_2]^{2-}$ was observed by ^1H NMR spectrum of a reaction mixture of $[Fe_2S_2(Z\text{-cys–Ala–OMe})_4]^{2-}$ with tdt-H_2 in Me_2SO-d_6. Two broad peaks at δ 10.8 and 10.2 were found and assignable to the phenyl protons of tdt ligand, since the authentic tdt complex exhibited two Ph–H signals at δ 10.2 and 10.5 in Me_2SO-d_6. The methyl proton peaks of the ligand were overlapped with *t*-Bu peaks at δ 4.7.

The addition of tdt-H_2 to $[Fe_2S_2(Z\text{–Ala–cys–OMe})_4]^{2-}$ results in formation of $[Fe_2S_2(tdt)_2]^{2-}$ with a smooth substitution reaction of Z–Ala–Cys–OMe with the tdt ligand. In this case, the $[Fe_2S_2]^{2+}$ core remains until the addition of over 5 equiv of tdt-H_2.

The addition of over 3—4 equivs of tdt-H_2 to **1** resulted in formation of another new species having absorption maxima at 495 nm (ε 14500), 375 nm (ε 27400), and 313 nm (ε 19500). The addition of tdt-H_2 to a DMF solution of $[Fe_2S_2(S_2\text{-}o\text{-xyl})_2]^{2-}$ (**3**) (Fig. 3) or $[Fe_2S_2(Z\text{-cys–Ala–Ala–cys–OMe})_2]^{2-}$ (**4**) (Fig. 4) at

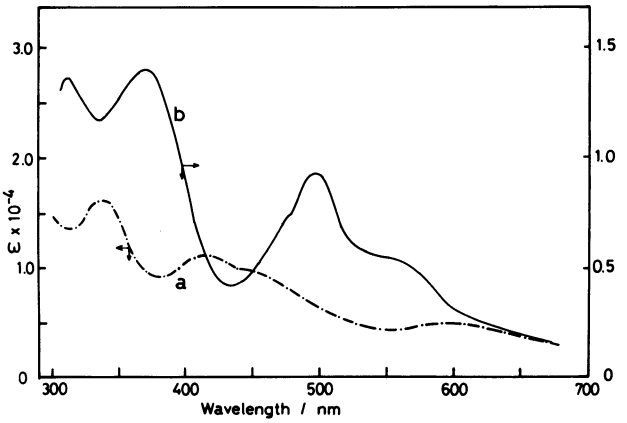


Fig. 3. Visible spectra of a) $[NEt_4]_2[Fe_2S_2(S_2\text{-}o\text{-xyl})_2]$ and b) the difference visible spectrum of the species forming from the ligand-exchange reaction (solution-(a)+tdt-H_2 (1 : 1)) in DMF at room temperature.

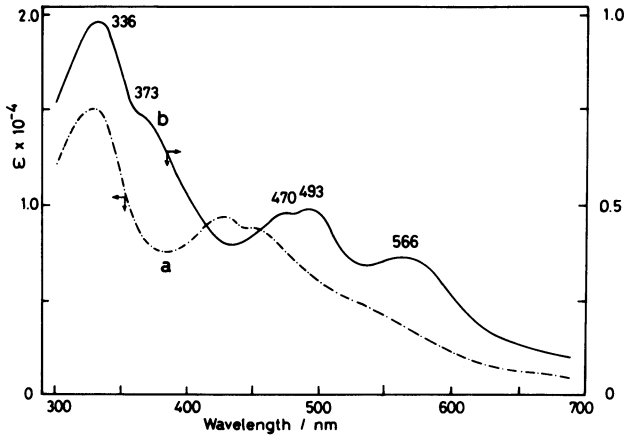


Fig. 2. Visible spectra of a) $[NEt_4]_2[Fe_2S_2(Z\text{–Ala–cys–OMe})_4]$ and b) the difference visible spectrum of the species obtained from the ligand-exchange reaction (solution-(a)+tdt-H_2 (1 : 1)) in DMF at room temperature.

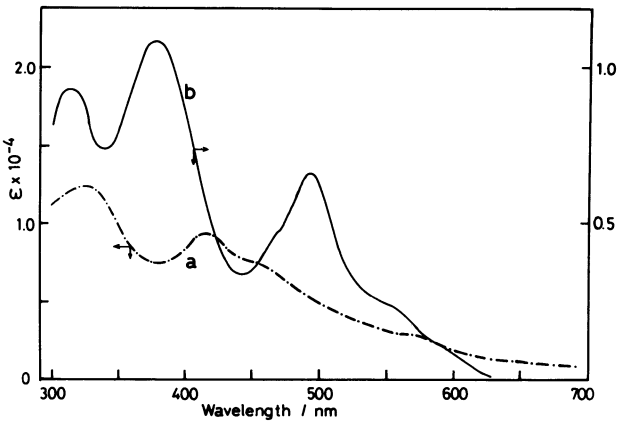
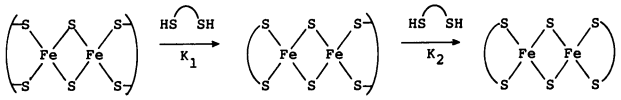


Fig. 4. Visible spectra of a) $[NEt_4]_2[Fe_2S_2(Z\text{-cys–Ala–Ala–cys–OMe})_2]$ and b) the difference visible spectrum of the species forming from the ligand-exchange reaction (solution-(a)+tdt-H_2 (1 : 1)) in DMF at room temperature.

(1 : 1) molar ratio gave another species which exhibits absorption maxima at 492 nm (ε 9700), 370 nm (sh, ε 13000), and 339 nm (ε 16000). The difference spectrum of the species is shown in Fig 4-b. The chelation of two tdt ligands is expected for Fe(III), but the absorption maxima of $[Fe(tdt)_2]^{1-}$ in DMF were reported to be observed at 645 nm (ε 100), 503 nm (ε 10000), and 386 nm (ε 17000).[18] The bis(peptide)Fe(III) complex, $[Fe(Z\text{-}cya\text{-}Ala\text{-}Ala\text{-}cys\text{-}OMe)_2]^{1-}$ has been found to have absorption maxima at 495 nm (ε 3400) and 353 nm (ε 5500).[5] Therefore, these two bis-chelated species are not present at (1 : 1) ratio. Then, the new species suggests the formation of [Fe(tdt)-(Z-cys-Ala-Ala-cys-OMe)]$^{1-}$. This ligand-exchange reaction gave hydrogen sulfide which was detected as silver sulfide in 68% yield. The addition of tdt-H_2 to a DMF soultion of **4** results in preferential cleavage of Fe-S* at (1 : 1) molar ratio.

Addition of *o*-Xylene-α,α′-dithiol (*o*-xyl-(SH)$_2$) to a Solution of 2Fe-2S Peptide Complex. *o*-Xyl-(SH)$_2$ is also one of effective chelating reagents for Fe(III) ion. The succesive addition of 1—5 equiv of *o*-xyl-(SH)$_2$ to a DMF solution of $[Fe_2S_2(Z\text{-}Ala\text{-}cys\text{-}OMe)_4]^{2-}$ and $[Fe_2S_2(Z\text{-}cys\text{-}Ala\text{-}Ala\text{-}cys\text{-}OMe)_2]^{2-}$ showed a visible maximum at 588 nm with high-molar absorption coefficient which is assignable to a LMCT absorption maximum (ε 4800) of $[Fe_2S_2(S_2\text{-}o\text{-}xyl)_2]^{2-}$ (Fig. 5). The addition of *o*-xyl-(SH)$_2$ to a solution of the peptide complexes results in formation of the same 2Fe-2S complex after the ligand substitution reaction. This titration was monitored by the CD spectral method and resulted in observation of 20% of the Δε value at 580 nm (Fig. 6). Clear isosbestic points in the CD spectra were observed at 350, 395, and 437 nm. The results indicate a monotonous substitution of Z-Cys-Ala-Ala-Cys-OMe with *o*-xyl-(SH)$_2$ giving mixed ligand [2Fe-2S] complexes as shown in the following scheme. The *K* value ($K_1 \cdot K_2$) obtained by the CD



method is 0.4 (K_1=0.2, K_2=2.0) in DMF. On the contrary, the successive addition of *o*-xyl-(SH)$_2$ to $[Fe_2S_2(Z\text{-}Ala\text{-}cys\text{-}OMe)_4]^{2-}$ results in gradual appearance of an absorption maximum at 592 nm. *K* value for the ligand-exchange reaction of $[Fe_2S_2(Z\text{-}Ala\text{-}cys\text{-}OMe)_4]^{2-}$ is 0.0007 mol^2 in DMF (by the CD

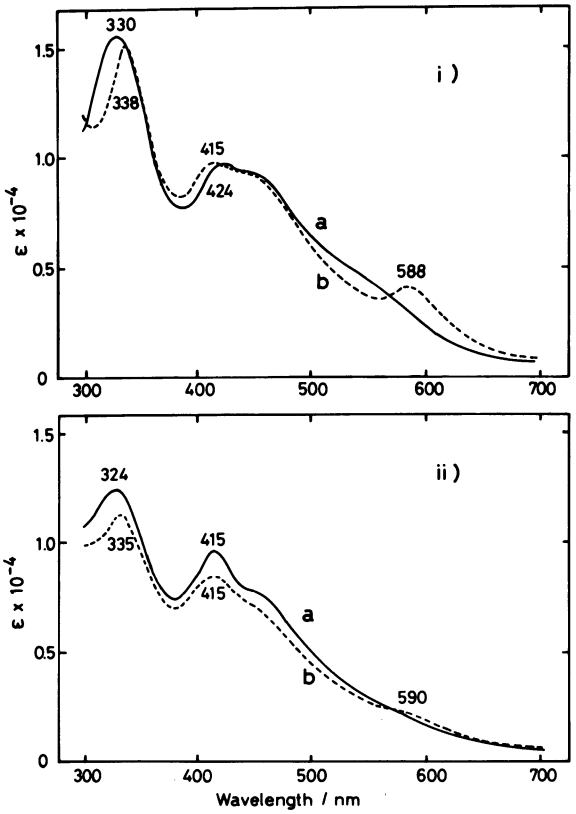


Fig. 5. Visible spectral change of the ligand-exchange reaction of i) (a) $[NEt_4]_2[Fe_2S_2(Z\text{-}Ala\text{-}cys\text{-}OMe)_4]$ and (b) solution-(a)+*o*-xyl-(SH)$_2$ (1 : 2) and ii) (a) $[NEt_4]_2[Z\text{-}cys\text{-}Ala\text{-}Ala\text{-}cys\text{-}OMe)_2]$ and (b) solution-(a)+*o*-xyl-(SH)$_2$ (1 : 2) in DMF at room temperature.

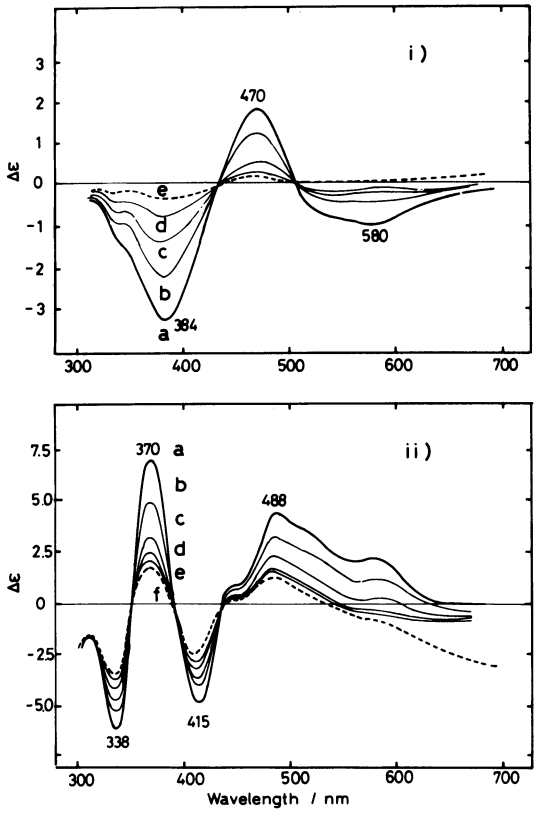


Fig. 6. CD spectral change of the ligand-exchange reaction of i) (a) $[NEt_4]_2[Fe_2S_2(Z\text{-}Ala\text{-}cys\text{-}OMe)_4]$, (b) solution-(a)+*o*-xyl-(SH)$_2$ (1 : 1), (c) (1 : 2), (d) (1 : 3), and (e) (1 : 4) and ii) (a) $[NEt_4]_2[Z\text{-}cys\text{-}Ala\text{-}Ala\text{-}cys\text{-}OMe)_2]$, (b) solution-(a)+*o*-xyl-(SH)$_2$ (1 : 1), (c) (1 : 2), (d) (1 : 3), (e) (1 : 4), and (f) (1 : 5) in DMF at room temperature.

method).

Chelating Ability of Z-Cys-Ala-Ala-Cys-OMe. The displacement reaction by tdt-H_2 with Cys-containing 2Fe-2S complexes in DMF was found to be different from that by *o*-xyl-$(SH)_2$. The addition of *o*-xyl-$(SH)_2$ to **1** and **2** results in formation of $[Fe_2S_2(S_2\text{-}o\text{-xyl})_2]^{2-}$ with a characteristic absorption maximum at 592 nm, which corresponds to the reported maximum at 590 nm (ε 4800) in DMF.[13] The difference between the K values (0.0007 for **2** and 0.4 mol^2 for **3**) is ascribed to the chelation effect of Z-Cys-Ala-Ala-Cys-OMe in **4**. The presence of the clear isosbestic point of the CD spectra with addtion of *o*-xyl-$(SH)_2$ to **3** indicates absence of a bridged Z-Cys-Ala-Ala-Cys-OMe ligand since the bridging ligand must be substituted first with tdt ligand. The successful employment of *o*-xyl-$(SH)_2$ for the core extrusion of [2Fe-2S]adrenal ferredoxin has already been reported.[1,10]

The addition of tdt-H_2 to a DMF solution of **2** and **3** results in the cleavage of Fe-S* bond in the $Fe_2S_2^{2+}$ core, giving $[Fe(tdt)(S_2\text{-}o\text{-xyl})]^{1-}$ or $[Fe(tdt)(Z\text{-cys-Ala-Ala-cys-OMe})]^{1-}$ with the following reaction scheme.

$$[Fe_2S_2(S_2\text{-}R)_2]^{2-} \xrightarrow{\text{tdt-}H_2} [Fe(tdt)(S_2\text{-}R)]^{1-} + H_2S \xrightarrow{\text{tdt-}H_2} 2[Fe(tdt)_2]^{1-} + (HS)_2\text{-}R \quad (2)$$

($S_2\text{-}R^{2-}$ = S_2-*o*-xyl^{2-}, Z-Cys-Ala-Ala-Cys-OMe^{2-})

Previously, we reported that $[Fe(Z\text{-cys-Ala-Ala-cys-OMe})_2]^{1-}$ exhibits CD extrema at 560 nm ($\Delta\varepsilon$ +1.3) and 500 nm ($\Delta\varepsilon$ −1.6) in DMF. The (1:1) species, $[Fe(tdt)(Z\text{-cys-Ala-Ala-cys-OMe})]^{1-}$, exhibits an absorption maximum at 494 nm (ε 12000) and CD extrema at 550 nm ($\Delta\varepsilon$ −0.6), 495 nm ($\Delta\varepsilon$ −1.0), and 335 nm ($\Delta\varepsilon$ −13.0). Formation of hydrogen sulfide was confirmed by precipitation of silver sulfide from silver nitrate. The satisfactory yield (68%) of silver sulfide indicates that the addition of tdt-H_2 to **3** preferentially cleavages the Fe-S* bonds according to the Eq 2. Averil et al. have reported the core extrusion of [2Fe-2S] adrenodoxin with tdt-H_2.[10] They speculated the formation of $[Fe(tdt)_2]^{2-}$ with addition of 19 equivs of tdt-H_2 to adrenodoxin. The reduction of $[Fe(tdt)_2]^{1-}$ should be involved in this case because the mononuclear bis(1,2-dithiolene)Fe(III) complex has a relatively positive redox potential and is easily reduced by the dithiol giving (2−) species ($[Fe^{II}(tdt)_2]^{2-}$). Our results suggest that, upon the core extrusion from iron-sulfur proteins, some of dithiols destroy a native cluster itself.

Thus we found that the addition of one equiv of tdt-H_2 to $[Fe_2S_2(SR)_4]^{2-}$ complex results in the formation of either $[Fe(tdt)(SR)_2]^{1-}$ or $[Fe_2S_2(tdt)_2]^{2-}$. The former complex is formed by cleavage of Fe-S* and the later product is obtained by the substitution of SR with tdt ligand. Thus, although we have examined only a few cases of the ligand exchange reactions for Cys-containing peptide complexes, the chelation of the peptide thiolate ligand to $[Fe_2S_2]^{2+}$ core was conveniently determined by using tdt-H_2.

In conclusion, the chelation of Z-Cys-Ala-Ala-Cys-OMe to each Fe(III) ion in the [2Fe-2S] complex is confirmed. The bridging coordination of this sequence in native [2Fe-2S]ferredoxins is thus enforced by the peptide sequence of the other parts.

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