A Rubredoxin-Like Mononuclear FeS_4 Derivative of Adrenal Iron-Sulfur Protein (Adrenodoxin)

Yokio Sugiura,* Kazuhiko Ishizu,** and Tokuji Kimura

Department of Chemistry
Wayne State University
Detroit, Michigan 48202

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Summary: A rubredoxin-like mononuclear iron-sulfur derivative of adrenodoxin was prepared from the apoprotein and $FeCl_3$ in the presence of dithiothreitol. The mononuclear compound displayed optical absorption maxima at 276, 350, and 500 nm, and exhibited electron paramagnetic resonance absorption at g = 4.27 with a shoulder at g = 4.28, which can be ascribed to high spin ferric ion. From p-chloromercuriphenyl sulfonate titration experiments the iron atom appears to contain approximately one g atom of iron per mole of protein. This rubredoxin-like derivative was very unstable at 22° (the half-life was approximately 10 minutes), whereas the native 2 Fe-2S* protein is known to be quite stable. This instability is believed to be intrinsic to the polypeptide sequence of adrenodoxin.

Adrenodoxin is essential as an electron carrier in a system which catalyzes the hydroxylation reactions of steroids in adrenal cortex mitochondria (1). This protein with a 2Fe-2S* redox center and 114 amino acid residues, contains 5 cysteine residues per molecule at the positions of 46, 52, 55, 92, and 95 (2). The two iron atoms are believed to be bonded to the 4 cysteine sulfur atoms and 2 labile sulfur atoms in binuclear tetrahedral coordination (3).

Rubredoxin has one or two iron atoms per molecule and contains no labile sulfur atom (4). Rubredoxin from aerobic bacteria is known to be a redox component in an enzyme system which hydroxylates fatty acids and hydrocarbons (5). From X-ray crystallography, it is known that the iron atom is bonded to the 4 cysteine residues in nearly tetrahedral symmetry (6).

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*On leave of absence from Kyoto University, Japan
**On leave of absence from Ehime University, Japan
The abbreviations used: S*, labile sulfur; PCMS, p-chloromercuriphenyl sulfonate; and EPR, electron paramagnetic resonance

Both adrenodoxin and rubredoxin are quite stable, when the samples are stored frozen or at 4°C.

A question is whether or not a rubredoxin-like mononuclear iron chromophore can be prepared from apoadrenodoxin and iron. From this study, we expect to learn the degree of constraint in the folded polypeptide chain of adrenodoxin molecule when the mononuclear iron center is formed instead of the native binuclear chromophore.

Materials and Methods

Adrenodoxin was prepared from bovine adrenal glands and the ratio of absorbance at 414 nm to that at 276 nm was 0.86. Apoadrenodoxin was parepared as described elsewhere (7). The resulting apoadrenodoxin had negligible amounts of labile sulfur and iron atoms. The mononuclear iron derivative of adrenodoxin was prepared by the following method. 1.0 ml of solution containing 15 mg of apoadrenodoxin in 0.5 M Tris-HCl buffer (pH 8.3) was placed in a reaction vessel. By the addition of dithiothreitol and urea, the solution was brought to 5-fold molar excess and 6 M, respectively. After standing for one hour, 20 fold molar excess of $FeCl_3$ in 0.5 ml of H_2O was added to the aproadrenodoxin solution. After three minutes, the reaction mixture was applied to a small DEAE-cellulose column (1.0x7.0 cm) and the cellulose was washed with 30 ml of 0.01 M phosphate buffer (pH 8.3). The protein fraction was eluted with 0.01 M phosphate buffer (pH 8.3) containing 0.5 M KCl. All manipulations on the DEAE-cellulose column should be finished within 30 minutes. The sample was stored in liquid N_2 immediately after the separation.

Protein and iron were determined by the biuret method and the o-phenanthroline method, respectively, EPR spectroscopy was performed by the use of a Varian spectrometer (E-4).

Results

Chemical analysis, optical absorption spectrum, and stability

Table I shows the chemical analysis of iron in the mononuclear iron samples. The ratios of iron to protein were in the range between 0.7 and 0.8, indicating that the derivative has approximately one iron atom per molecule. There was no detectable labile sulfur atom, as judged by the

Table I. Chemical Analyses of FeS_4 Derivative of Adrenodoxin

Sample No.	Protein	Iron	Fe/Protein
1	0.24 mM	0.17 mM	0.71
2	0.75	0.58	0.78
3	0.84	0.61	0.73

modified method of Fogo and Popowsky (8). The optical abosrption spectrum of the oxidized compound had maxima at 276, 350, and 500 nm. This spectrum closely resembles those of rubredoxins which have maxima at 280, 378, and 495 nm (9). The adrenal mononuclear iron protein had a millimolar extinction coefficient of 3.9 at 500 nm, which is a comparable value to that of clostridial rubredoxin (4.4 at 490 nm) (10). The large extinction coefficient is suggestive of the chelation of iron to sulfur atoms. The adrenal mononuclear protein was very unstable at room temperature. The half-life of this protein was approximately 10 minutes at 22°. For practical purposes, the sample was kept in liquid N_2, where the chromophore was reasonably stable for a week.

Electron paramagnetic resonance

Fig. 1 (a) illustrates the EPR spectrum of the oxidized sample. The spectrum had a signal at g = 4.27 with line width Δ H (peak to peak) of 30 gauss. A distinct shoulder at g = 4.28 was observed with reproducibility. No other significant signal was detected in the lower magnetic field range. Upon the reduction with dithionite, the signal at g = 4.3 completely disappeared. These EPR properties are similar to those of oxidized rubredoxin, which exhibits a EPR signal at g = 4.3, except their line shapes are somewhat different. The X band signal of rubredoxin is composed of wide and narrow components at g = 4.77, 4.31, and 4.02 in addition to the signal at g = 9.4 (11). The adrenal derivative had a sharp main signal at g = 4.27 with a distinct shoulder at g = 4.28. The Δ H is narrower than that of

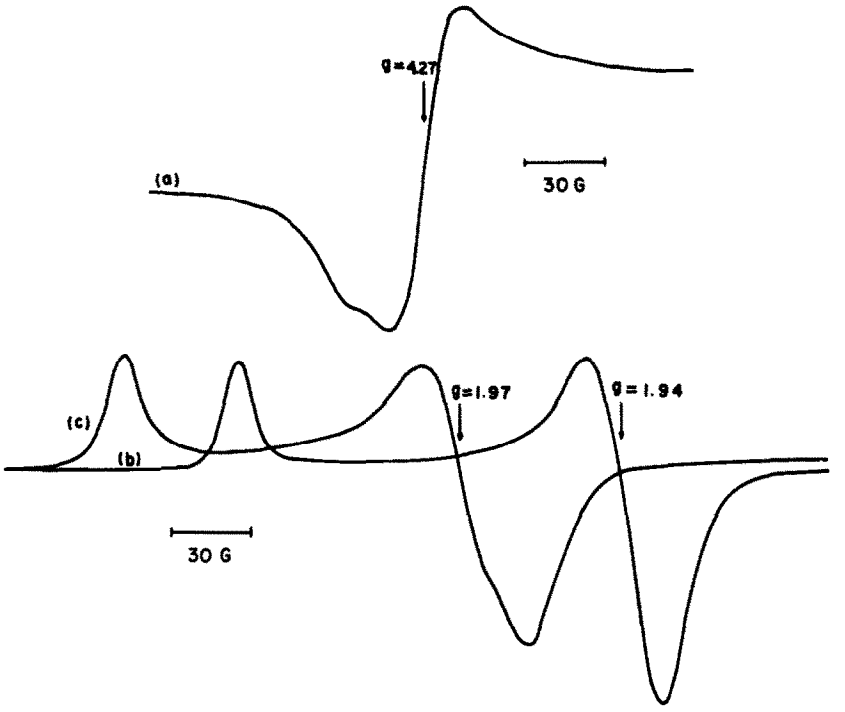

Figure 1. EPR spectra of FeS_4 derivative of adrenodoxin and the reconstitution upon addition of Na_2S or H_2Se

a) FeS_4 derivative of adrenodoxin (0.080 mM); oxidized
b) Sample (a) plus Na_2S (0.16 mM); reduced form
c) Sample (a) plus H_2Se (0.16 mM); reduced form

Conditions for EPR spectroscopy: microwave power, 20 mW (a) or 10 mW (b c); frequency, 9.27 GHz; modulation amplitude, 10 gauss; scanning rate, 25 gauss per minute; time constant, 1.0 second; temperature, 77°K.

rubredoxin. Our failure to detect a signal at g = 9 region would suggest that at 77°K, the observed signals are mainly due to the population of spins in the middle Kramer doublet (11).

p-Chloromercuriphenyl sulfonate titration

The protein ligands which are bonded to the iron atom were examined by EPR signal titration with PCMS. As shown in Fig. 2, the titration curve indicates that 4.5 moles of PCMS per mole of protein was required to diminish the signal intensity to completion. This strongly suggests that the iron atom is bonded to 4 cysteine sulfur atoms.

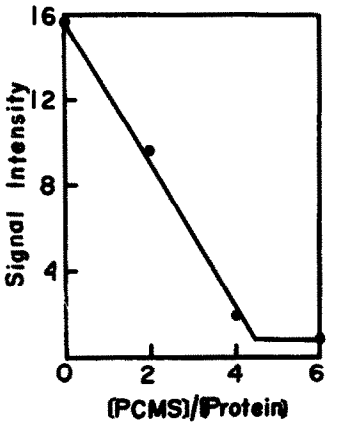

Figure 2. Titration of FeS_4 derivative od adrenodoxin with PCMS

To the reaction mixture containing FeS_4 derivative of adrenodoxin (0.080 mM), various amounts of PCMS were added. The signal intensity at g = 4.27 was measured at 77°K immediately after the addition of PCMS. The experimental conditions were the same as in Figure 1.

Reconstitution of native adrenodoxin from mononuclear derivative

An attempt to reconstitute the native protein from the mononuclear iron-sulfur compound was made by adding Na_2S or H_2Se. Fig. 1 (b, c) indicates that the EPR signals of the reconstituted and then reduced samples are identical to those of the reduced forms of the native protein and its Se derivative. The signal intensity of the reconstituted sample was able to account for its iron content by 83% of the signal intensity of the native protein. Therefore, we propose that without external addition of iron, this reconstitution reaction goes through the following equation:

$$2(FeS_4)\text{-adrenodoxin} + 2S^* \longrightarrow Fe_2S^*_2S_4)\text{-adrenodoxin} \quad +(S_4)\text{-apoadrenodoxin.}$$

It should be mentioned here that since the reconstitution occurs also by adding labile sulfur source first, and then iron, the validity of the above mechanism for in vivo reconstitution reaction has to await further investigation.

Discussion

The present results clearly demonstrate that the addition of iron ions to apoadrenodoxin, a mononuclear iron-sulfur derivative was obtained.

The optical absorption and EPR properties were similar to those of rubredoxin. At present, we believe that the high spin ferric ion is bonded to 4 cysteine sulfur atoms in tetrahedral symmetry. The mononuclear derivative was remarkably unstable at 22° relative to both native adrenodoxin, a binuclear complex, and rubredoxin, a mononuclear complex. Upon the addition of Na_2S to the adrenal mononuclear compound, the native binuclear chromophore was spontaneously formed.

Rubredoxin from Clostridium pasteurianum (12), Peptococcus elsdenii (13), and Micoccus aerogenesis (14) have genetically invariant cysteine residues at the positions of 6, 9, 39, and 42. Those are cys-thr (ser)-val (ile, leu)-cys, and cys-pro-leu (thr)-cys sequences. These four cysteine residues are responsible for the binding of iron atom in 15-membered ring. Although the iron binding site is unclear, bovine adrenodoxin has similar sequences at the position of 52-55, and 92-95: cys-ser-thr-cys, and cys-gln-ile-cys, respectively (15). In spite of the fact that the two proteins have two similar cys-X-Y-cys sequences being separated by 33, and 40 amino acid residues in respective molecules, rubredoxin has a stable mononuclear iron-sulfur complex, while adrenodoxin has a stable binuclear iron-sulfur complex and its mononuclear derivative is very unstable.

From our studies on the model iron-sulfur complexes (16), 1, 3-propanedithiol as a ligand forms vary stable six-membered chelate with iron, displaying a similar optical absorption spectrum in the visible region to that of rubredoxin, and did not afford any nFe-nS* protein-like spectrum by the addition of sulfide. In contrast, 1, 4-butanedithiol forms an unstable seven-membered ring, and has a similar spectrum to the 2Fe-2S* protein formed by the addition of sulfide. Since the above two ligands differ by only one CH_2 group, the carbon chain length plays an important role in the determination of whether the mononuclear or binuclear complex is stable.

In this context, we strongly feel that the three dimensional structure of adrenodoxin should accomodate two iron atoms together with two labile sulfur atoms, but not one iron atom alone. It appears that as in the case of the model iron-sulfur complexes, a slight difference in the protein ligands crucially determines whether or not the mononuclear or binuclear iron-sulfur complex is the stable chromophore of rubredoxin and adrenodoxin.

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