THE BINDING SITES OF IRON IN RUBREDOXIN FROM MICROCOCCUS AEROGENES*

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Although a number of nonheme iron proteins have been obtained in homogeneous form,[1] the nature of the chemical linkage between the iron and protein remains obscure. Rubredoxin appears to be a suitable protein for such investigations because it has a low molecular weight and contains one mole of iron per mole of protein.[2] Moreover, rubredoxin does not contain histidine or arginine,[2] thus eliminating the possibility of chelation with these amino acid residues.

Materials and Methods.—Preparation of rubredoxin: *Micrococcus aerogenes* was grown in 100-liter quantities in a 130-liter fermenter in a complex medium containing glutamate.[3] Approximately 500 ml of a cell-free preparation containing ferredoxin and rubredoxin was adsorbed to a column (3.2 × 10 cm) of *O*-(diethylaminoethyl) cellulose (DEAE-cellulose) previously equilibrated with 0.1 *M* Tris buffer, pH 7.0. The column was washed with 300 ml of 0.05 *M* phosphate buffer, pH 6.5, to remove nonadsorbed and weakly adsorbing materials. Adsorbed proteins, including ferredoxin and rubredoxin, were eluted with 0.15 *M* Tris buffer, pH 7.3, containing 0.67 *M* NaCl. The eluted material was passed through a column (3.2 × 50 cm) of Sephadex G-25 (Pharmacia Co., coarse grade) equilibrated and eluted with 0.1 *M* Tris buffer, pH 7.0, and was adsorbed to a second column (2.5 × 90 cm) of DEAE-cellulose. This column had been equilibrated with 0.15 *M* Tris buffer, pH 7.3, containing 0.15 *M* NaCl and was eluted with the same solution. Rubredoxin was removed by 0.5–1.0 liter of this solution. The rubredoxin-containing fraction was diluted 1:3 to 1:4 by the addition of distilled water and adsorbed to a third column (2.5 × 7 cm) of DEAE-cellulose equilibrated with 0.1 *M* Tris buffer, pH 7.0. Elution with a small volume of 0.15 *M* Tris buffer, pH 7.3, containing 0.67 *M* NaCl provided a concentrated solution of rubredoxin. The rubredoxin was precipitated by the addition of ammonium sulfate to full saturation. The 280:490 absorbance ratio was about 2.4. Crystalline rubredoxin could be obtained by gradual addition of powdered ammonium sulfate; at 5°C, crystals appeared after 1–2 weeks at 70–80% saturation with ammonium sulfate. The 280:490 absorbance ratio was about 2.3. For the experiments described, the rubredoxin preparations showed a 280:490 absorbance ratio of less than 2.4.

Preparation of derivatives: S-β-Aminoethylcysteinyl-rubredoxin (AEC-rubredoxin) was prepared by reacting apo-rubredoxin with ethylenimine[4] by the method used earlier in studies of ferredoxin.[5] Oxidized rubredoxin was obtained by treating the native protein with performic acid as described by Moore.[6]

Enzymatic hydrolysis of protein: About 7 μmoles of AEC-rubredoxin in 6–8 ml of water were treated two times with 2% w/w of either TPCK-trypsin[7] or TLCK-chymotrypsin[8] and the reaction was allowed to proceed for 5 hr and 16 hr, respectively, at 37° during which time the pH was adjusted to 8.0 by the addition of 0.1 *N* NaOH. The reaction mixtures were lyophilized and dissolved in appropriate buffers for the purification of the peptides on Dowex 50-X2 or Dowex AG 1X2 (Bio-Rad Lab.) columns (1.5 × 120 cm). In certain cases, the peptides were purified additionally by paper chromatography (details of the peptide separation will be published separately). The methods used for the amino acid analysis of the peptides, for the determination of the NH_2-terminal and COOH-terminal amino acids, and for sequence determination have been described previously.[5]

Metal analyses: Hg analyses were performed on the *p*-chloromercuribenzoate-treated protein (CMB-rubredoxin) which had been reacted with a fourfold and tenfold molar excess of CMB in 0.33 *M* Na-acetate buffer (pH 4.6). After extensive dialysis, the treated samples were analyzed in an atomic absorption apparatus similar to that described by Fuwa *et al.*[9] Iron was determined

on untreated samples of rubredoxin following wet ashing in 6 N HNO_3. The iron was determined microchemically using bathophenanthroline.[10]

Cysteine determinations: The cysteine content of rubredoxin was determined by performic acid oxidation and by conversion of the rubredoxin to AEC-rubredoxin as described above. The oxidized preparations and AEC-proteins were then analyzed for amino acid content with an amino acid analyzer.

Titration of rubredoxin with CMB: The titrations were carried out according to the method of Boyer.[11]

TABLE 1

CYSTEIC ACID AND AMINOETHYLCYSTEINE CONTENT

Protein derivative	Moles per mole
Oxidized rubredoxin	3.7 $CySO_3H$
Aminoethylcysteinyl rubredoxin	3.6 AEC

Results.—Sulfhydryl and iron content of rubredoxin: The results obtained from both methods of assay for cysteine (Table 1) suggest that *M. aerogenes* rubredoxin contains four cysteine residues per mole of protein. An iron content of one mole per mole of protein was found. The same quantities of cysteine and iron per mole of protein have been found for rubredoxin isolated from *Clostridium pasteurianum.*[2]

CMB-rubredoxin reactions: The CMB titration[11] of rubredoxin is shown in Figure 1. Noteworthy is the fact that excess CMB is required. We have attributed the need for excess reagent to the fact that the reactant, protein $(—S—)_4—Fe^{+++}$, must be converted to protein $(—S—)_4—(Hg—R)_4$ by CMB, and the Fe—S bonds must be broken and the S—Hg—R bonds formed. In order to quantitate the amount of CMB incorporated into protein, the reaction mixture was dialyzed after 18 hours of reaction against deionized water and the Hg content analyzed by

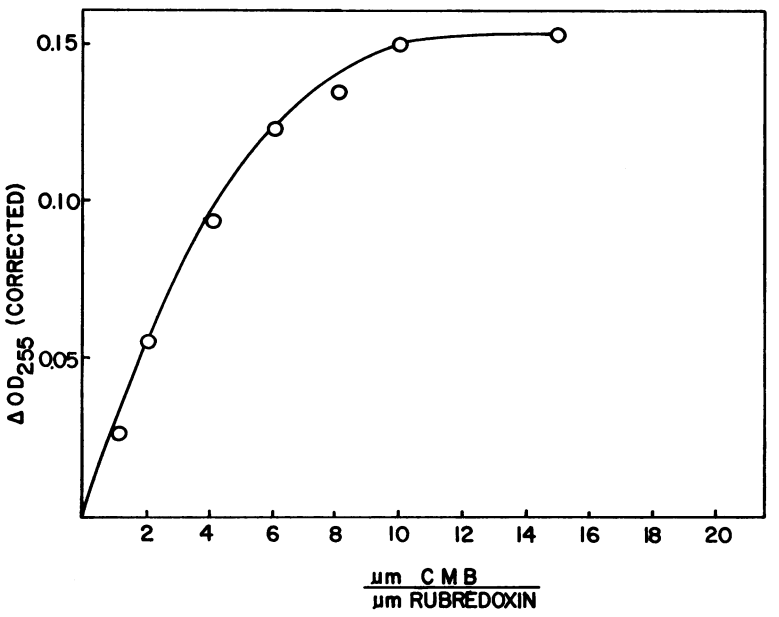

FIG. 1.—Titration of rubredoxin with CMB according to Boyer.[11] In separate experiments, 0.011 μmoles protein and the indicated molar ratios of CMB were reacted for 3 hr in 0.33 M acetate buffer, pH 4.6, at 25°C. Appropriate blank corrections were made.

atomic absorption.[9] At a CMB:protein molar ratio of 4, the product was found to contain 2.25 moles of Hg and at a molar ratio of 10, 3.78 moles of Hg were incorporated.

The fact that plot of change of optical density at 255 mμ versus moles of CMB/moles of protein was a continuous curve until the end point (Fig. 1) pointed to the fact that all of the sulfhydryl groups were probably tied up to the iron. If there were three sulfhydryl (SH) groups chelated to the iron and one free, the free sulfhydryl group would react more rapidly than the other three chelated to the iron and possibly a break in the titration curve would be expected. In order to obtain proof of the lack of free SH groups, the protein was reacted with a 20-fold molar excess of iodoacetic acid in 5 *M* guanidine hydrochloride at pH 7.0 for 30 minutes. The reaction mixture was desalted on a column of Sephadex G-25 in H_2O and aliquots were hydrolyzed for 24 hours in 6 *N* HCl. Amino acid analysis of the hydrolysate failed to reveal detectable amounts of carboxymethylcysteine indicating that free sulfhydryl groups were not present. Therefore, the four sulfhydryl groups appear to be chelated to the iron.

Spectral changes observed when CMB was added to rubredoxin also indicate that the four cysteine residues are chelated to the iron. Figure 2 shows that the absorption in the visible wavelength region, most probably attributable to the iron chelates, is abolished by the addition of CMB.

Tryptic and chymotryptic peptides: In order to determine the location of the

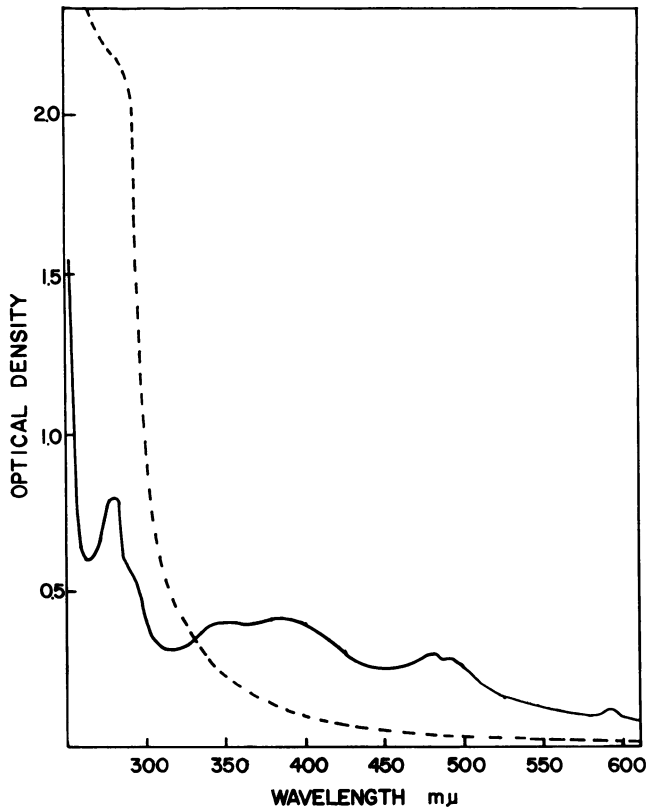

FIG. 2.—Absorption spectra of native rubredoxin (————) and CMB-rubredoxin (- - - -). The following concentrations were used: 0.05 μmoles/ml rubredoxin in 0.1 *M* Tris buffer, pH 7.0, and 0.055 μmoles/ml CMB-rubredoxin. The latter was prepared as described in the *Materials and Methods* section, at a CMB:rubredoxin ratio of 10:1.

TABLE 2

TRYPTIC AND CHYMOTRYPTIC PEPTIDES OF *M. aerogenes* RUBREDOXIN

Peptides	Position of peptide*	Amino acid composition
Tryptic		
T-1	5	(Gly_2, Ala)-Lys
T-2	1	(Met, Glu)-Lys
T-3	3	(Thr, Leu)-AEC
T-4	2	(Phe, Glu)-AEC
T-5	4	(AEC, Asp_6, Thr, Ser, Glu_3, Pro_4, Gly_3, Ala_2, Val_3, Ile, Leu_2, Tyr_2, Phe, Trp)-AEC
T-6	6	(Asp_2, Glu_3, Val, Tyr, Phe)
Chymotryptic		
C-1	5b	Val-AEC-Pro-Leu
C-2	1	(Lys, Glu, Met)-Phe
C-3a	2	(AEC, Thr, Glu, Leu)
C-3b	3	(AEC, Gly)-Tyr
C-4	6a	AEC-(Lys, Asp, Glu_2, Gly_2, Ala, Val, Tyr, Phe)
C-5	6	AEC-(Lys, Asp_2, Glu_3, Gly_2, Ala, Val, Tyr, Phe)
C-6	5	(AEC, Asp_2, Ser, Glu_2, Pro, Val_2, Leu, Trp)
C-7	6b	(Asp, Glu)
C-8a	4	(Asp_4, Thr, Glu, Pro_3, Gly_2, Ala_2, Val, Ile, Leu, Tyr, Phe)
C-8b	5a	(Asp_2, Ser, Glu_2, Val)-Trp

* The positions indicate the sequence in which peptides are located within the protein molecule starting from the NH_2-terminal end. The placing required sequence studies which will be discussed in a future publication.

cysteine residues, AEC-rubredoxin was hydrolyzed with trypsin and chymotrypsin in separate experiments and the peptides were fractionated. Table 2 summarizes the amino acid compositions of the various peptides. Partial sequences were determined in order to establish the overlapping sequences required for locating the cysteine residues in the protein. These experiments indicate that cysteine residues are located at positions 6, 9, 38, and 41. Figure 3 indicates the positions of these residues and their chelation by iron.

EPR spectra: The electron paramagnetic resonance (EPR) spectrum of rubredoxin at pH 7.0 shows an asymmetric resonance under oxidized conditions at $g = 4.432$ (Fig. 4) and a line width for the narrow component of 18.3 gauss. The spectrum did not change appreciably at pH 1.0 but at an alkaline pH, the general shape of the EPR spectrum changes but the total intensity remains constant (Fig. 4). Others[2] have reported on the acid stability of rubredoxin. The signal becomes more symmetrical, indicating that the remaining two ligands coordinated to the iron are modified. Such a pH dependence suggests that these remaining two ligands are possibly tyrosine or a free amine from one of the lysines.

The EPR signal intensity only accounts for approximately 15 per cent of the iron content assuming high-spin iron when calibrated against a standard Cu^{++} solution.

Discussion.—Due to the high molecular weight and the consequent complexity of structural analysis, it has not been possible to determine how iron is linked in most of the nonheme iron proteins isolated from microbial, plant, or animal sources. Considerable progress has been made, however, in the analysis of ferredoxin, a low-molecular-weight nonheme iron protein isolated from certain bacteria or plants. But, due to the presence of numerous sulfide and iron atoms in this molecule, the iron-protein chelate structure has not been established unequivocally.

The present studies with rubredoxin indicate that one iron atom is chelated to four cysteine residues. These cysteine residues have been shown to be present in positions 6, 9, 38, and 41 of the peptide chain. Two of the cysteine residues are located in the NH_2-terminal region and two in the COOH-terminal portion of the

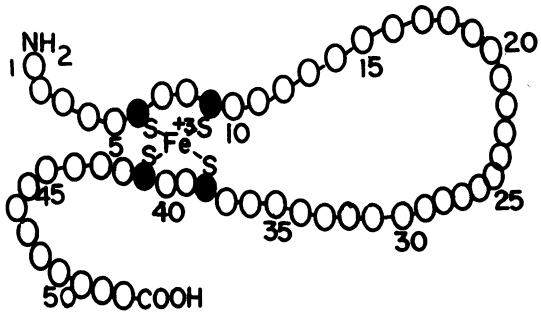

FIG. 3.—Schematic representation showing the iron binding sites in *M. aerogenes* rubredoxin. The circles represent amino acid residues. The black circles in positions 6, 9, 38, and 41 represent the cysteine residues.

molecule. Two other amino acid residues are spaced between each of the two sets of cysteine residues. The iron could then serve to hold the molecule together, as shown schematically in Figure 3.

Generally, nonheme iron proteins have resonances at approximately $g = 1.94$ or $g = 4.3$. Ferredoxins belong to the former class, giving resonances at approximately $g = 1.94$ under reducing conditions, whereas rubredoxin was found to give a resonance at approximately $g = 4.3$ under oxidizing conditions. The oxidation state of iron responsible for the 1.94 signal in ferredoxin remains unknown.[12] Brintzinger *et al.*[13] have suggested that it arises from Fe(III) in a strong ligand field of tetrahedral symmetry, whereas Blumberg and Peisach[14] proposed that it may arise from diamagnetic Fe(II) bonded to a free radical species with strong overlap between the two.

High-spin Fe(III) in rhombic fields characteristically shows EPR signals in the region of $g = 4.3$ and is therefore considered to be the source of the signal observed with rubredoxin. Such signals have been obtained for a number of nonprotein compounds.[15] A simple g tensor for iron in a rhombic field cannot be written. A general Hamiltonian equation, $H = -gj\,H + [S_z^2 + {}^1/_3S(S+1)] + E(Sx^2 - Sy)$, can be written where $E/D = {}^1/_3$ for a maximum rhombic character of the field and $E/D = 0$ for a completely axial field.

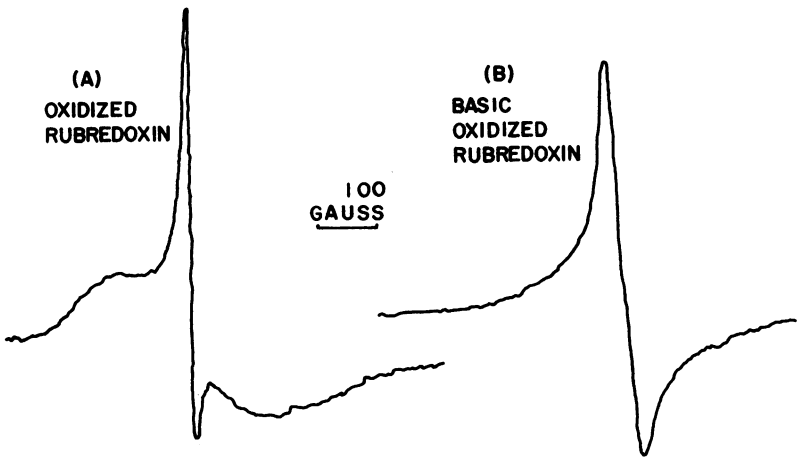

FIG. 4.—EPR spectra of rubredoxin. (*A*) The protein was dissolved in 0.1 *M* Tris buffer, pH 7.0 at a concentration of 2 mg/ml. (*B*) The solution used in (*a*) was adjusted to pH 10 with 4 *N* NaOH.

For the nonheme iron proteins Fe-conalbumin and Fe-transferrin,[16, 17] broad asymmetric resonances have been observed in the $g = 4.3$ region. It has been suggested that four ligand molecules with nitrogen and oxygen are on the corners of a rectangle and coordinately bonded to the iron. In addition, two more ligands are coordinated on the perpendicular axis through the iron. It is of interest that rubredoxin which probably has Fe(III) in a rhombic field gives an EPR signal from iron bound to four sulfur groups and not nitrogen or oxygen.

Summary.—From the EPR spectrum and its variation with pH, the sequence studies on the location of cysteine residues and the quantitative analysis of the number of sulfhydryl groups, it is suggested that the one ferric iron in rubredoxin is coordinately bonded in a rhombic symmetry to four sulfhydryl groups (provided by four cysteine residues) and possibly to tyrosine or lysine residues. The cysteine residues are located in positions 6, 9, 38, and 41 in the rubredoxin molecule. It is proposed that iron may function not only as an electron acceptor but also to stabilize the conformation of the molecule.

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