Effect of Hexacyanoferrate(III) on *Mycobacterium smegmatis* 7Fe Ferredoxin: Further Evidence for Formation of a 6Fe(2×[3Fe–4S]) Ferredoxin

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(Received February 28, 1995)

Addition of potassium hexacyanoferrate(III) to *Mycobacterium smegmatis* 7Fe ferredoxin resulted in the formation of a new 6Fe(2×[3Fe–4S]) ferredoxin, which was verified by the iron content and resonance Raman spectra of the highly purified preparation. The results suggest that the 4Fe cluster in the native ferredoxin was converted into a 3Fe cluster. The new 3Fe cluster produced was found to be similar in structure to the native 3Fe cluster as judged by the Raman spectra. The produced 6Fe(2×[3Fe–4S]) ferredoxin was further degraded into apoferredoxin upon application of additional hexacyanoferrate(III).

Mycobacterium smegmatis ferredoxin has been isolated from the bacterial cells' soluble fraction.[1] It contains 106 amino acid residues and the amino acid sequence of this ferredoxin[2] was found to be quite similar to those of several other bacterial ferredoxins, such as *Azotobacter vinelandii* ferredoxin I,[3] *Pseudomonas ovalis* ferredoxin,[4] and *Thermus thermophilus* ferredoxin.[5] It also exhibited a UV-visible absorption spectrum similar to those of many bacterial-type ferredoxins. It contains ca. 7 g-atoms of iron per 11400 grams of ferredoxin. The presence of two Fe–S clusters in the ferredoxin was suggested by potentiometric titrations. The two iron–sulfur clusters were identified as a 3Fe cluster and a 4Fe cluster by the EPR,[1] the ^{1}H NMR,[6] and by the resonance Raman spectra.[7]

Ferredoxins have been shown to participate in oxidation–reduction reactions. Their iron–sulfur clusters have central roles in electron transfer. Oxidation and reduction of ferredoxins have often been performed by the addition of several non-physiological oxidants and reductants. Among the oxidants, potassium hexacyanoferrate(III) has been used frequently for the oxidation of various ferredoxins. Addition of hexacyanoferrate(III) to *A. vinelandii* ferredoxin I, which contains one 3Fe cluster and one 4Fe cluster per molecule, resulted in the selective destruction of the 4Fe cluster,[8—10] i.e., hexacyanoferrate(III) addition led to the formation of a 3Fe ferredoxin. In contrast, our ^{1}H NMR spectral data with *M. smegmatis* ferredoxin suggested the conversion of the 4Fe cluster to the new 3Fe cluster upon hexacyanoferrate(III) addition, i.e., hexacyanoferrate(III) produced a 6Fe ferredoxin (2×[3Fe–4S]).[11] However, these two different experimental results were obtained from the observations on the hexacyanoferrate(III)-treated ferredoxins which were insufficiently[10] purified after the treatment or not purified at all.[11]

In this paper, we have re-examined the effects of hexacyanoferrate(III) addition to *M. smegmatis* ferredoxin, followed by careful separation of the produced components, and have confirmed that a new 6Fe(2×[3Fe–4S]) ferredoxin is formed initially from native *M. smegmatis* 7Fe ferredoxin by the action of hexacyanoferrate(III): The 4Fe cluster of *M. smegmatis* ferredoxin is converted into a 3Fe cluster by adding potassium hexacyanoferrate(III). Further treatment with hexacyanoferrate(III) destroys the new 3Fe cluster as well as the original 3Fe cluster. That is, further addition leads to the direct formation of the apoproteins without formation of any intermediate form such as 3Fe or 2Fe cluster-containing ferredoxin.

Experimental

Materials. DEAE-cellulose (DE32) was obtained from Whatman Chemical Separation Ltd., hydroxyapatite from Seikagaku Kogyo Co., potassium hexachloroplatinate (IV) from Kojima Chemicals, potassium hexacyanoferrate(III) from Koso Chemical Co., and *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES) from Dojin Chemicals. Bovine serum albumin was purchased from Sigma Chemical Company.

Methods. The isolation of *M. smegmatis* ferredoxin has been described.[1] Protein concentrations were determined by the use of millimolar extinction coefficient of 26.0 at 406 nm for the native ferredoxin[1] or by Folin–Ciocalteu reagent for the apoproteins[12] using bovine serum albumin as a standard. The absorption spectra were recorded with a Hitachi 3400 recording spectrophotometer at ambient temperature.

Each solution used for the ferredoxin iron or protein estimations was prepared as follows: The ferredoxin solution was denatured by addition of 5% trichloroacetic acid. After standing for ca. 60 min at 5 °C, the precipitate was separated from the solution by centrifugation at $10000\times g$ for 20 min. The supernatant was then used for the iron estimation, while the precipitate was dissolved in 20 mM sodium phosphate buffer, pH 7.0, and this solution was used for the protein estimation. The iron content was determined by atomic absorption spectrophotometry at 248.3 nm with a Dainiseikosha atomic absorption spectrophotometer model SAS/727 equipped with an Fe hollow-cathode lamp (Jarrel–Ash)[13] using a standard solution prepared by dissolving metal iron in dilute hydrochloric acid, followed by dilution with 5% trichloroacetic acid.

Polyacrylamide (20%) gel electrophoresis at pH 8.9 was performed according to the method of Davis.[14]

Raman spectra were recorded as described previously,[7] except that the measurement was conducted at 77 K: The sample was immersed into a liquid nitrogen reservoir made up of quartz glass and the scattered light at 90 ° to the incident beam was collected.

The effects of potassium hexacyanoferrate(III) on *M. smegmatis* ferredoxin were examined as follows: Various amounts of potassium hexacyanoferrate(III) dissolved in 0.1 M HEPES (1 M=1 mol dm^{-3}), pH 8.0, were added to a 0.5 mM ferredoxin solution in 0.1 M HEPES, pH 8.0. After incubation at 20 °C, each sample was analyzed by 20% polyacrylamide gel electrophoresis, absorption spectroscopy and Raman spectroscopy, or was chromatographed at 5 °C on a hydroxyapatite column before each analysis. The hexacyanoferrate(III)-treated ferredoxin (18 mg) was chromatographed on a hydroxyapatite column (1.5×15 cm) and eluted with a linear gradient of potassium phosphate (pH 8.0) from 5 to 150 mM in a total volume of 300 ml. The eluted samples were analyzed by absorption spectroscopy and the fractions containing proteins were then concentrated by ultrafiltration with an Amicon membrane (YM-5). These concentrated fractions were analyzed by polyacrylamide gel electrophoresis and Raman spectroscopy. In the cases where $K_2[PtCl_6]$ was used, the same method was applied as in the case of potassium hexacyanoferrate(III). A different buffer, 50 mM potassium phosphate, pH 8.0, was used in the case of $[Fe(bpy)_3](ClO_4)_3$, because the oxidant oxidizes Tris directly.

Results

The effect of hexacyanoferrate(III) on *M. smegmatis* ferredoxin was investigated by several physicochemical techniques. In the initial experiment, an absorbance change in the visible region was monitored at regular time intervals after the addition of hexacyanoferrate(III) at a molar ratio of 5 (hexacyanoferrate(III)/ferredoxin). The results shown in Fig. 1 indicate that the absorbance over the visible region decreased gradually as the incubation was continued and that the decrease ceased at 40 min. At this stage it was obvious that all hexacyanoferrate(III) was consumed in the reaction mixture. To follow the reaction, an aliquot of the incubated sample was picked up at each time and electrophoresed on 20% polyacrylamide gel up to 60 min after the addition of hexacyanoferrate(III). The results shown in Fig. 2 indicate that, at the initial stage of the reaction (1—3 min) few changes in the electrophoretic pattern could be observed. At incubation times over 5 min, a brown-colored band at R_f=0.67, the original band at R_f=1.0, and a broad non-colored, Coomassie blue-stainable protein band at R_f=ca. 0.47 were clearly visualized. Further-

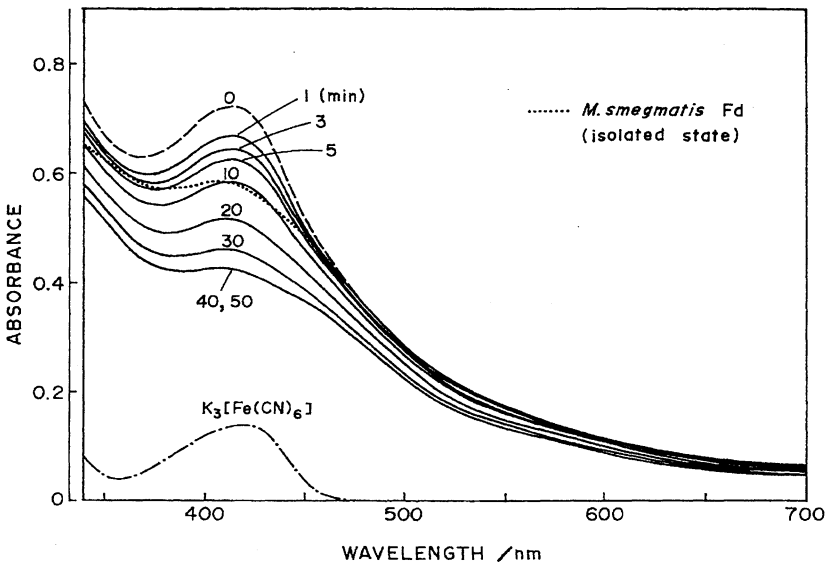

Fig. 1. The visible absorption spectra of hexacyanoferrate(III)-treated *M. smegmatis* ferredoxin. The purified ferredoxin (18 mg in 3.0 ml of 0.1 M HEPES, pH 8.0) was treated at 20 °C with 5 molar excesses of potassium hexacyanoferrate(III). Dotted line: native ferredoxin; dashed line: a spectrum composed of native ferredoxin (18 mg/3 ml) and potassium hexacyanoferrate(III) (2.5 mM); dotted broken line: potassium hexacyanoferrate(III) (2.5 mM). The number above each line represents the time (min) after the addition of hexacyanoferrate(III). The cell path length was 0.10 cm.

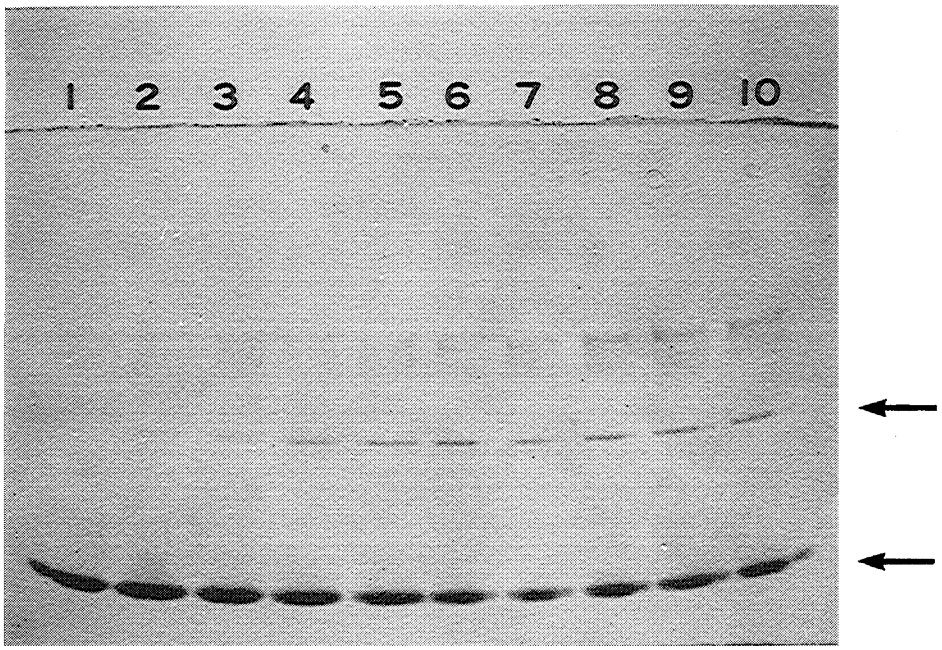

Fig. 2. Polyacrylamide gel electrophoresis of hexacyanoferrate(III)-treated *M. smegmatis* ferredoxin. Lane 1; native ferredoxin; lanes 2—10: *M. smegmatis* ferredoxins which were treated at 20 °C with 5 molar excesses of potassium hexacyanoferrate(III) for 1, 3, 5, 10, 20, 30, 40, 50, and 60 min. Two micrograms of the ferredoxin were taken up at each time and the reaction was stopped by the addition of a large excess of sodium ascorbate. Each mixture was then applied to the gel. After electrophoresis for 18 h at 5 mA, the proteins were stained with Coomassie brilliant blue R-250. Other details are described in "Experimental". Arrows indicate brown bands.

more, the intensities of these bands increased gradually as the incubation was continued. These results suggest that a new ferredoxin visualized at $R_f=0.67$ was produced along with several apoferredoxins (R_f=ca. 0.47) upon addition of hexacyanoferrate(III).

We then tried to separate the components produced by the addition of potassium hexacyanoferrate(III). Ferredoxin which had been incubated for 60 min with a 5 molar excess of hexacyanoferrate(III) was chromatographed on a hydroxyapatite column and four fractions were eluted (Fig. 3). Each fraction was separated and analyzed by UV-visible absorption spec-

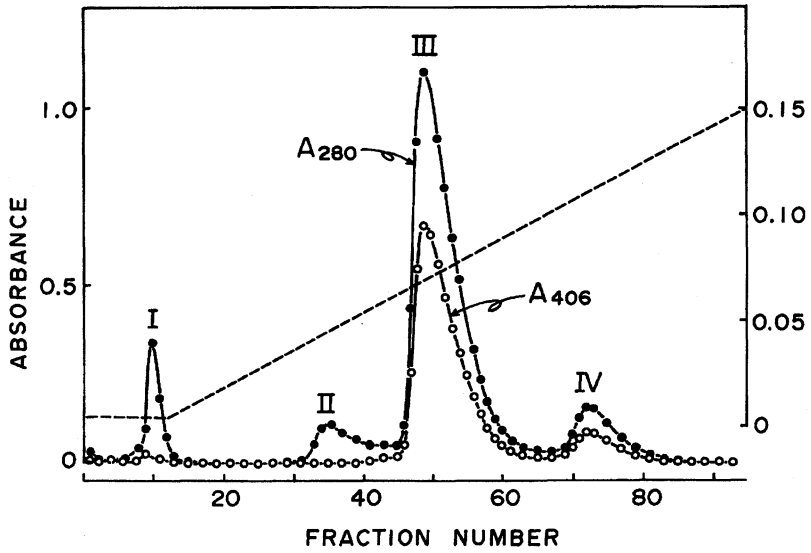

Fig. 3. Hydroxyapatite column chromatography of hexacyanoferrate(III)-treated *M. smegmatis* ferredoxin. The hydroxyapatite column (1.5×15 cm) was equilibrated with 5 mM potassium phosphate buffer, pH 7.0 and the hexacyanoferrate(III)-treated ferredoxin was then applied to the column. The column was washed with the same buffer (30 ml) and then eluted with a linear gradient of 5—150 mM potassium phosphate, pH 7.0 (150 ml). Solid line: absorbance at 280 nm; dashed line: concentration of potassium phosphate, pH 7.0.

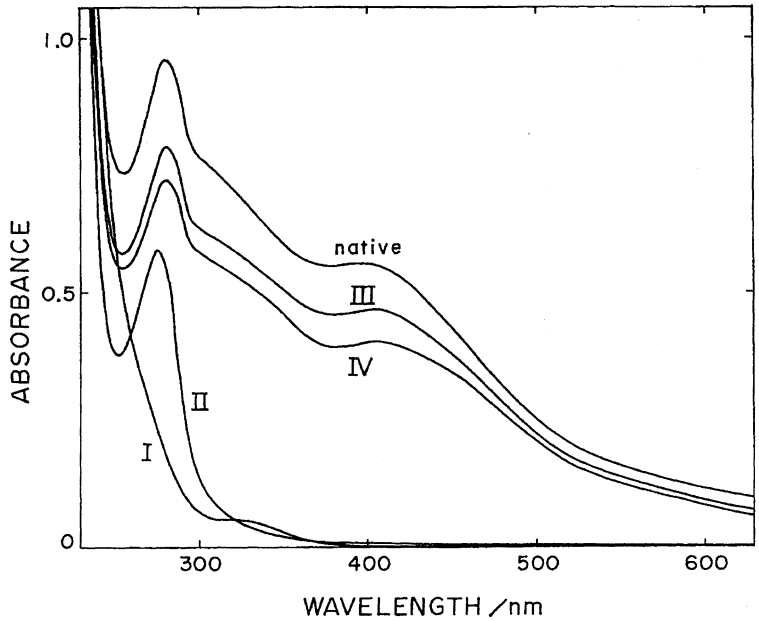

Fig. 4. UV-visible absorption spectra of fractions I—IV eluted from the hydroxyapatite column. Absorbance on the vertical axis is arbitrary. The spectrum of native *M. smegmatis* ferredoxin is also shown as a reference.

troscopy (Fig. 4), polyacrylamide gel electrophoresis (Fig. 5), resonance Raman spectroscopy (Fig. 6), and iron content estimation. As can be seen in Fig. 4, fraction I seemed to be hexacyanoferrate(II) ion and/or some non-protein component(s) because of the absorption spectral shape in the UV region. Fraction II was thought to be apoferredoxin(s), and fractions III and IV were both thought to be ferredoxins, because of their characteristic spectral shapes in the UV-visible region, which were quite similar to that of native *M. smegmatis* ferredoxin.[1] The electrophoretic patterns of fraction III and fraction IV displayed in Fig. 5 show that both fractions were separated from each other by the chromatography and were also highly purified.

Resonance Raman spectra is one of the best methods to identify the iron-sulfur clusters. Johnson et al. have established that resonance Raman spectrum in the low frequency region (200—450 cm^{-1}), in which several bands characteristic to iron–sulfur clusters are observed, can be used to identify the 3Fe and 4Fe clusters in the iron–sulfur proteins.[15] We remarked in their precise resonance Raman spectroscopy that the appearance of the bands at 336 and 359 cm^{-1} is a good indication of the presence of 4Fe cluster, although these bands' intensities are commonly weak. The resonance Raman spectra of fractions III and IV shown in Fig. 6 were different from each other: the Raman bands at 336 and 359 cm^{-1} detected both in the native ferredoxin and in fraction III disappeared completely in fraction IV, while in fraction III, these bands were slightly diminished. These results suggest that the ferredoxin in fraction III must contain both a 3Fe and a 4Fe cluster, while the ferredoxin in fraction IV has 3Fe clusters only. The slight difference in the ratio of the 359 cm^{-1} band intensity to that of 368 cm^{-1} band in native ferredoxin and in fraction III may indicate that some structural change occured in fraction III compared to the native ferredoxin.

The iron contents of fractions III and IV were determined to be 6.8 and 5.8 (mol/mol ferredoxin), respectively. These results indicate that fraction III must contain a 7Fe ferredoxin, while fraction IV must contain a new 6Fe(2×[3Fe–4S]) ferredoxin. EPR spectral data[16] also support the presence of 3Fe clusters in the hexacyanoferrate(III)-treated ferredoxins.

The effect of potassium hexacyanoferrate(III) was further investigated by changing the molar ratios (hexacyanoferrate(III)/ferredoxin) from 1 to 100; changes were followed by polyacrylamide gel electrophoresis and absorption spectroscopy. As shown in Fig. 7, the absorbances over the visible region, which were measured after cessation of the reactions, decreased in proportion to the amount of the oxidant added. The decreases in absorbance were clearly observed even at a molar ratio of unity, although the absorbances over ca. 450 nm decreased very little. On the other hand, the electrophoretic patterns displayed in Fig. 8 show that, at ratios of 3—5, a new brown-colored band at $R_f=0.67$ appeared. The intensities of these new bands gradually increased as the ratio increased. At ratios of 10 and 20, the intensities of the $R_f=1.0$ and 0.67 (brown-colored) bands were almost constant, and the intensities of the non-colored, $R_f=0.54$ and 0.58 bands were also constant. At molar ratios of 50 and 100, colorless protein bands' intensities increased, suggesting that some of the original ferredoxin was converted into several apoproteins. On the other hand, the resonance Raman spectrum at a molar ratio of unity was almost the same as that of native ferredoxin, and at a molar ratio of 10, the

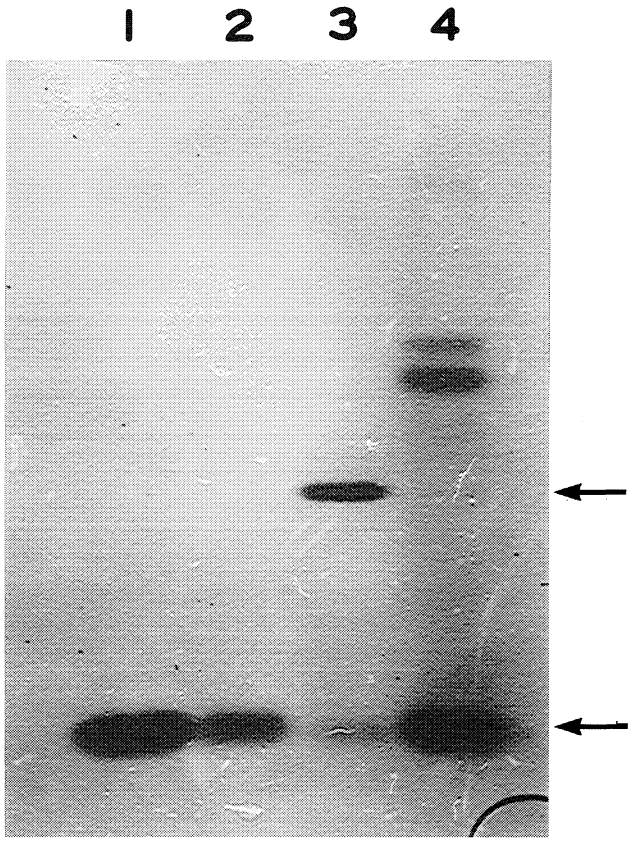

Fig. 5. Polyacrylamide gel electrophoresis of fractions III and IV eluted from hydroxyapatite column. Lane 1: native *M. smegmatis* ferredoxin; lane 2: fraction III; lane 3: fraction IV; and lane 4: hexacyanoferrate(III)-treated *M. smegmatis* ferredoxin (not purified). The experimental conditions were the same as in Fig. 2. Two micrograms of each sample were applied to the gel. Arrows indicate brown bands.

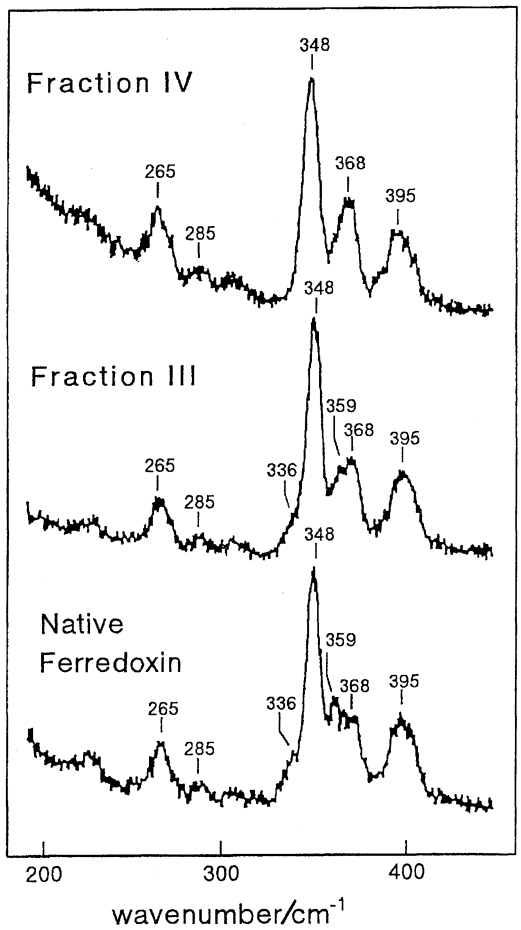

Fig. 6. Resonance Raman spectra of fractions III and IV produced by the action of potassium hexacyanoferrate(III). Upper: fraction IV; middle: fraction III; and lower: native *M. smegmatis* ferredoxin. The experimental conditions are described in "Experimental".

Raman spectrum was found to be quite similar to that at a molar ratio of 5 (spectra not shown).

Potassium hexacyanoferrate(III) was thus proved to be able to convert the 4Fe cluster to a 3Fe cluster in the experiments described above. Two other oxidants were also examined to determine whether they have any iron–sulfur clusters' conversion ability in ferredoxin. Potassium hexachloroplatinate(IV), $K_2[PtCl_6]$, and tris(bipyridine)iron(III) perchlorate, $[Fe(bpy)_3](ClO_4)_3$, were selected because of their higher oxidation–reduction potentials (E_m=680 mV for $K_2[PtCl_6]$, E_m=1040 mV for $[Fe(bpy)_3](ClO_4)_3$) than that of potassium hexacyanoferrate(III) (E_m=412 mV). Each oxidant was added to *M. smegmatis* ferredoxin at a molar ratio of 5 (oxidant/ferredoxin), and after incubation for 60 min at 20 °C, each sample was analyzed by polyacrylamide gel electrophoresis and by resonance Raman spectroscopy. The electrophoretic patterns suggest that these two oxidants produced no new brown-colored bands as in the case of potassium hexacyanoferrate(III), although $K_2[PtCl_6]$ caused the appearance of a colorless, Coomassie blue-stainable band at R_f=0.48, and $[Fe(bpy)_3](ClO_4)_3$ produced no protein bands. On the other hand, no detectable changes in the resonance Raman spectrum were observed upon addition of either oxidant (data not shown), suggesting that little conversion of the iron-sulfur clusters of *M. smegmatis* ferredoxin occurred upon addition of these two oxidants, although some degradation did occur.

Discussion

M. smegmatis ferredoxin has been shown to be a 7Fe ferredoxin containing both 4Fe and 3Fe clusters in one molecule.[1,7,8] Our previous ^{1}H NMR spectral data have suggested that the 4Fe cluster of the ferredoxin is converted to a new 3Fe cluster upon addition of potassium hexacyanoferrate(III).[11] On the other hand, in the case of *A. vinelandii* ferredoxin I, it has been reported that the selective destruction of the 4Fe cluster occurred to give a 3Fe ferredoxin after hexacyanoferrate(III) oxidation.[8—11] In the present study, we reexamined the effect of hexacyanoferrate(III) oxida-

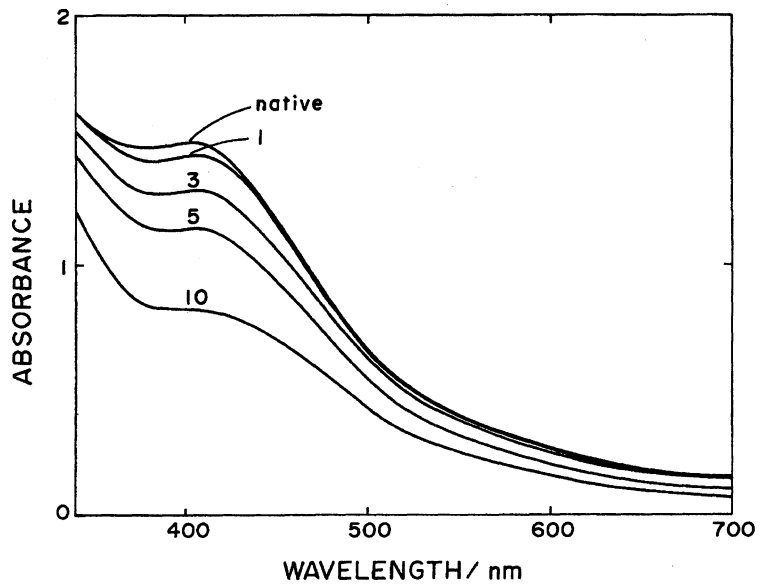

Fig. 7. UV-visible absorption spectra of *M. smegmatis* ferredoxin treated with potassium hexacyanoferrate(III) at several hexacyanoferrate(III)/ferredoxin ratios. Each ferredoxin solution (6 mg ml^{-1} in 0.1 M HEPES-Na, pH 8.0) was treated with potassium hexacyanoferrate(III) at molar ratios (hexacyanoferrate(III)/ferredoxin) of 1, 3, 5, and 10 and was incubated until no further absorbance changes were observed. The UV-visible absorption spectrum of each mixture was then recorded in a cell with a light path length of 0.1 cm.

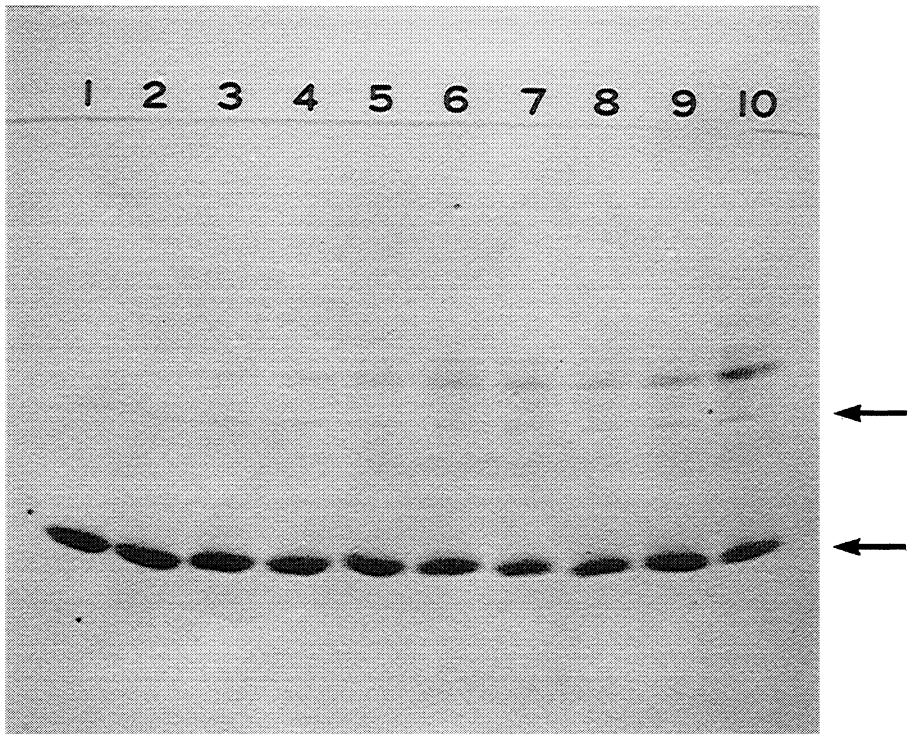

Fig. 8. Polyacrylamide gel electrophoresis of *M. smegmatis* ferredoxin treated with potassium hexacyanoferrate(III) at various oxidant/ferredoxin ratios. The experimental conditions were the same as in Fig. 2 except that the molar ratios of hexacyanoferrate(III)/ferredoxin were 1, 2, 3, 4, 5, 10, 20, 50, and 100, displayed in lanes 2—10, espectively. Lane 1: native ferredoxin. Arrows indicate brown bands.

tion on *M. smegmatis* ferredoxin in detail, by separating carefully the compounds produced upon addition, and analyzing these compounds by several physicochemical techniques. All experimental data obtained clearly indicated the production of a new 6Fe(2×3Fe) ferredoxin by the hexacyanoferrate(III) oxidation of *M. smegmatis* ferredoxin. No indication of production of the 3Fe ferredoxin was obtained in the present study. This observation is in line with our ^{1}H NMR result.[11]

Resonance Raman spectroscopy of ferredoxins has

elucidated that the band profile in the low frequency region (200—450 cm^{-1}) depends mainly upon the structure of the core iron–sulfur cluster,[15] while the small band change in frequency and intensity may be attributed to the deviation in the cluster structure and /or in the peptide conformation around it (vide infra). Thus, the new 3Fe cluster produced by hexacyanoferrate(III) action must have a structure quite similar to that of the intact 3Fe cluster, because the resonance Raman spectrum of Fraction IV remained unchanged as a whole, except for the absence of the bands at 336 and 359 cm^{-1}, which are characteristic to 4Fe cluster rather than the native ferredoxin (Fig. 6). On the other hand, a conformational difference in the peptide structure around the 3Fe clusters involved in the new 6Fe ferredoxin has been more sensitively indicated by our previous ^1H NMR data[11] in which the signals of cysteinyl α and/or β protons were classified into those from two 3Fe clusters in obviously different ligation structures.[18]

Morgan et al.[8] have identified a cysteinyldisulfide radical as an intermediate species upon hexacyanoferrate(III) oxidation of *A. vinelandii* ferredoxin I. We tried to detect this type of paramagnetic species by EPR spectroscopy at 77 K. However, no EPR signal of this type could be found in the case of *M. smegmatis* ferredoxin.

The different behavior upon hexacyanoferrate(III) oxidation for *M. smegmatis* ferredoxin and *A. vinelandii* ferredoxin I is attributable, firstly, to the stability difference between the 6Fe ferredoxin of *M. smegmatis* ferredoxin and that of *A. vinelandii* ferredoxin I, which might once be produced by the potassium hexacyanoferrate(III) addition. In fact, a slight difference can be mentioned in the resonance Raman spectra of *M. smegmatis* ferredoxin and *A. vinelandii* ferredoxin I: the band at 336 cm^{-1} is observed to be slightly lower in intensity in the spectrum of the former than in the latter. This experimental finding implies that some structural difference is involved, especially in the iron-sulfur clusters and/or the peptide structure near around the clusters, in these ferredoxins. Thus, we think that the 6Fe ferredoxin produced from *A. vinelandii* ferredoxin I must be fairly labile if it is produced, so that it will be rapidly converted to 3Fe ferredoxin which would be more stable in this case.

Secondly, some uncertainty is involved in the procedure of iron content estimation in the case of *A. vinelandii* ferredoxin I:[10] The estimation is based on the special assumption that the eluted sample from DE-52 column contains 40% of 3Fe- and 60% of apo-ferredoxins. The precise iron content estimation should be made on the isolated and purified sample.

A conversion scheme of *M. smegmatis* ferredoxin induced by potassium hexacyanoferrate(III) can be proposed as follows:

$$\begin{array}{ccccc} [3Fe,\ 4Fe] & & \xrightarrow[(1)]{-e} & & [3Fe,\ 4Fe'] \\ 7FeFd & & & & 7FeFd' \\ \xrightarrow[(2)]{-Fe^{2+}} & [3Fe,\ 3Fe'] & & \xrightarrow[(3)]{-6Fe^{3+}} & [—,\ —] \\ 6FeFd & & & & apoFd, \end{array}$$

where 4Fe′ indicates a possible oxidized state of the 4Fe cluster $[4Fe–4S]^{3+}$, 3Fe′ is a new $[3Fe–4S]^{+}$ cluster produced from the $[4Fe–4S]^{3+}$ cluster by hexacyanoferrate(III) addition, bar(—) means the absence of any iron–sulfur clusters, and Fd is an abbreviation of ferredoxin. Reaction (1) is an oxidative step of the $[4Fe–4S]^{2+}$ cluster, reaction (2) a conversion step of the $[4Fe–4S]^{3+}$ cluster and reaction (3) a degradation step of the $[3Fe–4S]^{+}$ and $[3Fe–4S]^{+\prime}$ clusters. This final step seems to be a direct degradation process without any stable intermediate cluster formation, because we did not detect any additional brown band or fraction in the native polyacrylamide or in the hydroxyapatite column chromatography.

Different behavior between *M. smegmatis* ferredoxin and *A. vinelandii* ferredoxin I must also be mentioned in the optical spectra change upon the hexacyanoferrate(III) oxidation: in the case of *A. vinelandii* ferredoxin I, a rapid increase in absorbance was detected just after the addition of potassium hexacyanoferrate(III), such phenomenon could not be followed in the case of the present *M. smegmatis* ferredoxin (Fig. 1). The first absorbance increase of *A. vinelandii* ferredoxin I could be ascribed to the oxidation process of $[4Fe–4S]^{2+}$ to $[4Fe–4S]^{3+}$ (corresponding to reaction (1) in the above scheme). Although the apparent absorbance increase could not be detected by the present instrumental arrangements, the presence of $[4Fe–4S]^{3+}$ species is highly probable also in the reaction of *M. smegmatis* ferredoxin because of its low midpoint $[4Fe–4S]^{+}/[4Fe–4S]^{2+}$ oxidation–reduction potential of the iron–sulfur clusters.[1]

The two other reagents, potassium hexachloroplatinate(IV) and tris(bipyridine)iron(III) perchlorate, used in the present study are well-known oxidants stronger than potassium hexacyanoferrate(III). It is presumed that these two reagents stimulate the oxidation of $[4Fe–4S]^{2+}$ cluster to give the 6Fe ferredoxin. However, the action of potassium hexachroloplatinate(IV) produced neither 6Fe ferredoxin nor 3Fe ferredoxin, and at the same time smaller amount(s) of apoferredoxin(s) could be obtained. In the case of tris(bipyridine)iron(III) perchlorate, no change was observed in the electrophoretic pattern after the addition of the reagent. Thus, the present experimental results showed that the efficiency of oxidant to promote the conversion reaction (1)—(3), could not directly be related to its oxidation–reduction potential.

The function of *M. smegmatis* ferredoxin is still unknown. Therefore, it is impossible to know whether

the 6Fe ferredoxin produced is active in vivo. However, we found that the spinach ferredoxin could be replaced by the present 6Fe ferredoxin in a spinach ferredoxin–$NADP^+$ reductase system. The activity of the 6Fe ferredoxin in the system was estimated to be 65% that of spinach ferredoxin.[1,19,20] A further study of the new 6Fe ferredoxin is now in progress.

The authors wish to thank Drs. K. Nagayama of University of Tokyo and D. Ohmori of Juntendo University for their helpful discussions. They are also grateful to Professor T. Takada and Ms. N. Kurihara of Rikkyo University for the iron determination using atomic absorption spectrophotometry, Dr. J. A. Gardner for reading the original manuscript, and to Ms. M. Taketsugu for preparing the figures.

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