Further Characterization of Ferredoxin Nitrite Reductase and the Relationship between the Enzyme and Methyl Viologen-dependent Nitrite Reductase

Masakazu Hirasawa-Soga, Shigeo Horie* and Goro Tamura

Department of Agricultural Chemistry, Faculty of Horticulture, Chiba University, Matsudo, Chiba 271, Japan

*Department of Biochemistry, School of Medicine, Kitasato University, Sagamihara, Kanagawa 228, Japan

Received November 20, 1981

Ferredoxin-dependent nitrite reductase of spinach has been further characterized and the relationship between this enzyme and methyl viologen-dependent nitrite reductase studied. Purified ferredoxin nitrite reductase, having a molecular weight of 86,000, showed 2.5 times higher ferredoxin-dependent activity than methyl viologen-linked activity. Besides 4 mol of labile sulfide the enzyme contained about 2 mol of siroheme per mol. When dithionite, methyl viologen and nitrite were added, ESR signals of a heme nitrosyl complex at \( g = 2.14, 2.07 \) and 2.02 were observed. Moreover, hyperfine splitting of the signal due to \(^{14}\)N nuclear spin was also observed at 2.033, 2.023 and 2.013. The sole addition of hydroxylamine to the ferric enzyme also caused the same but much less intense signals with the hyperfine splitting.

On treatment of the ferredoxin nitrite reductase (native enzyme) with DEAE-Sephadex A-50 chromatography, a modified nitrite reductase having a molecular weight of 61,000 and a protein fraction having an apparent molecular weight of 24,000 were separated. The modified enzyme contained about one mol of siroheme and 4 mol of labile sulfide per mol and showed essentially the same heme ESR signals as the native enzyme. Contrary to the native enzyme, this modified enzyme accepted electrons more efficiently from methyl viologen than ferredoxin and the reduction of nitrite to ammonia catalyzed by the modified enzyme was not stoichiometric. The observed nitrite to ammonia ratio was 1 to less than 0.6. Cyanide at concentrations between 0.02 to 0.2 mM inhibited the activity of the native enzyme almost completely but the modified enzyme was inhibited only partially.

From the results obtained, it is suggested that the native ferredoxin-linked nitrite reductase consists of two components (or subunits) and removal of the light component results in formation of a modified enzyme with increased relative affinity to methyl viologen.

Nitrite reductase of green plants (EC 1.7.7.1) has been extensively purified and characterized in detail. Reduced methyl viologen has been employed in purification studies for assaying of the enzymatic activity. The purified enzyme has a molecular weight of 60,000 to 64,0001~12) and the molecule can not be dissociated by sodium dodecyl sulfate (SDS), indicating that the molecule does not contain subunits.10,11) Two to six atoms of iron have been detected per molecule of the enzyme.1,3,4,10~15) Murphy et al.16) have identified the heme prosthetic group of spinach nitrite reductase as siroheme, an iron-tetraproporphyrin of the isobacteriochlorin-type with eight carboxylic acid side chains. All nitrite reductase preparations reported by other authors exhibited similar absorption spectra with peaks in the regions of 380~390 nm and 570~580 nm, characteristic of siroheme-containing proteins.5~11,16,17) Labile sulfide has also been found in this enzyme. Aparicio et al.17) showed by ESR studies at liquid helium temperature that spinach nitrite reductase contained an iron-sulfur center. Recently, Lancaster et al.12) reported that the spinach enzyme contains a tetranuclear iron-sulfur center (Fe₄S₄) and
suggested that the enzyme contained one siroheme and one Fe$_4$S$_4$ cluster per molecule.

We have described the isolation and some properties of ferredoxin-linked nitrite reductase from spinach leaves.$^{19-21}$ This enzyme had a molecular weight of 86,000, which is higher than that of methyl viologen-linked nitrite reductase reported so far. It contained 7 iron atoms and 4 atoms of labile sulfur per molecule. In this paper, we report further characterization of the enzyme and also the relationship between this enzyme and methyl viologen-linked nitrite reductase.

### MATERIALS AND METHODS

**Materials.** Fresh leaves of *Spinacia oleracea* (L. cv. Michinoku No. 2) were used in this study. The plants were grown in a field during the 1980 growing season. Spinach ferredoxin was prepared by the method of Tagawa and Arnon$^{22}$ ($A_{422}: A_{277}=0.42$) and dissolved in 30 mM Tris-HCl buffer, pH 8.0.

The following chemicals were purchased from commercial sources: RNA polymerase B (SDS-PAGE Marker 1); DEAE-cellulose (DE 52) (Whatman Ltd.). Other chemicals were of analytical grade.

**Assay of nitrite reductase activity.** The assay method for ferredoxin-linked nitrite reductase was the same as described in a previous paper.$^{21}$

**Other analytical methods.** ESR spectra were recorded at the temperature of liquid N$_2$ with a Japan Electron Optics Spectrometer, model JES-PE-1X, equipped with a 100 kHz field modulator. The modulation width was 2 gauss. SDS gel electrophoresis was performed by the method of Weber and Osborn,$^{23}$ using 5% and 13% polyacrylamide. Ammonia was determined by the Conway microdiffusion technique, followed by the phenol-hypochlorite test for ammonia as modified by Russell.$^{24}$ Protein was determined by the method of Bradford,$^{25}$ using bovine serum albumin as a standard. Iron content of the enzyme was determined by the procedure of Moss et al.$^{26}$ Siroheme was analyzed, qualitatively and quantitatively, with the acetone-HCl extract of the enzyme to which pyridine had been added, according to the procedure of Siegel et al.$^{27}$ Analysis of the enzyme for acid-labile sulfide was performed as described by Siegel et al.$^{27}$

**Preparation of nitrite reductase.** The procedure used for the preparation of native ferredoxin nitrite reductase was the same as described in a previous paper.$^{21}$ The modified enzyme that reacted more efficiently with methyl viologen than ferredoxin was also prepared from the native enzyme by DEAE-Sephadex A-50 chromatography as described previously.$^{28}$

### RESULTS

**The increase in the ratio of ferredoxin-linked to methyl viologen-dependent activity by the purification**

Our most recent results for the purification of ferredoxin nitrite reductase are summarized in Table I. The specific activity of ferredoxin nitrite reductase (A) increased more with purification than that of methyl viologen-dependent nitrite reductase (B), especially after ferredoxin-Sepharose affinity chromatography. Thus the ratio of (A)/(B) of the final preparation was 2.48, being the highest value reported so far. Moreover, when 2 mg of ferredoxin was added to the methyl viologen assay system, the observed activity was higher than (A) and (B) up to the step of DEAE-cellulose chromatography. After the step of gel filtration, however, the specific activity in the presence of both ferredoxin and methyl viologen was essentially equal to (A) (data not shown). These results suggested that a nitrite

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Fd-Activity (A) (total units)</th>
<th>Specific activity</th>
<th>MV-Activity (B) (total units)</th>
<th>Specific activity (A)/(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (35~75%)</td>
<td>29,905.2</td>
<td>1.01</td>
<td>30,911.4</td>
<td>1.05</td>
</tr>
<tr>
<td>1st DE-52</td>
<td>28,012.4</td>
<td>1.07</td>
<td>29,851.3</td>
<td>1.14</td>
</tr>
<tr>
<td>2nd DE-52</td>
<td>32,000.5</td>
<td>18.9</td>
<td>28,587.1</td>
<td>16.9</td>
</tr>
<tr>
<td>Ultrogel AcA 34</td>
<td>24,810.3</td>
<td>141.4</td>
<td>16,906.0</td>
<td>96.4</td>
</tr>
<tr>
<td>Fd-Sepharose 4-B</td>
<td>6,359.3</td>
<td>301.4</td>
<td>2,565.8</td>
<td>121.6</td>
</tr>
</tbody>
</table>
Ferredoxin–Nitrite Reductase

reductase fraction reacting more efficiently with methyl viologen had been removed during the purification.

**ESR Spectra**

As reported previously, native ferredoxin nitrite reductase in its ferric state showed ESR signals of a high spin heme at \( g = 6.9 \) and \( 5.2 \) at liquid N\(_2\) temperature. The ESR spectrum of the modified enzyme in its ferric high spin state shown in Fig. 1 is very similar to that of the native enzyme. The signals of the enzyme dissolved in potassium phosphate buffer (KP-buffer) had the same \( g \)-values, but were several times more intense than those of the same enzyme dissolved in Tris-NaCl buffer (TN-buffer). It has been reported that the pH of phosphate buffer in the frozen state decreases at extremely low temperatures but that of Tris buffer does not change appreciably. Therefore, at liquid nitrogen temperature, the enzyme dissolved in KP-buffer was not bound with hydroxyl ions and was mostly in the high spin state whereas the enzyme dissolved in TN-buffer was probably in thermal equilibrium between the high and low spin states of hemoprotein hydroxide.

The ferric high spin signals of the native enzyme disappeared on reduction with di-

![ESR Spectrum of Modified Nitrite Reductase in the Low Magnetic Field](image)

**Fig. 1.** ESR Spectrum of Modified Nitrite Reductase in the Low Magnetic Field.

The sample was concentrated by membrane filtration using a Bio-engineering G-10T membrane. Protein concentration, 3.26 mg/ml; solvent, TN-170 buffer; microwave frequency, 9176.8 MHz; power, 0.6 mW; response, 0.3 sec. Other conditions are described in MATERIALS AND METHODS.

![ESR Spectra of Native Nitrite Reductase in the High Magnetic Field](image)

**Fig. 2.** ESR Spectra of Native Nitrite Reductase in the High Magnetic Field.

Protein was concentrated as in Fig. 1. Protein concentration, approximately 7 mg/ml; solvent, KP-200 buffer. (A) Nitrite reductase was frozen after reaction with 1 mM NaNO\(_2\), 1 mM methyl viologen and 1 mM Na\(_2\)S\(_2\)O\(_4\) at room temperature for 10 min. Conditions of measurement were; microwave frequency, 9176.8 MHz; power, 0.6 mW; response, 0.3 sec. Other conditions are described in MATERIALS AND METHODS. (B) Nitrite reductase was frozen after the addition of 1 mM NH\(_2\)OH at room temperature for 10 min. Conditions of measurement were the same as in (A).
thionite and, when dithionite, methyl viologen and nitrite were added, signals of a heme nitrosyl complex at \( g = 2.14, 2.07 \) and 2.02 were observed. The intensity of these signals was dependent on the concentration of the enzyme. Moreover, hyperfine splitting of the signal with a coupling constant of 9 mT due to \(^{14}\)N nuclear spin was also observed at \( g = 2.033, 2.023 \) and 2.013 (Fig. 2A). These narrow splittings suggest that the electron density is localized principally on the iron atom of the siroheme, rather than on the nitrogen of the NO ligand. Very similar signals of a heme nitrosyl complex with \(^{14}\)N hyperfine splitting have been reported for methyl viologen-dependent nitrite reductase preparations by other authors.

We further found that the same but much less intense signals with the hyperfine splitting appeared on sole addition of hydroxylamine to the native ferric enzyme (Fig. 2B).

**Analysis of siroheme**

Chemical analysis of siroheme content of the native enzyme by the pyridine hemochromogen method showed that it contained about 1.6 mol of siroheme per mol of enzyme. In view of the relative instability of siroheme, the above result suggests that the actual siroheme content is 2 mol per mol of the native enzyme.

In fact, we observed a somewhat curious behaviour of the extracted heme chromophore. Treatment of the native enzyme dissolved in KP-200 buffer (200 mM potassium phosphate buffer, pH 7.7) with 9 volumes of acetone containing 0.01 N HCl followed by centrifugation gave a colored supernatant solution of heme with absorption maxima at 390, 578 and 700 nm and a shoulder at 510 nm. The absorption ratio of the Soret to \( \alpha \) band was 2.6. Almost no chromophore remained bound to the protein precipitate (Fig. 3). After adding pyridine to a final concentration of 20%, the acetone-HCl extract was concentrated to near-dryness under a stream of argon, and the residue was dissolved in a small volume of pyridine. The resulting pyridine ferrihemochrome solution showed the absorption spectrum shown in Fig. 4A. The absorption maximum in the visible region was at 560 nm, which was at a 3 to 4 nm longer wavelength than that of siroheme pyridine ferrihemochrome reported in the literature.

Then the pyridine ferrihemochrome solution was applied to a column of Sephadex LH-20 equilibrated with pyridine and elution was performed with the same solvent. The chromophore migrated in the column as two violet-red bands. The fraction eluted first contained about 5 times more siroheme than the fraction eluted later. The absorption spectra of the two...
chromophore fractions were identical and the maxima were at 401 and 556 nm (absorption ratio, 2.5) with a shoulder at 468 and 520 nm (Fig. 4B).

In view of the possible decomposition of a part of the siroheme during extraction and determination, the siroheme content was also estimated by titration with nitrite. Nitrite is known to react slowly with ferric siroheme of the enzyme forming a 1:1 complex.11) On binding with nitrite, the \( \alpha \)-maximum of the native enzyme shifts from 573 to 568 nm and the Soret maximum shifts from 388 to 396 nm.20) The result of titration of the native enzyme with nitrite is shown in Fig. 5. The observed initial decrease in absorbance at 568 nm probably corresponds to the process of reduction of nitrite by endogenous reducing equivalent of the enzyme. The enzyme preparation seems to become fully oxidized only after its endogenous reducing equivalent has been consumed. When the amount of nitrite was corrected for the probable initial reduction, the maximal increase (or decrease) in absorbance at 568 nm (or 573 nm) was achieved by binding of about 2 mol of nitrite per mol of native ferredoxin nitrite reductase. Similar results were obtained by measuring the minimum amount of nitrite required for the maximal shift of the \( \alpha \) or Soret band. These results also suggest that the enzyme contains 2 mol of siroheme per mol. On the other hand, the heme content of the modified enzyme was estimated also by the pyridine ferrihemo-

Fig. 4. Absorption Spectra of Extracted Heme in Pyridine.
Heme extracted with acetone-HCl was transferred to pyridine, and subjected to chromatography on a column of Sephadex LH-20. (A): Before subject to chromatography. (B): After subjection to Sephadex LH-20 column chromatography; (B)-I, first eluted fraction; (B)-II, later eluted fraction.

Fig. 5. Titration of Native Nitrite Reductase with Nitrite.
Nitrite reductase, 12 nmol in KP-200 buffer, was titrated at room temperature with nitrite. The increase (or decrease) in the absorbance at 568 nm was monitored. The spectra were recorded after the addition of nitrite.
TABLE II. STOICHIOMETRY OF NITRITE REDUCTION BY NITRITE REDUCTASE

Experimental conditions were the same as those for the standard assay described in a previous paper.21)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Electron donor</th>
<th>(\text{NO}_2^-) reduced ((\mu\text{mol}))</th>
<th>(\text{NH}_4^+) produced ((\mu\text{mol}))</th>
<th>(\text{NH}_4^+/\text{NO}_2^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native21)</td>
<td>Ferredoxin</td>
<td>2.00</td>
<td>1.93</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Methyl viologen</td>
<td>2.00</td>
<td>1.74</td>
<td>0.87</td>
</tr>
<tr>
<td>Modified</td>
<td>Ferredoxin</td>
<td>0.97</td>
<td>0.50</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Ferredoxin</td>
<td>0.96</td>
<td>0.47</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>Methyl viologen</td>
<td>1.21</td>
<td>0.45</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Methyl viologen</td>
<td>1.05</td>
<td>0.38</td>
<td>0.36</td>
</tr>
</tbody>
</table>

TABLE III. EFFECT OF SOME INHIBITORS

The reaction mixture was preincubated for 2 min at 30°C before the standard assay.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Electron donor</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>KCN</td>
<td>0.02</td>
<td>Methyl viologen</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>o-Phenanthroline</td>
<td>0.2</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>(NH₄)₂SO₄</td>
<td>0.4</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.8</td>
<td>Ferredoxin</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40.0</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Modified</td>
<td>KCN</td>
<td>0.002</td>
<td>Methyl viologen</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.02</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td></td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>o-Phenanthroline</td>
<td>0.2</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>p-CMB</td>
<td>0.2</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td></td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>p-CMS</td>
<td>0.2</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td></td>
<td>52</td>
</tr>
</tbody>
</table>

Chrome method. It was found that there was about 1.2 mol of siroheme per mol of the modified enzyme.

Iron and labile sulfide content

Analysis of the modified enzyme for total iron and acid-labile sulfide showed the presence of 5g-atoms of iron and 3.4 mol of sulfide per mol of the enzyme. When these data are taken together with the others, it can be deduced that the modified enzyme has one mol of siroheme and 4 mol of acid-labile sulfide per mol. The data thus indicate the presence of an iron-sulfur center in the modified enzyme.

Catalytic properties

The native enzyme catalyzed a 6-electron reduction of nitrite to ammonia, and the stoichiometry of this reaction clearly showed a 1:1 ratio between nitrite consumed and ammonia formed,21) whereas the modified enzyme did not catalyze the stoichiometric reduction of nitrite to ammonia (Table II). The apparent \(Km\) for methyl viologen of the native enzyme was calculated to be 100 \(\mu\text{M}\), whereas that of the modified enzyme was calculated to be 53 \(\mu\text{M}\). This latter value is in good agreement with the \(Km\) value described for the methyl viologen-dependent enzyme from spin-
ach. The apparent $K_m$ of the modified enzyme for nitrite was estimated to be 0.59 mM. This value was not so much different from those reported by other authors for the spinach enzyme and also from that estimated by us with the native enzyme (unpublished data).

**Effects of inhibitors**

The effects of various substances on the reaction catalyzed by the native or modified enzyme were examined (Table III). Cyanide was found to be an effective inhibitor. At a concentration of 0.2 mM, KCN inhibited the activity of the native enzyme completely, and, even at a concentration of 0.02 mM, nearly 90% inhibition was observed. The modified enzyme was less sensitive to cyanide and the observed inhibition was 90% at a concentration of 0.2 mM and 45% at a concentration of 0.02 mM. Ammonium sulfate was slightly inhibitory to the native enzyme with reduced ferredoxin or methyl viologen as electron donor, but required higher concentrations.

A metal chelating reagent, $o$-phenanthroline, showed no inhibition of the native enzyme at a concentration of 0.02 mM. However, the modified enzyme was inhibited at this concentration of $o$-phenanthroline by 18%.

The effects of these inhibitors suggest that the enzymatic mechanism of the native ferredoxin-linked nitrite reductase is not identical to that of the modified enzyme.

**Properties of coupling protein**

By applying the native enzyme to a DEAE-Sephadex A-50 column, equilibrated with TN-170 buffer (10 mM Tris-HCl buffer, pH 8.0, containing 170 mM NaCl), its activity was completely adsorbed. However, a catalytically inactive protein fraction could be removed from the column by washing with the same buffer. Then the activity (the modified enzyme) could be eluted slowly with the same buffer. The molecular weight of the modified enzyme thus obtained was found to be 61,000 by SDS-polyacrylamide gel electrophoresis. The molecular weight of the catalytically inactive protein (defined as coupling protein) was estimated to be 24,000 by SDS-polyacrylamide gel electrophoresis. This coupling protein itself has no nitrite reducing activity with either reduced ferredoxin or methyl viologen as electron donor, but required higher concentrations.

DISCUSSION

By precise examination of the results of purification studies summarized in Table I, we obtained a tentative explanation for the problem of donor specificity of nitrite reductase. The ratio of ferredoxin-linked (A) and methyl viologen-dependent (B) activity increased with purification. Because the physiological electron donor for the nitrite reductase in spinach tissues is ferredoxin, the increase in (A)/(B) ratio indicates that the methyl viologen-dependent nitrite reductase, which is a partially modified enzyme, had been removed by
the purification. This kind of purification could be achieved only by using ferredoxin as the electron donor for the activity determination throughout the purification process. Probably, the native enzyme receives electrons from ferredoxin and also, less effectively, from methyl viologen, whereas the modified enzyme has lost most of the ability to accept electrons from ferredoxin and interacts mainly with methyl viologen. There may be another possibility that the native enzyme receives electrons from methyl viologen through the mediation of ferredoxin, whereas the modified enzyme has become capable of receiving electrons directly from methyl viologen. Whatever the reason may be, however, complete separation of the native and modified enzymes may be difficult to achieve because the purified native enzyme is rather unstable and becomes altered or modified on standing. Therefore, the actual ratio of ferredoxin-linked and methyl viologen-dependent activity \((A)/(B)\) ratio may be even higher than the observed value.

The native ferredoxin nitrite reductase in its ferric state showed ESR signals of a high spin hemoprotein, which were very similar to those of the modified enzyme. Furthermore, in the presence of dithionite, methyl viologen and nitrite, the native enzyme showed ESR signals of a heme-nitrosyl complex. A hyperfine splitting due to \(^{14}\text{N}\) nuclear spin was also observed. The hyperfine splitting signal was much less intense compared with those observed with heme-NO complexes.\(^{38-43}\) These ESR properties were very similar to those reported for methyl viologen-dependent nitrite reductase.\(^{12,28-31}\) Therefore, the protein structure around the siroheme of the native ferredoxin nitrite reductase is probably very similar to that of the methyl viologen-dependent enzyme. As shown in Fig. 2B, almost exactly the same ESR signals of a heme-nitrosyl complex were observed when hydroxylamine was added to the ferric enzyme in the absence of the reducing system, although the signal intensity was much less. Since hydroxylamine is a reducing agent, probably a part of the ferric enzyme was reduced by hydroxylamine and bound to nitrite which was generated by the concomitant oxidation of hydroxylamine.

Siegel et al.\(^{27}\) reported that on Sephadex LH-20 chromatography, the pyridine ferrihemochrome of siroheme from \(E.\ coli\) sulfite reductase migrated as a single violet-red band with absorption maxima at 401 and 557 nm and a shoulder at 520 nm. The absorption ratio of \(A_{401}:A_{557}\) was reported to be 2.7. In our experiment, pyridine ferrihemochrome from the native spinach enzyme showed absorption maxima at 401 and 560 nm and a shoulder at 475 nm. The ratio of \(A_{401}:A_{560}\) was 2.5. On gel filtration on Sephadex LH-20, the pyridine ferrihemochrome migrated as two violet-red bands. The two fractions showed the same absorption spectrum with maxima at 401 and 556 nm and a shoulder at 468 nm. The shift of absorption maximum from 560 and 556 nm could not be attributed to the separation into two bands on gel filtration. The reason for the separation into two bands and the shift of the absorption maxima is not clear at present.

The inhibition of spinach nitrite reductase by cyanide has been reported by other authors\(^{5,11,34}\) and the extent of the inhibition was comparable to our results with the modified enzyme.\(^{34}\) Dalling et al.\(^{44}\) reported that \(o\)-phenanthroline, an iron-chelator, inhibited nitrite reductase by 3% at 1 mM. In our study, however, 15% inhibition was observed with the native enzyme and only 18% inhibition was observed with the modified enzyme at 0.2 mM. The inhibition by \(p\)-chloromercuribenzoate (\(p\)CMB) has also been reported for the enzyme of spinach.\(^{5,11,34}\) Ramirez et al.\(^{34}\) reported that the enzyme itself was inhibited by 1 mM cyanide (90% inhibition) but not by 0.1 mM \(p\)CMB with reduced methyl viologen as the electron donor. On the other hand, Ho and Tamura reported that 96% (0.25 mM KCN) and 25% (0.15 mM \(p\)CMS) inhibition was observed.\(^{5}\) In our present study, the modified enzyme was not inhibited by \(p\)CMB or \(p\)-chloromercuribenzenzene sulfonic acid (\(p\)CMS) at a concentration of 0.2 mM. As shown in Table III, the effect of inhibitors on native nitrite
Ferredoxin-Nitrite Reductase

1327

reductase was considerably different from that on the modified enzyme, suggesting the difference in the mechanism of catalysis of the two kinds of enzyme preparations.

The native nitrite reductase catalyzed the reduction of nitrite to ammonia stoichiometrically, whereas the modified enzyme did not. This difference along with the different sensitivity to inhibitors may be due to the lack of the coupling protein in the modified enzyme. The results of our analysis of the coupling protein indicated the presence of about one non-heme iron atom per molecule. Therefore, it may be assumed that the coupling protein mediates the flow of electrons from ferredoxin to the siroheme and iron-sulfur cluster of nitrite reductase. The non-heme iron of the coupling protein may be very sensitive to cyanide. Methyl viologen can supply electrons to the modified enzyme for the reduction of nitrite, but some of the redox components of the modified enzyme may be only slowly reducible without the coupling protein and thus incomplete reduction of nitrite may occur as indicated by the non-stoichiometric reduction of nitrite to ammonia. Further investigations are necessary to elucidate the role of the coupling protein.

The mode of resolution of the native enzyme into the modified enzyme and the coupling protein was rather unusual. The resolution did not occur at the stage of DEAE-cellulose chromatography or ammonium sulfate fractionation of the crude enzyme but occurred on DEAE-Sephadex A-50 chromatography of the purified enzyme. Thus the resolution could not be explained by simple dissociation of the ionic linkage between the coupling protein and the catalytic part of the enzyme. A possible explanation for the unusual mode of resolution into two components would be obtained by postulating that, in the purified state, the conformation of the native enzyme tends to change into a sort of "open conformation," from which the linking protein part can be released more easily by the DEAE-Sephadex A-50 treatment.

We have previously reported that the native nitrite reductase contained 4 mol of acid-labile sulfide and 7 g-atoms of total iron per molecule. From these results, we suggest that the native enzyme protein contains 2 mol of siroheme, 4 mol of iron of an iron-sulfur cluster and one mol of non-heme iron (not iron-sulfur cluster) per mol. The modified enzyme probably lacks one mol of siroheme and the one mol of non-heme iron.

Acknowledgments. We thank Mr. K. Takahashi for his technical assistance and the donation of the spinach by Mr. T. Shiina and Dr. T. Hiyoyasu of this faculty is also gratefully acknowledged. This work was supported by grants from the following companies: The Japan Carlit, Hodogaya Chemical and Showa Denko.

REFERENCES