Reconstitution of an Iron-Sulfur Cluster with Bound Sulfur: A Possible Source of Acid-Labile Sulfur in Biological Systems

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The reconstitution of the iron-sulfur cluster of spinach ferredoxin was examined in vitro using low and high molecular bound sulfur compounds as sulfur donors. Bound sulfur was rapidly converted to acid-labile sulfur to form an iron-sulfur center in the presence of dihydrolipoate and iron. Reconstitution yields of above 95% were obtained with cystine trisulfide (CT, 0.25 mM) and sulfur-bound albumin (SBA, 1.0 mM) at 37°C, pH 7.3, following 60 min incubation. Spectroscopic features and biological activity of the reconstituted ferredoxin were identical to those of the native holo-protein. The acid-labile sulfur content found in the isolated reconstituted ferredoxin was 2 atoms/mole protein, similar to the theoretical value.

A possible role for bound sulfur in mammalian cells is indicated and discussed.

Key words: bound sulfur; ferredoxin; iron-sulfur cluster; acid-labile sulfur; reconstitution

The origin of the inorganic sulfide present in many non-heme iron proteins has been the subject of extensive investigation. Ferredoxin is the first iron-sulfur protein to be chemically reconstituted from its apoprotein, iron (III) salts, reducing agents and sodium sulfide. However, it seems unlikely that exogenous sodium sulfide is the source of labile sulfur in vivo. Evidence has also been reported for the possible involvement of sulfurtransferases in the biosynthesis of iron-sulfur clusters. Taniguchi and Kimura found that adrenodoxin could be reconstituted upon treatment of its apoprotein with 3-mercaptopropionate and 3-mercaptopropionate sulfotransferase (E.C. 2.8.1.2); in another system involving rhodanese (E.C. 2.8.1.1) and thiosulfate as a substitute for inorganic sulfide in the reaction medium for restoring the iron-sulfur center of ferredoxin. Although the role of the sulfurtransferase enzymes in these reconstitutions has been well studied, little is known about the physiological substrates used as sulfur donors. Indeed, no quantitative data for the commonly used substrates, 3-mercaptopropionate and thiosulfate, in mammalian tissues have been published to date.

Recently, we found that sulfide was released from human serum following reduction with dithiothreitol (DTT). Furthermore, we measured these releasable sulfur atoms in various mammalian sera and rat tissues. This sulfide species is referred to as bound sulfide, which is defined as divalent sulfur that is easily liberated as sulfide following reduction with excess thiols. Bound sulfide is widely distributed in tissues, but its physiological role is still uncertain.

In this paper, we have investigated the possibility that bound sulfur compounds contribute to the formation of iron-sulfur clusters using an in vitro reconstitution system with apoprotein prepared from spinach ferredoxin.

MATERIALS AND METHODS

Standard solutions of analytes and reagents for acid-labile sulfur determination were prepared according to our previous report. Other chemicals of reagent grade were from Sigma (St. Louis, MO) and Wako Pure Chemical (Osaka, Japan). NADPH-cytochrome c reductase was purchased from Sigma. Spinach ferredoxin was prepared according to the method of Petering and Palmer. Apoferredoxin and D,L-dihydrolipoate (DHL) were obtained by the method of Pagani et al. DHL concentrations were determined by the method of Elman. Cystine trisulfide (CT) was prepared as described by Feltcher and Robson.

Sulfur-bound albumin (SBA) was prepared as previously described with minor modification. A solution of crystalline bovine serum albumin containing 200 mg protein/10 ml phosphate buffered saline (PBS, pH 7.4) was prepared with 0.01 M sodium sulfide and incubated for 8 h at 37°C. The reaction mixture was chromatographed on a column of Sephadex G-25 (Pharmacia, 1.5 x 25 cm) to remove excess sulfide. The bound sulfur content in SBA solution was determined by a method we described previously. The concentration of SBA was expressed as its bound sulfur content. The reconstituted ferredoxin in the reaction mixture was evaluated by the HPLC method of Sakihama et al. with some modifications as follows. A 10μl sample of reaction mixture was loaded on to a TSK-gel Phenyl-5PW column (0.75 x 7.5 cm, Tosoh Co., Ltd.). The mobile phase was 0.1 M phosphate buffer, pH 7.0, containing 1.55 M ammonium sulfate, delivered at a flow rate of 1.0 ml/min using a Spectrophysics SP 8800 pump. Detection involved a Hitachi L-4250 UV-VIS spectrophotometer set at 420 nm. Peaks were plotted and integrated using a Hitachi Model L-2500 integrator.

The activity of ferredoxin was assayed as described by Petering et al. For reconstitution, unless otherwise specified, 0.1 mm apoferredoxin in 0.15 M Tris/HCl (pH 7.3) was incubated in the presence of 20 mM DHL and 1 mM ferroc nitrate at 37°C. The reaction was started by addition of bound sulfur compound (0.25—1.0 mM CT or SBA). When isolation of the reconstituted ferredoxin was required for characterization, the reaction mixture was chromatographed on a column of Sephadex G-25 (1 x 15 cm) and the proteins subsequently absorbed on a cartridge column of Toyopak DEAE M (Tosoh Co.)

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equilibrated in 0.15 M Tris/HCl buffer (pH 7.3). Ferredoxin was eluted with 1 M NaCl in 0.15 M Tris/HCl buffer (pH 7.3).

Protein concentrations were determined using ferredoxin (ε₂₈₅ = 9680 M⁻¹ cm⁻¹) or by a biuret method.

Absorbance spectra were recorded on a Hitachi U-2000 spectrophotometer and circular dichroism (CD) was measured on a Jasco J 20 instrument.

RESULTS

Spinach ferredoxin highly purified by ion-exchange chromatography contains two components (ferredoxin type I and type II). For microdetermination of holoferredoxin, we modified the hydrophobic HPLC method which had been devised originally to separate ferredoxin into two distinct molecular species in their native form. Good separation of ferredoxin type I (Fd I) and type II (Fd II) was achieved when 0.1 m phosphate buffer (pH 7.0) containing 1.55 m ammonium sulfate was used as the isocratic eluent. Figure 1A shows a typical chromatogram obtained with a purified native spinach ferredoxin solution. The retention times for Fd I and Fd II were about 12 and 18.5 min, respectively.

Because each standard curve for the peak areas of Fd I and Fd II, plotted against concentrations of standards, was linear over the range of 5—100 μM with correlation coefficients of 0.99 and the peaks (corresponding to Fd I and Fd II) showed the exactly same absorbance spectra as described previously, a standard curve for the determination of holo-ferredoxin was prepared using the sum of the two peak areas for Fd I and II (see Fig. 2).

As shown in Table 1, the levels of reconstitution using CT (low molecular type of bound sulfur) and SBA (high molecular type of bound sulfur) as sulfur donors were investigated using various biological thiols. In the presence of DHL (5 mM) and ferric nitrate (1 mM), reconstitution was found to be 98.8% for SBA and 61.0% for CT. Very low reconstitution yields were observed with other biological thiols, such as reduced glutathione (GSH) and cysteine, at the same concentration.

The concentration of bound sulfur in the reaction mixture affected the extent of reconstitution. In the presence of 0.1 mM apoferredoxin and 5 mM DHL, more complete reconstitution was obtained with 0.25 mM CT and 1 mM SBA, respectively (Fig. 3).

![Fig. 1. Typical Chromatograms of Native Ferredoxin in Solution and Reconstitution Reaction Mixtures Using CT or SBA as a Sulfur Donor](image)

![Fig. 2. Calibration Curves for Determination of Holo-Ferredoxin from Spinach](image)

![Fig. 3. Reconstitution of Ferredoxin Depended on the Concentration of Bound Sulfur Using CT or SBA as a Sulfur Donor](image)

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Table 1. Effect of Various Reducing Compounds on the Reconstitution Reaction Using Bound Sulfur Compounds

<table>
<thead>
<tr>
<th>Reducing compound</th>
<th>SBA (%)</th>
<th>CT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>DHL</td>
<td>98.8</td>
<td>61.0</td>
</tr>
<tr>
<td>GSH</td>
<td>2.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.4</td>
<td>0.9</td>
</tr>
</tbody>
</table>

a) Incubation: 37°C, 60 min. b) Final concentration of reducing compounds: 5 mM. c) Each set of data is the average of two experiments with duplicate determinations. d) Final concentration of bound sulfur: 1 mM.
Typical chromatograms, obtained from each optimum reconstitution system (containing 0.1 mM apoferrredoxin) with SBA or CT as the sulfur donor, are shown in Fig. 1C, D.

Figure 4 shows the time-course of reconstituted ferredoxin in the reaction system with CT. The acid-labile sulfur content of isolated ferredoxin was also determined. It was found under these reaction conditions that bound sulfur in CT was rapidly converted to acid-labile sulfur to form an iron-sulfur cluster in the reconstituted ferredoxin. The value for the acid-labile sulfur found in isolated ferredoxin agreed with the theoretical sulfur content (2 atoms/mol protein) for holo-ferredoxin which contained a 2Fe-2S cluster as an active site.

The acid-labile sulfur content and biological activity of reconstituted ferredoxin isolated from the reaction mixture with CT are shown in Table 2. For comparison, data for native ferredoxin and apoferrredoxin are also shown. Ferreredoxin reconstituted with CT proved identical with native ferredoxin as judged by all the experimental data. The optical and CD spectra are also shown in Figs. 5 and 6, respectively.

DISCUSSION

Non-heme iron-sulfur proteins, containing labile sulfide are widespread in plants and animals and essential for health. The iron-sulfur proteins, as represented by ferredoxin, are functionally linked to the electron transport system, which is located in organized structures such as chloroplasts or mitochondria.

Most studies have been performed using an in vitro reconstitution system to investigate the biological mechanism for the formation of the prosthetic group in iron-sulfur proteins. However, the physiological source of labile sulfide is still unclear. Although a high reconstitution yield had been obtained with sodium sulfide in earlier studies, the physiological meaning of these results was not regarded as significant because the existence of toxic inorganic sulfide was doubtful. Indeed, although we could not detect free sulfide in vivo, it has been proved that reduction-labile sulfur is easily converted to sulfide and widely distributed in mammalian tissues and fluids.8-10

On the other hand, it seems likely that sulfurtransferase participates in the formation of iron-sulfur clusters in higher animals. The localization of rhodanese or 3-mercaptopyruvate sulfurtransferase is well known, however, their physiological substrates remain uncertain. Therefore, we selected bound sulfur for study and investigated its utilization as acid-labile sulfur in non-heme iron-sulfur protein.

Owing to the instability of iron-sulfur clusters in the
reconstitution reaction mixture, and the limited amount of protein that could be synthesized by this system, a rapid and sensitive assay was required to determine holoferrodoxin. The HPLC method that we used in this work is very useful for the practical estimation of reconstituted ferrodoxin yield. Furthermore, another of our methods is also more sensitive and specific than the conventional method for the determination of acid-labile sulfur in reconstituted and isolated ferrodoxin.

We employed exogenous model compounds, CT and SBA, as low and high molecular types of bound sulfur components in this study. It appeared that the sulfur atoms which came from CT and SBA were efficiently utilized to form iron-sulfur clusters of ferrodoxin in the presence of DHL.

From the chromatograms of reconstituted ferrodoxin (see Fig. 1), it was observed that spinach Fd I and Fd II were proportionally reconstituted, reflecting the constitution of apoproteins in the reaction mixture.

The lower reaction yields observed in the CT system, when CT was added at a 5—10 fold concentration relative to the apoprotein in the reaction mixture, may indicate that excess CT either inhibits the formation of iron-sulfur clusters or interacts with the reconstituted clusters.

Bound sulfur is released by reduction not only with DTT but also with GSH; however, a low reconstitution yield was found with 5 mM GSH. The role of the reducing agent in the reconstitution reaction is regarded as contributing to the release of the sulfur atom and reducing the disulfide in apoferrodoxin. In particular, a specific effect of DHL on the reconstitution system has been suggested by Cerletti. We presume that GSH and cysteine have insufficient ability to reduce intermolecular cysteine disulfide, which should contribute to cluster formation, in the apoferrodoxin artificially prepared.

By comparing native ferrodoxin with the reconstituted ferrodoxin using bound sulfur as sulfur donor, it was shown that bound sulfur could be used as a source of acid-labile sulfur in non-heme iron protein.

In previous work, we found high molecular and low molecular components containing bound sulfur and about 25% of the total bound sulfur was located in the mitochondrial fraction of rat tissues. It was also found that the capacity of bound sulfur production was enriched in the cytosol fraction and depended on γ-cystathionase (E.C. 4.4.1.2) activity. It would seem that the iron-sulfur center formation occurs in the mitochondrial matrix. Thus, we have shown that bound sulfur produced in the cytosol is transferred to the mitochondria by some transport mechanism. Although it is unclear whether enzymes are involved in this reaction, the transported bound sulfur may be used for the formation of iron-sulfur clusters. Further investigation to identify the bound sulfur component in vivo may clarify the physiological sulfur donor involved in iron-sulfur cluster assembly.

REFERENCES