THE RECONSTITUTION OF CLOSTRIDIAL FERREDOXIN

Richard Malkin* and Jesse C. Rabinowitz
Department of Biochemistry, University of California, Berkeley

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Previous experiments from this laboratory (Lovenberg, *et al.*, 1963; Malkin & Rabinowitz, 1966) indicated that when bacterial ferredoxin is treated with a mercurial, the color of the protein is bleached, and both the iron and the acid-labile sulfide of the protein are released. Regeneration of ferredoxin could be accomplished by the addition of 2-mercaptoethanol to the bleached reaction mixture. Apoproteins were isolated by chromatography of the reaction mixture on DEAE-cellulose or passage over Sephadex. However, it was not possible to reconstitute ferredoxin from the isolated proteins by the addition of iron and inorganic sulfide under any conditions tested. In the present communication, we wish to describe two methods for the preparation and isolation of apoferredoxins, and their use in the reconstitution of ferredoxin by the addition of ferrous ions and 2-mercaptoethanol and ferrous ions, sodium sulfide, and 2-mercaptoethanol respectively. The reconstituted materials are identical to native ferredoxin with respect to enzymic activity, acid-labile sulfide and iron content, and ultraviolet and visible absorption spectra.

Materials and Methods--Chelex-100 was purchased from Bio-Rad Laboratories. ^{59}Fe(II)-citrate was a gift from Dr. L. Winchell of the Donner Laboratory of this campus. The crystalline ferredoxin used was prepared from *Clostridium pasteurianum* by the procedure of Mortenson (1964) as modified by Lovenberg & Sobel (1966). ^{59}Fe-Ferredoxin was prepared as previously described by Lovenberg, *et al.* (1963). Protein concentrations were determined using a modifi-

*Predoctoral trainee of the United States Public Health Service.

cation of the phenol method previously described by us, and have been corrected so that all values are expressed as dry weight of protein. Iron was determined by the *o*-phenanthroline method (Lovenberg, *et al.*, 1963). It was not possible to use this assay in the presence of a mercurial, and ferredoxin labeled with ^{59}Fe was used in such experiments. All samples were counted using a Packard Tri-Carb Scintillation Counter in 10 ml of Bray's solution using 8% gain. The quenching due to the protein color was negligible. Acid-labile sulfide was determined as previously described (Lovenberg, *et al.*, 1963). In experiments where the protein had been treated with a mercurial, the acid-labile sulfide, also called "inorganic" sulfide, was first diffused by treating the sample with 2-mercaptoethanol at pH 5.4 as described by Malkin and Rabinowitz (1966). Other materials and methods used have been described by Lovenberg, *et al.*, (1963) and by Malkin and Rabinowitz (1966).

Results--Ferredoxin was treated with sodium mersalyl and the bleached reaction mixture was applied to a column of Chelex-100. The elution pattern is shown in Fig. 1. Less than 2% of the original iron present remained with

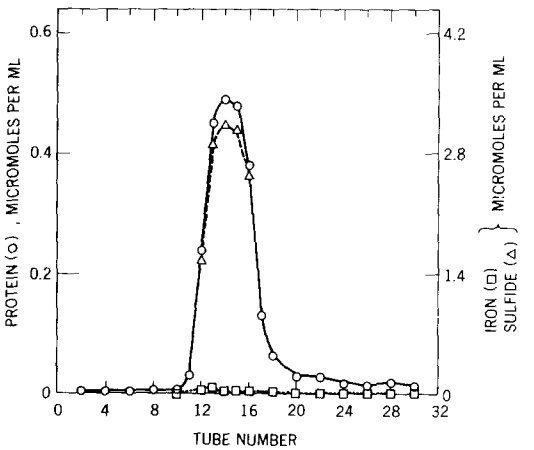

Fig. 1. Preparation of Apoferredoxin-(Mersalyl-Chelex). 15 mg of ^{59}Fe-ferredoxin (specific activity = 13,600 cpm/μmole iron) was treated with 90 μmoles of sodium mersalyl in 0.1 *M* Tris-HCl, pH 7.4, in a final volume of 4.5 ml at 25° for two minutes. The small amount of precipitate that formed was removed by centrifugation. The solution was passed over a column of Chelex-100 (2 x 17 cm) that had been equilibrated with water. The protein was eluted with water at room temperature, and 1.5 ml fractions were collected Fractions were assayed for protein, acid-labile sulfide, and radioactive iron as described in *Materials and Methods*. Fractions 12-16 were pooled, lyophilized and redissolved in distilled water to a concentration of 4.0 mg/ml.

the protein. No "inorganic" sulfide could be detected by direct analysis of any of the fractions. However, "inorganic" sulfide could be detected in some fractions after reduction and diffusion at pH 5.4, as described in *Materials and Methods*. The release of the "inorganic" sulfide after reduction with 2-mercaptoethanol suggests that the sulfide is present as the mersalyl derivative.

The Chelex-treated protein was then passed through a column of Sephadex-G-25 (Fig. 2). The fractions containing protein were free of "inorganic" sulfide even when this was determined after reduction of the protein fractions with 2-mercaptoethanol. However, the second ultraviolet absorbing peak, which contained no protein, was found to contain the "inorganic" sulfide after the samples had been reduced and diffused. The iron content of the pooled protein-containing fractions was approximately 1% of the original, as determined by radioactivity measurements. The analyses of the two apoferredoxin samples are shown in Table I.

Ferredoxin could be regenerated from both apoproteins described above, under the particular conditions to be described. Ferredoxin could be

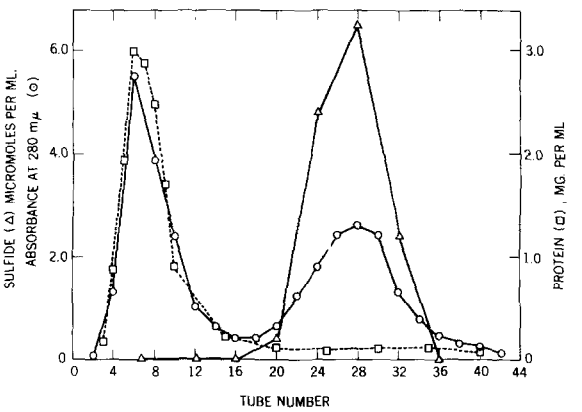

Fig. 2. Preparation of Apoferredoxin-(Mersalyl-Chelex-Sephadex). A solution (2 ml) containing 8 mg of protein isolated from the Chelex column described in Fig. 1 was passed over a column of Sephadex G-25 (1 x 45 cm) which had been equilibrated with water. The protein was eluted with water at room temperature and 0.5 ml fractions were collected. Absorbancies at 280 mμ were determined in micro cells in a Zeiss PM QII spectrophotometer. Protein, "inorganic" sulfide, and radioactive iron were determined as described in *Materials and Methods*.

Table I. The Reconstitution of Ferredoxin

Sample	Iron	Acid-Labile Sulfide	A_{390}	Activity
	μmoles/mg protein		*A/mg/ml*	*Units/mg*
1. Ferredoxin	1.09	1.09	2.59	68
2. Apoferredoxin-(Mersalyl-Chelex)	0.01	1.07	--	0
3. 2 + Fe(II) + 2-Mercaptoethanol	1.00	1.06	2.42	68
4. Apoferredoxin-(Mersalyl-Chelex-Sephadex)	0.01	0.00	0.11	0
5. 4 + Na_2S + 2-MET	0.00	0.07	0.55	0
6. 4 + Fe(II) + 2-MET	0.06	0.07	0.00	0
7. 4 + Na_2S + Fe(II) + 2-MET	1.01	1.24	2.63	78
8. 4 + $(R\text{-}Hg)_2S$ + Fe(II) + 2-MET	1.00	1.18	2.53	72

One mg of the indicated protein sample was incubated at 37° for 10 min with 1 μmole of $^{59}FeSO_4(NH_4)_2SO_4$ [specific activity = 8023 cpm/μmole], 1 μmole sulfide derivative when present, and 50 μmoles 2-mercaptoethanol in 0.1 *M* Tris-HCl, pH 7.4, in a final folume of 1.0 ml. The proteins were reisolated by chromatography on DEAE-cellulose columns (0.5 x 8 cm) equilibrated with 1 *M* potassium phosphate, pH 6.5, and washed with water prior to use. After applying the protein, the column was washed with water and 0.15 *M* NaCl to remove excess reagents. The protein was eluted with 0.80 *M* NaCl. Assays of enzymic activity were carried out with 1-2 μg of samples 1, 3, 7, and 8. No activity could be detected using 10-20 μg of the other samples. $(R\text{-}Hg)_2S$ was prepared by treatment of sodium sulfide with a 2-fold molar excess of sodium mersalyl in water.

reconstituted from the apoferredoxin-(Mersalyl-Chelex) by the addition of a ferrous salt and 2-mercaptoethanol. The reconstituted protein showed full enzymic activity and contained the same amounts of iron and sulfide as the native material and had an identical absorption spectrum. No added source of sulfide was required, and presumably the sulfide present in the protein fractions was reincorporated into the protein.

Ferredoxin could also be reconstituted from the apoferredoxin-(Mersalyl-Chelex-Sephadex) when both a ferrous salt and a source of sulfide were added together in the presence of 2-mercaptoethanol, but not when either component was added separately with 2-mercaptoethanol. The source of sulfide could be satisfied equally effectively with sodium sulfide itself or with the mersalyl-sulfide complex formed by treating sodium sulfide with an excess of mersalyl.

In both cases, the product is identical with the native material.

These experiments have been repeated using native ferredoxin in place of the ^{59}Fe-ferredoxin reported here with identical results with respect to formation of the apoproteins and their activity in the reconstitution of ferredoxin. However, it was not possible to analyze for Fe in mersalyl-containing components, and the results have therefore not been described in detail.

Discussion--The ability to reconstitute ferredoxin from the apoferredoxin derivatives prepared in these experiments appears to be associated with the protection of the apoprotein sulfhydryl groups by the mercurial, sodium mersalyl. In previous studies (Lovenberg, *et al.*, 1963; Malkin & Rabinowitz, 1966), when mersalyl was removed from the protein by treatment with excess 2-mercaptoethanol, the sulfhydryl groups of the apoprotein were oxidized to the disulfides during isolation. Similarly, when the apoferredoxins were prepared by treatment of the native protein with an iron chelating agent, the resulting protein contained no free sulfhydryl groups. Ferredoxin could not be reconstituted from these derivatives under the conditions described for reconstitution in this report (Malkin & Rabinowitz, 1966). In the experiments described here, the sulfhydryl groups of the apoproteins are combined with mersalyl, and this reagent is not removed unless the other components of ferredoxin, iron salts and "inorganic" sulfide, are also present. The source of sulfide can be the mersalyl-sulfide complex, as well as sodium sulfide. In the case of reconstitution from the apoferredoxin-(Mersalyl-Chelex), the source of sulfide is the mersalyl-sulfide complex formed from the protein.

Summary--Apoferredoxin derivatives have been prepared by treatment of *Clostridium pasteurianum* ferredoxin with the mercurial, sodium mersalyl. An apoprotein has been isolated by passage of the mixture over a column of Chelex-100. Ferredoxin can be reconstituted from this preparation by the addition of ferrous ions and 2-mercaptoethanol. No added source of "inorganic" sulfide is required for reconstitution with this derivative. When this apoprotein is passed over a Sephadex G-25 column, reconstitution of ferredoxin from the

isolated apoprotein requires the addition of both iron and a source of sulfide, as well as 2-mercaptoethanol. The reconstituted materials are identical to native ferredoxin in their activity in an enzymic assay, their iron and acid-labile sulfide content, and in their spectral characteristics.

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