ACID-LABILE SULFIDE OF CLOSTRIDIAL FERREDOXIN AND ITS ENZYMATIC EXCHANGE WITH SODIUM SULFIDE-^{35}S

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Ferredoxin (Fd) from Clostridium pasteurianum contains 8 non-heme iron atoms and from 6-8 acid-labile sulfide groups (Lovenberg et al, 1963; Mortenson, 1964; Malkin and Rabinowitz, 1967). Malkin and Rabinowitz (1966a) have concluded that the sulfide released by acid treatment is an independent component of Fd rather than a byproduct of acid degradation of its cysteinyl residues, a suggestion of Bayer et al, (1964), Bayer & Parr (1966) and Gersonde & Druskeit (1968). Evidence presented in this paper supports the conclusion that the acid-labile sulfide released from Fd is a different entity from the -SH group of the cysteinyl residues. This paper also reports on a new enzymatic reaction for the exchange of sodium sulfide-^{35}S with acid-labile sulfide of clostridial Fd.

MATERIALS AND METHODS

Sodium sulfate-$^{35}SO_4$ and sodium sulfide-^{35}S were purchased from New England Nuclear Corp. Iron was analyzed by use of a Perkin-Elmer 303 atomic absorption spectrophotometer. Inorganic sulfide was determined by a modification of the method of Sheppard and Hudson (1930). The molar extinction coefficient as measured in a Hitachi spectrophotometer was taken as 2.85×10^4 at 665 mμ. The radioactivity was measured with a Packard Tri-carb liquid scintillation spectrometer.

Clostridial Fd with an absorbancy ratio 390 mμ/285 mμ of 0.79 and a molar extinction coefficient of 3.0×10^4 at 390 mμ was isolated and purified by the method of Mortenson (1964). The purity was further established by disc electrophoresis on polyacrylamide gel at pH 9.0. Cells and crude extracts were prepared as previously described (Mortenson et al, 1967) except that, to improve the yield of protein, 1.0 mg lyso-

zyme was added per g dry cells. All treatments were performed under anaerobic conditions. Nucleic acids in crude extracts were partially hydrolyzed by nucleases and polynucleotide fragments removed by precipitation with 6% by weight protamine sulfate of extracted protein. The sulfide exchange enzyme activity was not removed from solution by this treatment or by further treatment with 40% DEAE-cellulose (w/w of protein) followed by chromatography on a Sephadex G-25 column. Further purification is in progress.

RESULTS AND DISCUSSION

When ^{35}S uniformly labeled clostridial Fd (isolated and purified from C. pasteurianum grown on $Na_2{}^{35}SO_4$ as a sole sulfur source) was treated with strong acid, 46% of the ^{35}S was released as H_2S, identified by trapping it with alkaline cadmium chloride as a yellow CdS precipitate (Table I). Mercaptoethanol was added to prevent sulfide oxidation and

TABLE I Demonstration of two different sulfur-containing groups of clostridial ferredoxin

Treatment	Total Counts (cpm)	% of Original Radioactivity	Moles of S per mole of Fd[c]
^{35}S uniformly labeled Fd	15,480	100	14.8
As above, treated with acid[a]	8,370	54	8.0
Chemically reconstituted Fd	290,000	100	—
As above, treated with acid[b]	39,000	13	—

a. Add 0.1 ml 2-mercaptoethanol (14.1 M) and 0.3 ml 5.5 N HCl to 0.23 μmole of ^{35}S uniformly labeled Fd. Incubate at 25° for one hour. Neutralize the bleached solution and measure the radioactivity.

b. Same conditions as (a) except 0.0167 μmole of chemically reconstituted Fd-^{35}S was used.

c. Based on 8 cysteinyl residues per Fd found 1) by amino acid analysis, 2) by the amino acid sequence (Tanaka et al, 1966) and 3) by the titration of combined -SH and sulfide with p-chloromercuribenzoate (Lovenberg et al, 1963). The 390/285 ratio of our Fd was 0.79 with a molar extinction coefficient of 3.0×10^4 at 390 mμ comparable to that of the above authors.

to insure maximum release of H_2S. Since clostridial Fd contains 8 cysteinyl residues by amino acid analysis (Tanaka et al, 1966), and the cysteinyl sulfur accounts for 54% of the radioactivity of ^{35}S uniformly labeled Fd, the number of acid-labile sulfide groups is 7. When chemically reconstituted radioactive Fd, prepared by the method of Malkin and Rabinowitz (1966b) using sulfide-^{35}S instead of ^{32}S, was treated with strong acid, 87% of the ^{35}S was released (Table I). This evidence suggests that acid-labile sulfide is a different entity from the -SH group of the cysteinyl residues.

Furthermore, a total sulfur analysis of lyophilized Fd (Table II) showed that Fd contained at least 14 sulfur atoms per molecule. This value, when corrected for loss of extinction at 390 mμ during drying, is 15. Since Tanaka et al (1966) found that Fd contains 8 cysteinyl residues, one can only conclude that the sulfide released from Fd by acid does not arise from cysteinyl sulfur, but rather from some other sulfur complex.

TABLE II. Total sulfur analysis of clostridial ferredoxin

Sample	Weight (mg)	%S[a]	moles S/mole Fd	
			observed	corrected[b]
1	2.050	7.28 ± 0.30%	13.6	14.6
2	1.532	7.35 ± 0.30%	13.8	15.2

a. Determined by the volumetric method of Schöniger (Quantitative Organic Microanalysis, 1961).

b. Corrected by the molar extinction coefficient and protein concentration before and after drying.

An enzymatic exchange of acid-labile sulfide of clostridial Fd with radioactive sodium sulfide-^{35}S was demonstrated (Fig. 1a); the optimum pH for the exchange was 8.0. The reaction mixture (total volume was 2.0 ml) contained 100 μmoles of Tris-HCl at pH 8.0, 0.12 μmole of clostridial Fd, 400 mμmoles of sodium sulfide-^{35}S and 27.0 mg of a desalted protein fraction (a cell-free extract was treated with protamine sulfate (6% by

weight of protein) and then was desalted by passage through a Sephadex G-25 column). The reaction mixture was incubated at 25° under hydrogen gas for 2 hours and then chromatographed anaerobically on a Sephadex G-100 column. Fractions of 5.0 ml were collected and assayed for protein concentration, iron content, O. D. at 390 mμ and radioactivity. Fd and other unidentified proteins in the enzyme fraction were found to be labeled. A control experiment without Fd added to the reaction mixture showed that no radioactive peak was present at elution position of Fd in the Sephadex G-100 elution profile (Fig. 1b). In other words the exchange was with Fd since other component(s) in the enzyme fraction used that eluted from Sephadex G-100 column at the same Rf as Fd, did not bind sulfide.

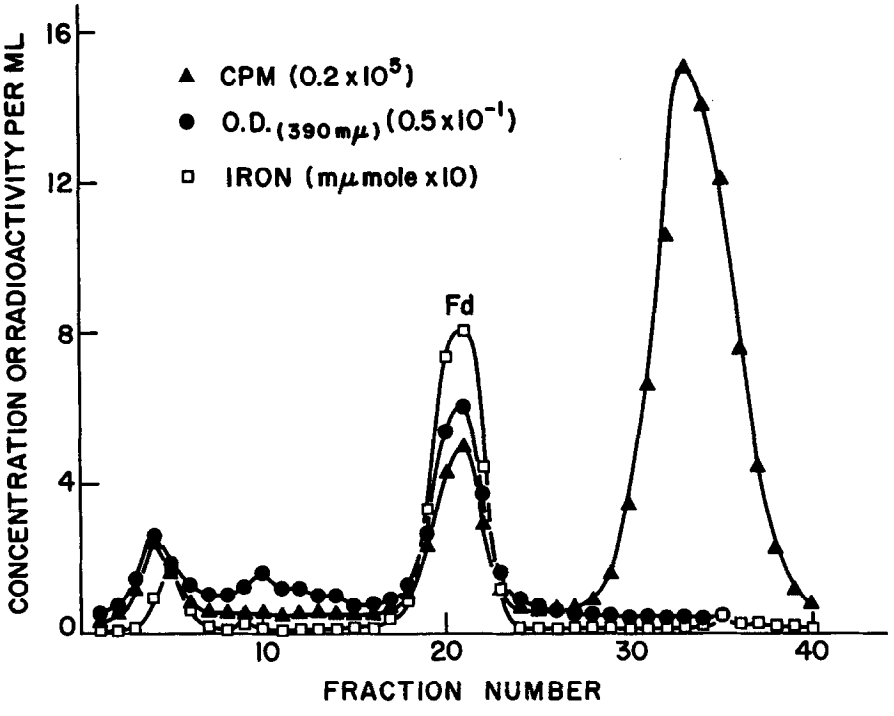

Fig. 1a. Chromatography on a Sephadex G-100 column of the enzymatic exchange reaction mixture to which unlabeled clostridial Fd was added. The volume of the fractions collected was 5 ml.

A percent exchange of 8.7 was calculated by dividing the specific activity of the isolated Fd by the specific activity of the added Na_2S and multiplying by 100 (Fig. 1a). In contrast, the percent exchange without the added enzyme fraction was only 0.32. In order to rule out possible non-specific binding of free sodium sulfide-^{35}S to Fd or incor-

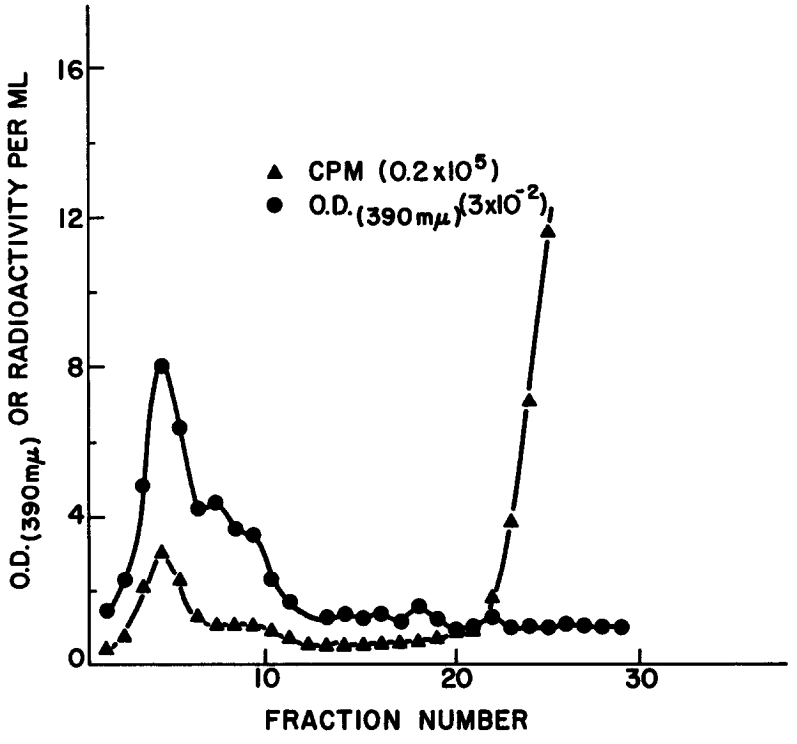

Fig. 1b. Chromatography on a Sephadex G-100 column of the enzymatic exchange reaction mixture to which no clostridial Fd was added. The protein concentration was twice as high as in Fig. 1a.

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poration of sodium sulfide-^{35}S into any apoferredoxin that might have been present, the protein in the reaction mixture was first incubated for two hours in the presence of excess unlabeled sodium sulfide. Next the excess sulfide-^{32}S was removed by chromatography on a Sephadex G-25

TABLE III. Release of the exchanged sulfide-^{35}S from ferredoxin

Treatment Conditions	Total O. D. per ml at 390 mμ	cpm/mg Fd
^{35}S exchanged Fd	2.45	66,200
^{35}S exchanged Fd + acid[a]	0 (bleached)	2,300

a. Add 0.1 ml 2-mercaptoethanol (14.1 M) and 0.05 ml HCl (5.5 N) to 0.082 μmole of isolated ^{35}S-exchanged Fd. Same conditions as shown in Table I.

column and then radioactive sulfide-^{35}S was added. There was little or no difference in the percent exchange when these precautions were taken.

Sodium sulfide-^{35}S exchange with acid-labile sulfide of Fd and not with the -SH group of its cysteinyl residues since in contrast with the result with sulfur-^{35}S uniformly labeled Fd, all radioactivity was released by acid treatment (Table III).

A plot of exchange activity versus time (Fig. 2) showed that exchange occurred up to 2 hours. Further incubation after 2 hours did not increase the percent exchange in the isolated Fd.

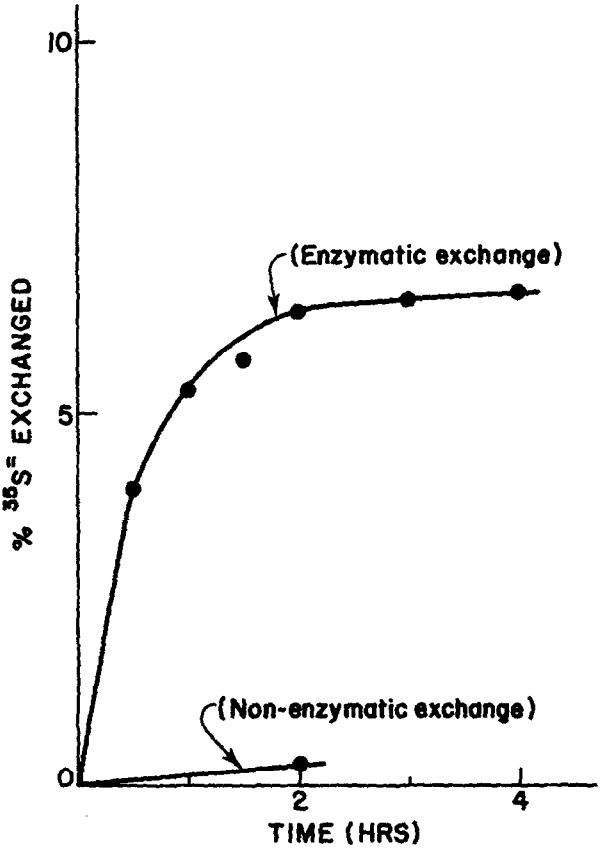

Fig. 2. Time course of sulfide-^{35}S exchange with Fd. The reaction mixture at 25° in a total of 2.5 ml, contained: 100 μmole of Tris-HCl (pH 8.0); 400 mμmoles of sulfide-^{35}S; 0.034 μmole of clostridial Fd and 29.2 mg of 40% DEAE-cellulose treated protein fraction (w/w of protein), and an atmosphere of H_2. Samples were taken at the time intervals indicated and DEAE-cellulose was used to isolate the Fd for analysis.

The origin of the acid-labile sulfide present in many non-heme iron proteins has been the subject of extensive investigation. Recent data on the amount of -SH group and sulfide of Fd obtained by p-chloromercuribenzoate titration at different pH values and on the total sulfur content of Fd suggested that acid-labile sulfide was of amino acid origin; it was released from the cysteinyl residues of Fd (Gersonde & Druskeit, 1968). This was supported by the results of Suzuki and Kimura (1967) who synthesized a non-heme iron protein by the interaction of iron and bovine serum albumin in the presence of 2-mercaptoethanol and found it contained equimolar amounts of iron and acid-labile sulfide. They concluded from this that the acid-labile sulfide of the artificial non-heme iron protein arose by β-elimination of the cysteinyl residues of the polypeptide chain. Lovenberg and McCarthy (1968) however, refuted this by demonstrating that when sulfide-free mercaptoethanol was used, inorganic sulfide had to be added to the reaction mixture to obtain the artificial non-heme iron protein and concluded that the acid-labile sulfide of this protein did not arise from the cysteinyl residues. Chemical reconstitution (Malkin & Rabinowitz, 1966b; Hong & Rabinowitz, 1967) of clostridial Fd from apoferredoxin required inorganic sulfide and iron. Our analyses of 1) uniformly labeled Fd-^{35}S, 2) of Fd made radioactive by chemical reconstitution with sodium sulfide-^{35}S, 3) of Fd labeled by "enzymatic" exchange with sodium sulfide-^{35}S, and 4) of the total sulfur in Fd show that acid-labile sulfide is distinct from the sulfhydryl groups of the cysteinyl residues of the protein. If acid-labile sulfide came from the cysteinyl residues, one would have to postulate cysteine resynthesis on addition of sulfide and iron in the method of Malkin and Rabinowitz (1966b) and one would have to account for the presence of only 8 cysteinyl residues in Fd when chemical analysis shows 14-15 sulfur atoms per molecule.

The exchange of sulfide-^{35}S into Fd of 8.7% suggests that only one of the 7 acid-labile sulfide groups of Fd exchanged. One would expect an exchange of about 14% if 7 sulfide groups are present in Fd and if one sulfide exchanged. The exchange of 8.7% is a minimum figure, however, since when sulfide-^{35}S exchanged with the sulfide containing proteins, unlabeled sulfide was released and diluted the radioactivity of the sulfide pool. A correction for the dilution cannot be made since the added sulfide-^{35}S exchanged with sulfide-containing proteins other than Fd, perhaps even with the sulfide exchange enzymes(s) itself, and the decrease in the specific activity of the pool is not predictable.

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