PREPARATION AND PROPERTIES OF CLOSTRIDIAL APOFERREDOXINS

Jen-shiang Hong and Jesse C. Rabinowitz
Department of Biochemistry, University of California, Berkeley

Received September 26, 1967

A procedure has previously been described for the removal of the iron and inorganic or "acid-labile" sulfide from clostridial ferredoxin, the isolation of the resulting protein, apoferredoxin, and the conditions for the reconstitution of ferredoxin from this protein (Malkin and Rabinowitz, 1966b). The apoprotein was prepared by treatment of ferredoxin with the mercurial, mersalyl, and was purified by chromatography on Chelex and Sephadex columns. Reconstitution required the addition of a source of ionic iron, inorganic sulfide, and a mercaptide reducing agent. These could be supplied by $Fe(SO_4)(NH_4)_2SO_4$, sodium sulfide, and 2-mercaptoethanol. No reconstitution was obtained if any one of the three components was omitted, nor was any iron or sulfide incorporated into the protein if either or both other components were omitted from the reconstitution incubation mixture. In this communication we wish to describe two alternate methods for the preparation of apoferredoxins, compare the chemical properties of these apoferredoxins, and describe the requirements for reconstitution of ferredoxin from these proteins. These preparations employ acid to remove the iron and inorganic sulfide from ferredoxin, rather than treatment with a mercurial, as previously described.

Methods and Materials--Protein concentrations were determined by a modification of the phenol method as described by Lovenberg, *et al.* (1963), and have been corrected so that all values are expressed as dry weight of

protein. The radioactivities of ^{59}Fe-ferredoxins and ^{14}C-apoferredoxins were determined with a Nuclear Chicago Mark I Scintillation Counter in 10 ml of Bray's solution. Iron was determined with bathophenanthroline as described by Doeg and Ziegler (1962). Acid-labile sulfide was determined as previously described (Lovenberg, *et al.*, 1963). The free sulfhydryl groups of apoferredoxins were determined by carboxymethylation using iodoacetic acid-1-^{14}C. The radioactive iodoacetic acid used had a specific activity of 63,500 cpm per μmole. Carboxymethylation was performed at room temperature for 15 minutes in 0.1 M Tris·HCl, pH 8.5, by treating apoferredoxin anaerobically with 4000-fold excess iodoacetic acid which had been neutralized with concentrated NaOH before use. The protein was isolated by chromatography on Sephadex G-25. The enzymic activity of ferredoxins was determined using the phosphoroclastic reaction as previously described by Lovenberg, *et al.* (1963).

Crystalline ferredoxin used was prepared from *Clostridium acidiurici* by the procedure of Buchanan, *et al.* (1963).

Apoferredoxin-I: The protein was prepared according to the method described by Malkin and Rabinowitz (1966b) for the preparation of apoferredoxin-(mersalyl-Chelex-Sephadex).

Apoferredoxin-II: Ferredoxin, 14 mg, in 11 ml of 0.015 M Tris·HCl, pH 7.4, was made 4% in TCA with a 15% TCA solution. This mixture was allowed to stand for 1 hour at 0° C. Evolution of H_2S gas was noted throughout this period and the brown color of ferredoxin was completely bleached at the end of this period. The white suspension was centrifuged at 17,000 rpm for 10 minutes and the supernatant solution was discarded. The precipitate was washed with 3 ml of 0.023 M formic acid and allowed to stand for 30 minutes at 0° C and centrifuged. The washing was repeated once. The precipitate was finally taken up in 4 ml of 0.1 M Tris·HCl, pH 9.0, and the insoluble material was removed by centrifugation.

The almost colorless supernatant was then passed over a Sephadex G-25

column (1.2 x 38 cm) which had been previously equilibrated with 0.02 M Tris·HCl, pH 8.5, and the protein was eluted with the same buffer. The apoferredoxin prepared in this way is designated apoferredoxin-II.

Apoferredoxin-III: Apoferredoxin-III was prepared using the same procedure as for apoferredoxin-II, but all solutions were 0.05 M with respect to 2-mercaptoethanol (2-MET).

Reconstitution of Ferredoxin from Apoferredoxin-II: Apoferredoxin-II, 1.8 mg, was incubated anaerobically by flushing N_2 gas for 4 hours at room temperature in 0.1 M Tris·HCl, pH 8.5, 8 M urea, 0.07 M 2-mercaptoethanol and a small amount of antifoam in a final volume of 4 ml. At the end of the incubation, 3 µmoles of $^{59}FeSO_4(NH_4)_2SO_4$ (specific activity = 330,000 cpm/µmole), 3 µmoles of Na_2S when present, and 140 µmoles of 2-mercaptoethanol were added while still flushing with N_2 gas. The reaction mixture was then diluted 3 fold with deaerated 0.1 M Tris·HCl, pH 8.5, and incubated for 15 minutes at 37° C. The reconstituted ferredoxins were reisolated by chromatography on DEAE-cellulose column (0.8 x 8 cm) equilibrated with 1 M potassium phosphate, pH 6.5, and washed with water prior to use. After applying the reaction mixture, the column was washed successively with 10 ml of 0.15 M Tris·HCl, pH 7.4, and 20 ml of 0.05 M Tris·HCl, pH 7.4, containing 0.23 M NaCl to remove excess reagents. The ferredoxin was finally eluted with 0.005 M Tris·HCl, pH 7.4, containing 0.58 M NaCl.

Reconstitution of Ferredoxin from Apoferredoxin-III: Apoferredoxin-III, 1.8 mg, in 0.1 M Tris·HCl, pH 8.5, and 0.05 M 2-mercaptoethanol in a volume of 1.6 ml was incubated for 15 minutes at 37° C with 3 µmoles of $^{59}FeSO_4(NH_4)_2SO_4$ and 3 µmoles of Na_2S when present. The reconstituted ferredoxin was reisolated as above.

Results--The analyses of native ferredoxin and apoferredoxins II and III for sulfide, iron, free sulfhydryl groups and enzymic activity are shown in Table I. The apoproteins were essentially free of iron and

Table I. Properties of Apoferredoxins and Reconstituted Ferredoxins

Sample	Iron	Acid-labile sulfide	Free -SH	A_{390}	Enzymic activity	Yield
	moles/mole protein			*A/mg/ml/cm*	%	%
1. Native ferredoxin	6.5	6.0	0.2[a]	3.94	100	--
2. Apoferredoxin-I[b]	0.06	0.00	0.0	0.11	0	75
3. Apoferredoxin-II	0.16	0.01	0.7	0.00	0	67
4. Apoferredoxin-III	0.11	0.01	7.5	0.00	0	70
5. Apoferredoxin-II + urea + $S^=$ + Fe^{++} + 2-MET	6.0	5.9		3.74	108	70
	6.6	6.8		2.65	114	75
	6.4	6.0		4.02	119	89[c]
6. Apoferredoxin-II + $S^=$ + Fe^{++} + 2-MET[d]	0.58	0.52		0.00	7	55
7. Apoferredoxin-II + urea + Fe^{++} + 2-MET[e]	0.95	0.13		0.00	0	77
8. Apoferredoxin-II + urea + $S^=$ + 2-MET[e]	0.05	0.10		0.00	0	75
9. Apoferredoxin-III + $S^=$ + Fe^{++} + 2-MET	5.8	5.8		3.24	108	72
	6.8	7.1		3.27	106	78
	5.8	5.1		3.94	85	71
	6.2	5.6		4.25	124	82[c]
10. Apoferredoxin-III + Fe^{++} + 2-MET[e]	2.1	0.1		0.49	3	79
11. Apoferredoxin-III + $S^=$ + 2-MET[e]	0.00	0.26		0.00	3	86

[a] Previously described (Lovenberg, *et al.*, 1963).

[b] Apoferredoxin-I contained 4.3 moles of mercury per mole protein as determined by colorimeter analysis (Tyuma, *et al.*, 1966); Malkin, R. and Rabinowitz, J. C., unpublished observations.

[c] Double the amounts of the iron salt and Na_2S used in the standard procedure were added.

[d] Protein was reisolated by gel filtration on Sephadex G-25 and then chromatographed on DEAE-cellulose column.

[e] Protein was reisolated by gel filtration on Sephadex G-25 instead of by chromatography on DEAE-cellulose.

acid-labile sulfide. Apoferredoxins II and III are similar in their properties and show no enzymic activity but differ in their content of free sulfhydryl groups. Apoferredoxin-II contains 0.7 moles of free sulfhydryl groups per mole of protein while apoferredoxin-III contains 7 free sulfhydryl groups per mole of protein. The presence of 2-mercaptoethanol in the preparation of apoferredoxin-III apparently prevents the free sulfhydryl groups of the apoprotein from oxidation. The yield of apoferredoxins II and III from ferredoxin is about 70%.

The properties of reconstituted ferredoxins are shown in Table I. Ferredoxin could be reconstituted from apoferredoxin-II if the apoprotein was reduced with 2-mercaptoethanol in 8 M urea and 0.1 M Tris·HCl, pH 8.5, for 4 hours at room temperature prior to the addition of an iron salt and sodium sulfide. Ferredoxin could not be reconstituted from apoferredoxin-II by the addition of an iron salt, sodium sulfide and 2-mercaptoethanol in the absence of urea. Nor could ferredoxin be reconstituted from the apoferredoxin previously treated with 2-mercaptoethanol in 8 M urea if only the iron salt or the sodium sulfide was added to the protein separately. About 75% of the apoprotein from incubation mixtures containing either the iron salt or sodium sulfide and the other components required for reconstitution was recovered, however it did not contain significant amounts of either iron or acid-labile sulfide.

Ferredoxin could be reconstituted from apoferredoxin-III by the addition of both an iron salt and a source of sulfide. Prior treatment of the apoferredoxin-III with 2-mercaptoethanol and urea was not required for reconstitution. The reconstituted ferredoxins formed from both apoferredoxin-II and apoferredoxin-III showed full enzymic activity and contained the same amounts of iron and sulfide as the native ferredoxin, and had identical absorption spectra. The yield of ferredoxin reconstituted from either apoferredoxins was from 70-90%.

In contrast to the behavior of apoferredoxins-I and II, apoferre-

doxin-III binds some iron when it is incubated with an iron salt in the absence of Na_2S. The amount of iron bound to the recovered protein is lower than that found in native ferredoxin. However the iron-containing protein isolated does not contain significant amounts of acid-labile sulfide, nor does it exhibit activity in the enzymic test equivalent to its iron content. No acid-labile sulfide could be detected in the protein isolated after incubation with Na_2S in the absence of an iron salt.

Discussion--It appears from these experiments that ferredoxin can be reconstituted from apoferredoxin if the apoferredoxin possesses free sulfhydryl groups, but not if they are oxidized to disulfides. Prior reduction of the disulfides to free sulfhydryl in 8 M urea with 2-mercaptoethanol is necessary for reconstitution in the case of apoferredoxin-II. In previous studies, apoferredoxin prepared from 2-mercaptoethanol-treated apoferredoxin-mersalyl derivatives (Lovenberg, *et al.*, 1963) and apoferredoxin prepared from α',α'-dipyridyl-treated ferredoxin (Malkin and Rabinowitz, 1966a) contained no detectable sulfhydryl groups. Ferredoxin could not be reconstituted from these apoferredoxins. However, ferredoxin could be reconstituted from isolated apoproteins in which the free sulfhydryl groups had been protected as the S-mersalyl derivatives if the mercury-containing apoprotein were treated with an excess of 2-mercaptoethanol in the presence of an iron salt and Na_2S. The apoproteins described here differ from the preparation previously described in that they are free of mercury.

The recent report by Suzuki and Kimura (1967) in support of cysteine as the source of "acid-labile" sulfide in non-heme iron proteins is not supported by the findings reported in our previous investigations (Malkin and Rabinowitz, 1966a), nor by the observation reported here, since the reconstitution of ferredoxins containing acid-labile sulfide requires the addition of sodium sulfide as well as of iron to the three apoproteins described.

Supported, in part, by Research Grant A-2109 from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

REFERENCES

Buchanan, B. B., Lovenberg, W., and Rabinowitz, J. C., *Proc. Natl. Acad. Sci. U. S.*, 49, 345 (1963).

Doeg, K. A., and Ziegler, D. M., *Arch. Biochem. Biophys.*, 97, 37 (1962).

Lovenberg, W., Buchanan, B. B., and Rabinowitz, J. C., *J. Biol. Chem.*, 238, 3899 (1963).

Malkin, R., and Rabinowitz, J. C., *Biochemistry*, 5, 1262 (1966a).

Malkin, R., and Rabinowitz, J. C., *Biochem. Biophys. Res. Commun.*, 23, 822 (1966b).

Suzuki, K., and Kimura, T., *Biochem. Biophys. Res. Commun.*, 28, 514 (1967).

Tyuma, I., Benesch, R. E., and Benesch, R., *Biochemistry*, 5, 2957 (1966).