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ISOLATION AND CHARACTERIZATION OF TARO FERREDOXIN*†

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Abstract—Ferredoxin was isolated and purified from taro, a tropical monocotyledonous plant. The absorption spectrum of the protein has maxima at 465, 420, 330 and 277 nm. The molecular weight of the protein determined by gel filtration and sedimentation analysis is 12,000 ± 1000. The protein contains two atoms of iron, two molecules of labile sulfide and five cysteinyl groups per molecule. The amino acid composition is Lys_{4-5}, His_1, Ang_1, Cys_5, Asx_{10}, Thr_6, Ser_8, Glx_{16}, Pro_4, Gly_{9-10}, Ala_7, Val_{10}, Ile_4, Leu_6, Tyr_4, Phe_2, and Trp_1. The amino and carboxy terminals of the protein are alanine.

INTRODUCTION

FERREDOXIN is the name given to non-flavin, non-heme iron-containing, electron carrier proteins found in certain anaerobic bacteria and in chloroplasts of plants.[1,2] The physical and chemical properties and amino acid composition of ferredoxins from two dicotyledonous plants, spinach[3] and alfalfa[4] have been studied in detail. A study of ferredoxins from different plant species could prove useful in elucidating phylogenetic relationships in higher plants and algae and aid in establishing the structure-function relationships of these important proteins. I wish to report the purification, properties, and amino acid composition of ferredoxin from a monocotyledonous plant *Colocasia esculenta* (Araceae), commonly known as the Hawaiian taro.

RESULTS

Isolation of Ferredoxin

Taro leaves were harvested from the farm adjacent to the University of Hawaii campus. All the steps in the isolation procedure were carried out at 4°. Tris-HCl buffer of pH 7·5 was used throughout the extraction. The following procedure, which is a modification of the procedure of Tagawa and Arnon[5] for the isolation of spinach ferredoxin, gave the best yield of taro ferredoxin.

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† Abbreviations used: CMB, *p*-chloromercuribenzoic acid; Dansyl, 1-dimethyl aminonaphthalene-5-sulfonyl; Fd, ferredoxin; DNP, dinitrophenyl; mersalyl, O-(3 hydroxymercuri-2-methoxy propyl) carbamyl phenoxyacetic acid.

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1 L. E. MORTENSON, R. C. VALENTINE and J. E. CARNAHAN, *Biochem. Biophys. Res. Commun.* **7**, 448 (1962).

2 K. TAGAWA and D. I. ARNON, *Nature* **195**, 538 (1962).

3 H. MATSUBARA, R. M. SASAKI and R. K. CHAIN, *Proc. Natl Acad. Sci.* **57**, 439 (1967).

4 S. KERESZTES-NAGY and E. MARGOLIASH, *J. Biol. Chem.* **241**, 5955 (1966).

5 K. TAGAWA and D. I. ARNON, in *Modern Methods of Plant Analysis* (edited by H. F. LINSKENS, B. D. SANWAL and M. V. TRACEY), Vol. VII, p. 595, Springer-Verlag, Berlin (1964).

Step 1. About 1 kg of leaves were homogenized in a Waring blendor with 3 l. 0·05 M buffer containing 0·05 M NaCl. The homogenate was filtered through cheese cloth.

Step 2. Acetone, at $-15°$, was added to the filtrate to obtain a final concentration of 35% acetone. The mixture was centrifuged at $1000 \times g$ for 15 min. The supernatant was stirred with two successive batches of 3 g of DEAE cellulose for 30 min. The brown resin containing the adsorbed ferredoxin was washed with 0·2 M buffer. Most of the ferredoxin was then eluted from the resin by stirring successively with two 50-ml volumes of 0·3 M buffer containing 0·5 M NaCl and filtering through glass wool.

Step 3. The filtrate was diluted four-fold and passed through a DEAE cellulose column equilibrated with 0·15 M buffer containing 0·15 M NaCl. The ferredoxin adsorbed in the column was eluted using the same buffer. The eluate was concentrated by readsorption on DEAE cellulose and development with 1 M buffer.

Step 4. Ammonium sulfate was added to the ferredoxin solution to give a final concentration of 0·6 g of salt per ml of solution. The mixture was centrifuged at $27,000 \times g$ for 15 min. The supernatant was dialyzed against 0·05 M buffer in an atmosphere of hydrogen.

Step 5. Ferredoxin was finally purified by the method of Bendall *et al.*[6] The dialyzate was adsorbed on a Sephadex G-75 column equilibrated with 0·05 M buffer. The column was developed with the same buffer and effluent fractions having a 420 nm to 280 nm ratio greater than 0·45 were pooled and concentrated.

Purification

The yield of ferredoxin was about 25 mg of pure protein per kilogram of stripped leaves. The protein showed absorption maxima at 465, 420, 330 and 277 nm; the relative absorbances are 0·39, 0·43, 0·64 and 1·00 respectively. The visible absorption of the protein decreases considerably on storage in air at 4° and also on lyophilization. So the protein was stored at $-15°$ in an atmosphere of hydrogen.

The photoreduction activity[7] of pure taro ferredoxin was 29 units per mg of protein corresponding to the reduction of 139 μmoles of NADP per mg of chlorophyll, in 10 min illumination.

Electrophoresis

The protein moved as a single red band in standard polyacrylamide gel (7·5%)[8] along with the marker dye. However, on staining the gel with amido black three very faint bands lying close together appeared. The total absorbance due to these three bands (by densitometer tracing) was less than 1% of the red band. The homogenity of the red band was further confirmed by electrophoresis in 30% acrylamide gel. In the 30% gel the ratio of the distance moved by ferredoxin to that by the marker dye was 0·75. Identical band patterns were observed on gel electrophoresis of taro and spinach ferredoxins. The electrophoretic mobility of taro ferredoxin at 0°, in 0·1 M buffer, pH 7·0, was $10·0 \times 10^{-5}$ cm^2 sec^{-1} V^{-1}.

Molecular Weight

Taro ferredoxin was eluted by tris buffer from a Sephadex G-100 column[9] just before cytochrome *c*. The molecular weight calculated from the standard curve was $12,000 \pm 1000$.

[6] D. S. BENDALL, R. P. F. GREGORY and R. HILL, *Biochem. J.* **88**, 29 (1963).
[7] A. SAN PIETRO, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. VI, p. 439, Academic Press, New York (1963).
[8] L. ORNSTEIN and B. J. DAVIS, *Disc Electrophoresis*, Canalco Industrial Corp., Bethesda, Md. (1961).
[9] J. R. WHITAKER, *Anal. Chem.* **35**, 1950 (1963).

This value was confirmed by sucrose gradient centrifugation studies.[10] The sedimentation constant of ferredoxin with respect to cytochrome *c* ($S_{20}W$ = 1·83S) was 1·8S.

Chemical Composition

The dry weight of taro ferredoxin was only 74 per cent of the weight of protein as determined using Folin-Ciocalteau reagent.[11] The protein contained 14·4 per cent nitrogen and 3·6 per cent dry ash. The iron content, estimated as ferrous iron,[12] was 0·93 per cent and labile sulfide content[13] was 0·53 per cent. These correspond to about 2 g. atoms each of iron and sulfide for 12,000 g of ferredoxin. Table 1 gives the extinction coefficients of taro ferredoxin at various wavelengths.

TABLE 1. MOLAR EXTINCTION COEFFICIENTS OF PLANT FERREDOXINS

Source of ferredoxin	Wavelength (nm) 277	330	420	465
Spinach*	20,160	12,780	9,680	8,800
Alfalfa†	19,000	12,200	9,020	8,240
Taro‡	22,500	14,400	9,700	8,800

The values are presented as the absorbance of a solution of protein containing 2 moles of iron per l.

* Calculated from the data given in Ref. 26.

† Calculated from the data given in Ref. 4

‡ Values calculated from absorbancy measurements and direct iron analysis of the same ferredoxin solution with a 420 to 277 nm absorbance ratio of 0·43.

Action of Sodiumdithionite and Urea

Taro ferredoxin lost about 50 per cent of its absorption at 420 nm when treated with low concentrations (one to five molar equivalents) of $Na_2S_2O_4$. The protein was bleached completely and irreversibly by a large excess of $Na_2S_2O_4$. The spectrum of the protein, after removal of the dithionite by dialysis against water, showed a shift in the u.v. absorption peak from 277 to 267 nm. Addition of 8 M urea (purified by passage through a mixed bed exchange resin) caused an immediate shift of the 465 nm absorption peak to 455 nm. The protein retained about 40 per cent of its original visible absorbance when incubated anaerobically with 8 M urea and 15 per cent of its visible absorbance when incubated aerobically for 40 hr. Urea did not produce any change in the u.v. spectra of ferredoxin.

Reaction with Mercurials

A molecule of taro ferredoxin contains two molecules of labile sulfide and five equivalents of half cysteine residue determined by amino acid analysis. Since two molecules of sulfide

[10] R. G. MARTIN and B. N. AMES, *J. Biol. Chem.* **236**, 1372 (1961).

[11] E. W. SUTHERLAND, C. F. CORI, R. HAYNES and N. W. OLSEN, *J. Biol. Chem.* **180**, 825 (1949).

[12] H. DIEHL and G. F. SMITH, in *The Iron Reagents*, p. 13, G. Frederick Smith Chemical Company, Columbus, Ohio (1965).

[13] W. LOVENBERG, B. B. BUCHANAN and J. C. RABINOWITZ, *J. Biol. Chem.* **238**, 3899 (1963).

react with four equivalents of mercurial, a molecule of ferredoxin should combine with nine equivalents of mercurial. A maximum of eight equivalents of CMB[14] or mersalyl[15] combined with a molecule of taro ferredoxin as shown in Table 2.

TABLE 2. TITRATION OF FERREDOXIN WITH MERCURIALS

Mercurial used	A $\frac{420\text{ nm}}{277\text{ nm}}$ of Fd	Mercurials reacted mole/mole of Fd
CMB	0·38	7·1 ± 0·2
CMB	0·41	7·6
CMB	0·43	8·1
CMB*	0·44	9·0
Mersalyl	0·39	7·3
Mersalyl	0·42	7·7
Mersalyl*	0·43	9·0

Protein concentration of ferredoxin solution was measured by the phenol color reaction[11] and was corrected for the enhanced color given by ferredoxins. The molecular weight of ferredoxin was assumed to be 12,000 in the calculations.

* Titration with spinach ferredoxin.

The values given are average of duplicate determinations.

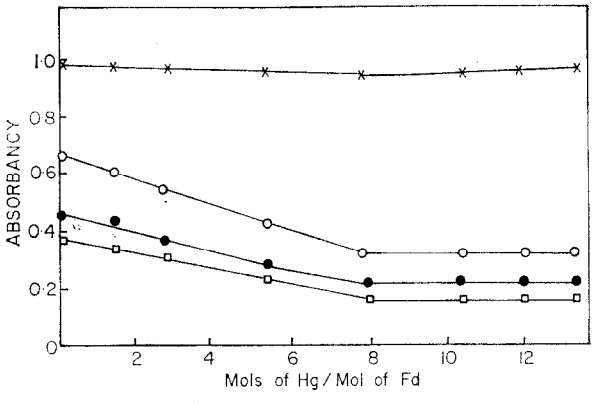

FIG. 1. EFFECT OF CMB ON THE ABSORBANCY OF FERREDOXIN.

A standard solution of CMB was added in aliquots to a solution of taro ferredoxin in 0·05 M phosphate buffer, pH 6·5. After 20 min incubation at 27°, the absorbancy of the reaction mixture was recorded at 255, 280, 330, 420 and 465 nm against the phosphate buffer as reference. The absorbancy values have been corrected for dilution effect and for the absorbancy of CMB at the respective wavelengths. Calculation from the absorbancy increase at 255 nm (not shown in the figure) showed that a maximum of 8 moles of CMB reacted with 1 mole of ferredoxin.

—×—×— 280 nm
—○—○— 330 nm
—●—●— 420 nm
—□—□— 465 nm.

[14] P. D. BOYER, *J. Am. Chem. Soc.* **76**, 4331 (1954).
[15] I. M. KLOTZ and B. R. CARVER, *Arch. Biochem. Biophys.* **95**, 540 (1961).

The correlation between the loss in visible absorbance and the amount of CMB reacting with the protein is shown in Fig. 1. It is evident from the curves that the presence of sulfide groups is essential for maintaining the color of ferredoxin. Table 3 gives the number of atoms of mercury bound to a molecule of taro ferredoxin, as determined by atomic absorption spectrophotometry.[16] The maximum number of atoms bound to a molecule of taro ferredoxin was four. The protein after complete reaction with CMB was found to be free from iron and inorganic sulfide.

TABLE 3. BOUND MERCURY IN APO FERREDOXIN

Moles of CMB added/mole of Fd (before dialysis)	Moles of Hg/mole of Fd (after dialysis)
4	1·2
5	1·8
8	3·9
10	4·0
10	3·9
10·6	4·1
15	3·9

Values given are average of duplicate analyses uncorrected for experimental losses of protein during dialysis: Bound mercury was determined by atomic absorption spectrophotometry as described under "Methods". Ferredoxin concentration was determined from the absorbancy of the protein at 420 nm using an extinction coefficient of 9·7 per micromole per ml at this wavelength. Ferredoxin used had a 420 nm to 277 nm absorbancy ratio of 0·43.

Amino Acid Composition

Due to the absorbance of ferredoxin in the 290 to 330 nm region it was not possible to obtain accurate values for the tryptophan content of the protein by the usual spectrophotometric assays. Basic hydrolysis of ferredoxin for 72 hr gave 0·5 residues of tryptophan per molecule with leucine as standard.[17] A two-dimensional paper chromatogram made from a chymotryptic digest of taro ferredoxin gave a single Ehrlich positive spot. The results suggest that a molceule of taro ferredoxin contains one residue of tryptophan.

Both oxidized[18] and carboxymethylcysteinyl ferredoxin were used to determine the amino acid composition. The results are shown in Table 4. For a comparison the compositions of spinach and alfalfa ferredoxins are also given in the table. It is quite likely that the taro ferredoxin molecule also comprises of 97 amino acid residues.

[16] K. FUWA, P. PULIDO, R. H. MCKAY and B. L. VALLEE, *Anal. Chem.* **36**, 2407 (1964).
[17] E. A. NOLTMAN, T. A. MOHOWALD and S. A. KUBY, *J. Biol. Chem.* **237**, 1146 (1962).
[18] M. TANAKA, T. NAKASHIMA, H. F. MOWER and K. T. YASUNOBU, *Arch. Biochem. Biophys.* **105**, 570 (1964).

TABLE 4. AMINO ACID COMPOSITION OF PLANT FERREDOXINS

Amino acid	Taro: Time of hydrolysis 24 hr mole/mole	22 hr mole/mole	72 hr mole/mole	Mean* value mole/mole	Nearest integer	Spinach‡	Alfalfa§
Lysine	4·55	4·10	4·59	4·41	4–5	4	5
Histidine	1·10	1·00	1·00	1·03	1	1	2
Arginine	1·00	0·98	0·96	0·98	1	1	1
Aspartic acid + asparagine	9·08	9·86	10·04	9·90	10	13	10
Threonine	5·75	5·72	5·20	6·00	6	8	6
Serine	7·20	7·32	5·08	8·20	8	7	8
Glutamic acid + glutamine	16·65	16·00	16·61	16·42	16–17	13	17
Proline	4·00	4·48	4·22	4·23	4	4	3
Glycine	9·65	9·41	9·62	9·56	9–10	6	7–8
Alanine	6·85	7·28	6·81	6·98	7	9	10
Half cysteine†	5·10	5·30	5·06	5·15	5	5	6
Valine	9·60	10·31	10·10	10·03	10	7	9
Methionine	0	0	0	0	0	0	0
Isoleucine	4·10	4·0	4·0	4·03	4	4	4
Leucine	6·0	5·85	5·92	5·92	6	8	6
Tyrosine	3·40	3·58	3·00	3·85	4	4	4
Phenylalanine	1·90	1·97	1·89	1·92	2	2	2

The 24-hr hydrolysis results are the average of duplicate analysis with S-carboxy-methylated ferredoxin. The 22- and 72-hr hydrolyses were performed with oxidized ferredoxin.

* The values of threonine, serine, and tyrosine were obtained after extrapolation to zero hour hydrolysis the respective values at 22- and 72-hr hydrolysis.

† Calculated as carboxymethyl cysteine in 24-hr hydrolysate as and cysteic acid in 22- and 72-hr hydrolysates.

The calculation of the number of residues was based on an assumption of arginine 1, histidine 1, proline 1, valine 10 and leucine 6.

‡ From Ref. 3.

§ From Ref. 4.

Terminal Amino Acid Residues

The DNP amino acid liberated from the DNP derivatives[19] of native and oxidized taro ferredoxin was identified as DNP alanine by TLC and paper chromatography. Alanine was confirmed as the amino terminal residue by identifying dansyl alanine as the only amino acid liberated by acid hydrolysis of dansyl ferredoxin.[20]

Hydrazinolysis of ferredoxin[21] liberated 0·36 mole of alanine per mole of the protein. Low yields of COOH terminal alanine have been observed in the hydrazinolysis of other proteins.[22] Carboxypeptidase B did not react with the protein. Carboxypeptidase A liberated alanine, threonine and alanine successively suggesting the carboxyterminal sequence to be Leu·Thr·Ala.

[19] H. FRAENKEL-CONRAT, J. I. HARRIS and A. L. LEVY, in *Methods of Biochemical Analysis* (edited by D. GLICK), Vol. 2, p. 359, Academic Press, New York (1955).

[20] W. R. GRAY and B. S. HARTLEY, *Biochem. J.* **89**, 59P (1963).

[21] J. H. BRADBURY, *Biochem. J.* **68**, 482 (1958).

[22] V. SHORE and B. SHORE, *Biochemistry* **6**, 1962 (1967).

DISCUSSION

The yield of ferredoxin from taro leaves compares favourably with that from spinach[7] and alfalfa.[4] The taro protein was more than 99 per cent pure judged by gel electrophoresis. The three faint bands that appeared on staining the gel may either be due to traces of colorless proteins or polymers of taro ferredoxin. The absorption spectra of the three plant ferredoxins are identical and they have comparable specific activities in the photoreduction assay.

The dry weight of taro ferredoxin was only three-fourths of the weight as determined by the colorimetric method. A higher value for the protein concentration when measured by the Folin colorimetric assay has been reported for bacterial ferredoxins also.[13]

Taro ferredoxin, like spinach and bacterial ferredoxins,[23] loses half its visible absorbance when treated with $Na_2S_2O_4$. The exact mechanism of this reaction is not yet understood except that dithionite causes a rearrangement in the electronic configurations of the iron and some of the sulfur atoms in ferredoxin. The blue shift in the u.v. absorbance when taro ferredoxin was treated with excess of sodium dithionite has been observed with spinach ferredoxin after reduction with hydrogen and *Clostridium pasteurianum* hydrogenase.[2] The chemical nature of the group giving this 267 nm peak is still not known. The spectral perturbation observed with 8 M urea should be due to a conformational change occurring around the iron–sulfur chromophoric group of ferredoxin. Garbett *et al.*[24] have reported that urea causes a change in the CD spectra of spinach ferredoxin.

In the reactions with CMB and mersalyl only four of the five cysteine groups of ferredoxin are accounted for. It is possible that in the course of the titration some of the cysteinyl groups (especially those linked to the iron) are partly oxidized to cystine bridges.

The amino acid compositions of taro, alfalfa and spinach ferredoxins show many similarities. The terminal amino acids of all the three proteins are alanine. They contain the same number of tryptophan, tyrosine, phenylalanine, isoleucine and arginine residues. However, a fingerprint of spinach and taro ferredoxin hydrolysates showed certain differences in the number and R_f values of the peptides from the two proteins. This suggests that there are differences in the primary structures of the two ferredoxins. Taro ferredoxin is the only ferredoxin studied from a monocotyledonous plant. The mono- and di-cotyledons are believed to have evolved from the Ranales order, one of the earliest fossil angiosperms.[25] Spinach belongs to the order Chenopodiales, alfalfa to the Rosales, and taro to the Arales: Despite morphological differences, the ferredoxins from mono- and di-cotyledonous plants show nearly the same physical and chemical characteristics. A comparative study of the primary structures of ferredoxins from more species of plants might give results which are consistent with the evolutionary history of plants.

EXPERIMENTAL

Electron transfer activity was determined by measuring the rate of photoreduction of NADP by ferredoxin, as described by San Pietro,[7] in the presence of Swiss chard chloroplasts.

Polyacrylamide gels of 7·5% and 30% concentration were prepared and run in Tris-glycine buffer, pH 8·3, according to Ornstein and Davis.[8] Free boundary electrophoresis of the protein in 0·1 M buffer, pH 6·5, was carried out in a Perkin–Elmer Model 38 Electrophoresis apparatus fitted with a Polaroid Land Camera.

Gel filtration was performed on Sephadex G-100 columns prepared and run as described by Whitaker.[9]

[23] R. MALKIN and J. C. RABINOWITZ, *Ann. Rev. Biochem.* **36**, 113 (1967).
[24] K. R. GARBETT, R. D. GILLARD, P. F. KNOWLES and J. E. STANGROOM, *Nature* **215**, 824 (1967).
[25] C. L. PORTER, *Taxonomy of Flowering Plants*, p. 125, W. H. Freeman, San Francisco (1959).
[26] K. TAGAWA and D. I. ARNON, *Biochem. biophys. Acta* **153**, 602 (1968).

Beef-heart cytochrome *c*, trypsin, beef-heart lactic dehydrogenase, and bovine serum albumin were used as reference standards and "Blue Dextran" was used to determine the void volumn of the column. Sucrose density gradient centrifugation was carried out by the method of Martin and Ames.[10] Cytochrome *c* and trypsin were used as protein standards.

The protein concentration of a freshly prepared solution of ferredoxin was determined by the colorimetric procedure of Sutherland *et al.*[11] using bovine serum albumin as standard. The same solution was used to measure the absorbance of the protein at various wavelengths and for the determination of dry weight.

Nitrogen content of the solution was determined by standard Kjeldahl procedure. Iron content was determined, after solution in HCl, using bathophenanthroline,[12] and inorganic sulfide was estimated by conversion to methylene blue as described by Lovenberg *et al.*[13]

The sulfide and sulfhydryl groups of ferredoxin were estimated by spectrophotometric titration using CMB[14] and mersalyl.[15] Cysteine, sodium sulfide, and reduced glutathione were used as references and each gave the expected stoichiometric titre value. The absorbancies of the reaction mixture at wavelengths of maximum absorption for ferredoxin were also recorded in the course of the titration with mercurials.

For the estimation of bound mercury, ferredoxin was incubated with varying amounts of CMB in 0·05 M phosphate buffer, pH 6·5, for 30 min. Excess CMB was then removed by dialysis. The mercury bound to the protein was determined by atomic absorption spectrophotometry as described by Fuwa *et al.*[16] A Westinghouse WL 22847 hollow cathode discharge tube operated at 10 mA was the emission source. The absorption of the solution was measured at 2537 A. Solutions of $HgCl_2$ and CMB were used as standards.

Oxidized ferredoxin was prepared by treating the protein first with TCA and then with performic acid as described by Tanaka *et al.*[18] Carboxymethylated ferredoxin was prepared by the procedure of Keresztes-Nagy and Margoliash.[4] About 1 mg each of the derivative was heated with 0·5 ml of 6 N HCl in vacuum for 22–72 hr at 105°. The mixture was then analyzed for their amino acid content in a Beckman Spinco Model 120B amino acid analyzer at 50° using standard techniques. Tryptophan was estimated by basic hydrolysis of ferredoxin for 72 hr as described by Noltman *et al.*[17]

The amino terminal residue was determined by preparing the dinitrophenyl derivative[19] and dansyl derivative[20] of ferredoxin. After hydrolysis of the respective derivatives with 6 N HCl, the DNP amino acid and the dansyl amino acid liberated were identified by TLC in standard solvent systems. The carboxy terminal amino acid was determined by hydrazinolysis of ferredoxin and its derivatives by the procedure of Bradbury.[21] Carboxypeptidase digestion was also carried out as described by Fraenkel-Conrat *et al.*[19] In both cases the carboxy terminal amino acids liberated were identified by analysis in the Beckman analyzer.

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