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RAO, K. Krishna, 1928-
ISOLATION AND CHARACTERIZATION OF TARO
FERREDOXIN.

University of Hawaii, Ph.D., 1968
Biochemistry

Please Note: School lists author's name as
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University Microfilms, Inc., Ann Arbor, Michigan

ISOLATION AND CHARACTERIZATION OF
TARO FERREDOXIN

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOCHEMISTRY
JUNE 1968

By

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DEDICATION

To Retnam and Ranji

ACKNOWLEDGMENTS

To the East-West Center, University of Hawaii, for
a generous grant.

To Mr. E. H. Higa for assistance in the preparation
of ferredoxin.

To Dr. J. Tsunoda for many valuable suggestions and
helpful discussion.

To my colleagues, W. W. Philleo, R. N. Asato, A. D.
Kidman, L. S. R. K. Rao, and A. M. Benson for
their help in many phases of this work.

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ABBREVIATIONS

A	Absorbancy
ADP	Adenosine 5'- diphosphate
ATP	adenosine 5'- triphosphate
CMB	p- chloromercuribenzoic acid
DEAE	O- (diethyl aminoethyl)
DFP	Di-isopropyl phosphofluoridate
DTNB	5,5'- dithiobis (2- nitrobenzoic acid)
Dansyl	1- Dimethylaminonaphthalene-5-sulfonyl
EDTA	Ethylenediaminetetraacetate
EPR	Electron paramagnetic resonance
Fd	Ferredoxin
FDNB	1-fluoro-2,4- dinitobenzene
M	Molar concentration
mu	Milli micron
Mersalyl	o- (3-Hydroxymercuri-2-methoxypropyl) carbamyl phenoxyacetic acid
NAD	Nicotinamide- adenine dinucleotide
NADP	Nicotinamide- adenine dinucleotide phosphate
Pi	Inorganic orthophosphate
PPNR	Photosynthetic pyridine nucleotide reductase
- SH	Sulfhydryl group
Tris	Tris (hydroxymethyl) aminomethane

ABSTRACT

Ferredoxin, a non-heme iron, electron carrier protein, was isolated from taro leaves. The protein was found to be pure as judged by starch and polyacrylamide gel electrophoresis and by end group amino acid analysis. The absorption spectrum of taro ferredoxin is similar to the absorption spectra of other plant ferredoxins and exhibits maxima at 465, 420, 330, and 277 mu. The ratio of absorbancies at 420 and 277 mu is 0.43.

The protein reduces NADP to NADPH in the presence of illuminated chloroplasts. The specific activity of the ferredoxin in the photoreduction was 29 enzyme units when assayed by standard procedure. Taro ferredoxin is about 25% as active as bacterial ferredoxin, on a mole basis, in the phosphoroclastic reaction with bacterial extracts.

The protein contains 14.4% nitrogen and has an ash content of 3.6%. A molecule of ferredoxin contains two atoms of iron and two atoms of labile sulfur. Spectrophotometric titrations with CMB and mersalyl indicate that up to eight moles of mercurial react with one mole of the protein. Treatment with mercurials results in the loss of color and the absorption maxima in the visible region of ferredoxin. The CMB treated protein, after extensive dialysis, was found to contain four atoms of bound mercury per mole of protein, as determined by atomic absorption analysis. The protein has a molecular weight of approximately 12,800 as determined by gel filtration and sucrose density gradient centrifugation methods. The molecular weight calculated from amino acid composition is between 10,700 and 11,000.

The amino acid composition of taro ferredoxin as determined by acid hydrolysis of oxidized ferredoxin and carboxy methyl cysteinyl ferredoxin is: Lys₄₋₅, His₁, Arg₁, Cys₅, Asp+Asn₁₀, Thr₆, Ser₈, Glu+Gln₁₆₋₁₇, Pro₄, Gly₉₋₁₀, Ala₇, Val₁₀, Ile₄, Leu₆, Tyr₄, Phe₂. Spectrophotometric titrations of the performic acid-treated protein indicate the presence of one tryptophan residue per mole of ferredoxin. The protein contains no methionine.

The amino terminal residue of the protein is alanine and the carboxyterminal sequence is (Leu. Thr) Ala. The terminal amino acid residues of spinach and taro ferredoxins are iden-

tical. Finger prints of chymotryptic digests of spinach and taro ferredoxins also show many similarities.

Treatment with sodium dithionite results in the loss of about 50% of the absorption of ferredoxin at 420 mμ. The EPR spectrum of dithionite-treated ferredoxin, at liquid nitrogen temperature, is similar to the EPR spectra of other reduced non-heme iron proteins.

INTRODUCTION

The first successful demonstration of a photosynthetic reaction in a cell-free system was made in 1939 by Hill (1) who showed that illuminated chloroplasts evolved oxygen in the presence of a non physiological electron acceptor like ferric oxalate. The conversion of carbon dioxide to phosphoglyceric acid was achieved in 1952 (2). Within a few years, Calvin and associates (3) were able to isolate a number of intermediates formed during the conversion of carbon dioxide to carbohydrate in photosynthetic algae and to propose that a "reductive pentose phosphate pathway" is operative in photosynthesis.

Arnon and coworkers have established (4) that photosynthesis consists of two phases: (a) a photochemical phase in which radiant energy is trapped and converted into chemical energy and (b) a chemical phase in which the chemical energy (stored as ATP and NADPH) released by phase a is utilized to convert carbon dioxide into organic compounds by a series of reactions that are independent of light. For each molecule of carbon dioxide that is assimilated to the level of carbohydrate in plants, energy released from three molecules of ATP and two molecules of NADPH is required (5). These two energy donors are formed during the photosynthetic phosphorylation according to the reactions:

It has been known since 1951 that illuminated chloroplasts can reduce pyridine nucleotides (6, 7, 8). The actual accumulation of reduced pyridine nucleotides in an illuminated grana suspension was first reported by San Pietro and Lang (9). These authors measured the reduced pyridine nucleotides by spectroscopic methods and indicated the presence of a soluble factor in chloroplasts which stimulated the pyridine nucleotide reduction. Subsequently, Arnon et al. (10) reported that NADP and a NADP-reducing factor with some properties of a protein, present in aqueous extracts of chloroplasts, acted as catalysts of photosynthetic phosphorylation. In 1958, San Pietro and Lang (11) isolated and purified a soluble protein from spinach chloroplasts which catalyzed the reduction of pyridine nucleotides by illuminated chloroplasts and names it photosynthetic pyridine nucleotide reductase (PPNR).

As early as 1952, Davenport et al. (12) had reported the presence of a water soluble protein factor in chloroplasts which acted as a catalyst for the reduction of methemoglobin by illuminated chloroplasts. Further studies (13) showed that

this methemoglobin reducing factor (MRF) catalyzed the reduction of a number of heme proteins and NADP by illuminated chloroplasts. Comparison of the spectral and catalytic properties of purified preparations of PPNR and MRF revealed that the two proteins are identical. The two proteins were shown to contain non-heme iron and labile sulfur.

In 1962, Mortenson et al. (14) reported the isolation of a non-heme, non-flavin, protein from Clostridium pasteurianum which functioned as an electron carrier in the phosphoroclastic reaction of the bacterium. These authors named the protein "ferredoxin". At the same time, Tagawa and Arnon (15) isolated from spinach leaves, a non-heme iron protein with electron carrier properties similar to that of bacterial ferredoxin. Both proteins had a very low oxidation reduction potential (E'_0 -0.43 V at pH 7.55) and both were reversibly oxidized and reduced with characteristic changes in absorption spectra. Bacterial ferredoxin was found to be capable of mediating in the dark reduction of pyridine nucleotides in the presence of hydrogen and bacterial hydrogenase. Due to the similarity in properties between clostridial ferredoxin and the pyridine nucleotide reducing factor of spinach, the name chloroplast ferredoxin was suggested for the latter by Arnon and associates. These authors also pointed out the identity of PPNR and MRF to chloroplast ferredoxin. The name ferredoxin was suggested for iron proteins which function as electron carriers on the "hydrogen side" of pyridine nucleo-

tides (16).

The chloroplast enzyme responsible for the reduction of NADP, ferredoxin-NADP reductase, was prepared in a crystalline form by Shin et al. (17). This enzyme was a flavoprotein specific for NADP with transhydrogenase properties. A similar protein had been isolated earlier by Keister et al. (18) and by Arnon and Jagendorf (19). The mechanism of ferredoxin mediated NADP reduction as envisaged by Shin and Arnon (20) is

where f_p stands for Fd-NADP reductase.

In addition to acting as an electron carrier in the reduction of NADP, chloroplast ferredoxin can mediate in the reduction of nitrate to nitrite, and nitrite and hydroxylamine to ammonia (23). Recently, Arnon et al. (4) have shown clearly that ferredoxin participates in both cyclic and noncyclic photophosphorylation, the two photochemical reactions that jointly account for the evolution of oxygen and for the assimilatory power made up of NADPH and ATP. Also, reduced chloroplast ferredoxin was shown to activate a specific fructose 1,6-diphosphatase indicating the role of ferredoxin in the regulation of carbohydrate metabolism in plants (24).

Thus it is evident that ferredoxin plays an important role in the photosynthetic energy conversion process in plants.

In the few years, since the discovery of ferredoxins and establishment of their role in the electron transfer mechanism of plants and bacteria (14, 15), there has been a tremendous interest in various laboratories in the study of these proteins. The relatively low molecular weight of ferredoxins has prompted protein chemists to study the amino acid sequence of ferredoxins from various bacteria and plants (26, 97, 75). It is the biochemist's eagerness to trace the evolution of life and desire to look for diversity in unity that resulted in the isolation of proteins like cytochrome c and hemoglobin from numerous species and in the determination of the amino acid sequence of these proteins (98). The enunciation of the genetic code has enabled biochemists to understand some of the amino acid substitutions that are found in a particular protein from different or the same species. The development of computer technology has been helpful to predict within reasonable limits the time lapse that would have occurred between the evolution of each species based on the amino acid sequence determinations of certain proteins from the respective species (99). Comparative biochemistry of proteins is still an open and promising field capable of making many future contributions.

Ferredoxins from plants and bacteria contain iron and a form of acid-labile sulfide which can be estimated as hydro-

gen sulfide. Simultaneous with the discovery of ferredoxins, the presence of electron transfer proteins with iron and labile sulfide was observed in mammalian mitochondria (100). Unlike the cytochromes and hemoglobin, the iron in the ferredoxins is extremely labile and the determination of the mode of binding of iron in these proteins has become a challenging problem for biochemists and biophysicists. Due to the presence of paramagnetic chromophore in the molecule, techniques like Mossbauer Spectroscopy (101, 102), optical rotatory dispersion (76), circular dichroism (103), proton relaxation (104), near infrared spectroscopy (105) and electron paramagnetic resonance spectroscopy (92-96) are applied along with chemical investigations to elucidate the structure of ferredoxins. Though the molecule of ferredoxin is smaller than molecules of ribonuclease and myoglobin, the presence of labile iron and sulfur makes the determination of ferredoxin structure by X-ray crystallography, after isomorphic replacement of heavy metals, difficult (22). The importance of, and interest in, the study of these non-heme iron proteins is illustrated by the special Symposia on these proteins held in Hawaii (Honolulu, 1963) and in Yellow Springs (Ohio, 1965). The applications of magnetic resonance techniques in the elucidation of non-heme iron protein structure was discussed by scientists from laboratories in an International Symposium held at Stockholm, Sweden, in 1966.

Though many bacterial ferredoxins were isolated between 1962 and 1965, (106, 25), the only plant ferredoxins adequately characterized by 1965 were those of spinach and parsley (27, 107). Bacterial ferredoxin is available commercially but the commercial production of plant ferredoxins has never been accomplished and samples are difficult to obtain from other laboratories for detailed studies.

The proposed objects of the present research were:

- 1) To devise a convenient method for the isolation of ferredoxin in a pure state from a plant readily available on the Islands of Hawaii.
- 2) To study the chemical composition and properties of the protein and compare them with those of other plant and bacterial ferredoxins.
- 3) To determine the molecular weight and optical and electron paramagnetic resonance spectra of the protein.
- 4) To determine the amino acid composition and terminal amino acid residues of the protein and compare these with those of other plant ferredoxins.

With these objects in view, preliminary investigations were started with leaves of Amaranthus gangeticus and conditions necessary to get the best yield of ferredoxin were worked out. The plant finally chosen was taro (Colocasia esculenta) which belonged to a different class from spinach. In the later stages ferredoxin was prepared from spinach (Spinacia oleracea), flown in from California, and from taro leaves, under identical conditions, and their physical and chemical properties were compared.

MATERIALS AND METHODS

MATERIALS

Guanidine hydrochloride, crystalline iodoacetic acid, mersalyl acid (sodium salt), N-bromosuccinimide, pyridine-2-azo-p-dimethyl aniline, and cytochrome c were purchased from Sigma Chemical Company, St. Louis, Missouri.

G. Frederick Smith Chemical Company, Columbus, Ohio, supplied standard iron solution and all the reagents used in iron analysis. Acrylamide, N,N'-methylene bisacrylamide and N,N',N'-tetramethyl ethylene diamine, reagents used in acrylamide gel electrophoresis, were obtained from Eastman Organic Chemicals, Rochester, New York. The same source supplied mercaptoethanol, hydrazine, and p-dimethyl aminobenzaldehyde.

Coenzyme A, crystalline bovine serum albumin, NADP and p-chloromercuribenzoic acid (sodium salt) were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

Cal Biochem, Los Angeles, California, was the source for Cellex D (DEAE-cellulose), Biogel P, dansyl chloride and standard dansyl amino acids.

Amberlite MB-1 and MB-3 were purchased from Mallinkrodt Chemicals, St. Louis, Missouri.

Matheson Coleman and Bell, East Rutherford, New Jersey, supplied N,N-dimethyl-p-phenylenediamine sulfate and ammonium persulfate.

FDNB was purchased from Pierce Chemical Company, Rockford, Illinois.

Silica gel was purchased from Warner-Chilcott Laboratories, Richmond, California

DFP carboxypeptidases A and B, and lyophilized trypsin were supplied by Worthington Biochemical Corporation, Freehold, New Jersey.

Sephadex was supplied by Pharmacia Fine Chemicals Inc., Piscataway, New Jersey.

DTNB was purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin.

Standard DNP amino acids and TLCK-Chymotrypsin were the gift of Dr. Joyce Tsunoda of this department, University of Hawaii.

Compressed hydrogen and nitrogen were obtained from Gaspro Ltd., Honolulu, Hawaii

All other chemicals used were standard laboratory reagents.

Distilled water or deionized water was used for making aqueous solutions.

Urea was always purified as described by Bensch et al. (34).

The leaves of taro plant were purchased from a farm near the University of Hawaii Campus. Chinese spinach was purchased from a local grocer. Spinach (*Spinacia oleracea*) was purchased from the Blue and Gold Grocery, Berkeley, Califor-

nia, and was flown immediately to Honolulu in refrigerated containers.

Swiss chard was grown outside the laboratory from seeds packed by the Ferry-Morse Seed Co., Mountain View, California.

METHODS

Preparation of adsorbent columns for chromatography.

Diethylaminoethyl cellulose (commercial Cellex-D) was processed and packed into columns by the procedure described by Peterson and Sober (35). Sephadex gels and Biogel P-10 were processed for column chromatography as recommended by the manufacturer.

Extraction of ferredoxin.

Fresh taro leaves, harvested in the morning, were freed of their mid-ribs, weighed, packed in plastic bags, and stored for five to fifteen days in the freezer. The frozen leaves were thawed at a convenient time in the cold room at 4°, before homogenization. At times the leaves were cooled to 4° immediately after harvest and homogenized without freezing and thawing. The procedure of Tagawa and Arnon (36) was used for isolation of ferredoxin, with some modifications. The entire operation was carried out at 4°.

Preparation of aqueous extract.

About 1 kg of leaves was homogenized with 3 liters of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.05 M NaCl, for two minutes, in a Waring Blendor (one gallon capacity) at low speed. The homogenate was filtered through a double layer of cheese cloth and a single layer of glass wool. The last portions were removed by mechanical compression of the filter cake.

Acetone treatment.

Ice cold acetone, stored in a freezer (-15°), was added to the filtrate, gradually with stirring, till the concentration of acetone in the mixture reached 35%. The mixture was then centrifuged at 1,000xg, for 15 minutes. The green residue of chloroplast was discarded and the brown supernatant was saved.

Preparation of DEAE-cellulose extract.

Three grams of Cellex-D was added to the supernatant, the mixture was stirred well for 30 minutes and then filtered through a Buchner funnel under suction. The filtrate was again treated with 3 g of resin, stirred and filtered. Most of the acidic proteins were removed from the supernatant by this procedure. The resin containing the absorbed proteins was washed on the funnel with 0.2 M Tris-HCl buffer. It was then suspended in 50 ml of 0.8 M Cl^{-} buffer (30 ml of 1 M Tris-HCl + 55 ml of 1 M NaCl diluted to 100 ml), stirred well for 30 minutes and filtered. The filter cake was stirred with another 50 ml of 0.8 M Cl^{-} buffer and filtered again. Most of the ferredoxin is eluted out of the resin by this process.

Column Chromatography.

The combined DEAE-cellulose extract was diluted five times with water and passed through a column of DEAE-cellulose, 10x2.2 cm, equilibrated with 0.2 M Cl^{-} buffer (15 ml of 1 M Tris-HCl + 8 ml of 1 M NaCl diluted to 100 ml). The column

was then washed with the same buffer and the proteins were eluted with 0.8 M Cl^- buffer. A concentrated protein solution is thus obtained. This eluate was diluted four times with water and passed through a DEAE-cellulose column, 8 x 2.2 cm, equilibrated with 0.3 M Cl^- buffer (15 ml of 1 M Tris-HCl + 18 ml of 1M NaCl diluted to 100 ml). The column was washed with 0.2 M Cl^- buffer and then developed with 0.3 M Cl^- buffer. A red band of ferredoxin could be seen, during elution, moving ahead of the rest of the colored proteins. The reddish brown eluate, containing the ferredoxin, was concentrated by diluting 2.5 times with water, adsorbed on a DEAE-cellulose column equilibrated with 0.1 M Tris-HCl buffer, and eluting with 1 M Tris-HCl buffer.

Salt Fractionation:

Ammonium sulfate crystals were added to the eluate from the previous step, (0.6 g of crystals per ml), and stirred well. The mixture was centrifuged at 27,000xg for 15 minutes. The brownish black residue was discarded and the pink supernatant was saved for isolation of ferredoxin by one of the methods mentioned below. All the operations mentioned hitherto were finished within 36 hours after starting homogenization of leaves.

Separation of ferredoxin:

Method 1: Solid ammonium sulfate was added to the supernatant taken in a beaker, gradually with stirring, till the solution became turbid. The mixture was stored in the cold

(-5°) for a few days. The ferredoxin precipitated and collected at the bottom of the beaker. A few crystals floated at the top of the liquid. The precipitate was separated by centrifugation and then dissolved in the minimum volume of 0.1 M Tris-HCl buffer. The ratio of absorbancies at 420 mμ and 280 mμ of the sample was about 0.35.

Method 2: The supernatant was diluted 40 times with water and passed through a DEAE-cellulose column 4 x 2.2 cm equilibrated with 0.1M Tris-HCl buffer. The absorbed protein was washed on the column with the same buffer. The ferredoxin was then eluted with a linear sodium chloride gradient of 0.2M to 0.5M chloride concentration. The gradient was prepared with a mixing solution of 0.1M NaCl in 0.1M Tris-HCl buffer and a reservoir of 0.4M NaCl in 0.1M Tris-HCl buffer. Eluate fractions were collected and the absorbancy of each fraction at 280 mμ and 420 mμ was measured in a Beckman DB Spectrophotometer. The ratio of absorbancies at 420 mμ and 280 mμ was calculated and fractions with a ratio higher than 0.3 were pooled. The pooled solution was frozen in dry ice-acetone mixture and concentrated by evaporation under reduced pressure.

Purification by gel filtration:

The ferredoxin prepared by either method was further purified according to Bendall et al. (37). A concentrated solution of ferredoxin was absorbed on a Sephadex G-75 column 3.3 x 33 cm equilibrated with 0.05 M Tris-HCl buffer.

Effluent fractions of 5 ml volume were collected and their $\frac{420\text{ m}\mu}{280\text{ m}\mu}$ absorbancy ratio determined. Fractions having the ratio above 0.44 were pooled and concentrated as before. Usually, pure ferredoxin elutes out first leaving the impurities behind. The concentrated ferredoxin solution was stored in the freezer, in serum bottles, in an atmosphere of hydrogen. When the ferredoxin was used in experiments in which the Beckman Spinco amino acid analyzer was to be used, the gel filtration was carried out in 0.05M phosphate buffer, pH 6.8 instead of Tris-HCl buffer since Tris may interfere in amino acid analysis.

In some later experiments Biogel P-10 was substituted for Sephadex G-75. The ferredoxin concentrate from the NaCl gradient elution was adsorbed on the top of a Biogel column equilibrated with 0.05M phosphate, pH 6.8. When the same buffer was passed through the column, pure ferredoxin moved as a red band ahead of a dark fraction which was eluted later.

Tris-HCl buffer used in all steps had a pH of 7.3 except for the buffer used to homogenize the leaves. The procedure employed was the same for the isolation of ferredoxin from spinach leaves and also from leaves of Chinese spinach. When larger batches of taro were used the sizes of the DEAE columns were increased proportionately.

Determination of electron transfer activity of ferredoxin.

The activity of ferredoxin was measured by determination

of the rate of ferredoxin-catalyzed photoreduction of NADP in the presence of chloroplasts. The NADPH formed was estimated by measuring the absorbancy at 340 mμ.

Chloroplasts were prepared from Swiss chard leaves by a modification of the method of Turner et al. (30). About 50 g of freshly harvested leaves were cooled to 4° and ground in a mortar with a little sea sand and 75 ml of a homogenizing medium containing 0.35M NaCl, 0.05M Tris-HCl buffer, and 0.001M ascorbic acid. The mixture was filtered through cheese cloth and the filtrate was centrifuged at 200xg for 1 minute. The residue consisting of sand and debris from the leaves were discarded and the supernatant was centrifuged at 700xg for 8 minutes. This residue was suspended in 30 ml of Tris-NaCl solution, prepared by a ten fold dilution of the homogenizing medium, and centrifuged again at 700xg for 8 minutes. The supernatant containing ferredoxin was discarded and the pellet was resuspended with stirring in 10 ml of the diluted Tris-NaCl solution. The suspension was filtered through a single layer of glass wool. The chlorophyll concentration in the chloroplast was determined by the method of Arnon (39).

The reagents used for the assay were:

NADP, 0.01M	0.05 ml
Tris-HCl buffer, pH 7.2, 0.5M	0.30 ml
Ferredoxin solution + Tris buffer pH 8.0, 0.005M	2.55 ml

Chloroplast suspension 0.10 ml

The chloroplast was added just before illumination. The assay was performed in a dark room at ambient temperature by the procedure of San Pietro (40).

Reaction mixtures containing, 0.5 micromole of NADP, 0.15 millimole of Tris buffer, chloroplast suspension equivalent to about 50 microgram of chlorophyll, and varying quantities of ferredoxin, were taken in 13 x 100 mm test tubes and mixed well. The tubes were placed around a 1,000 ml beaker containing water. Light from a 100 watt tungsten lamp, immersed in the water, was passed through the tubes for 5 minutes. The absorbancy of the supernatant was measured at 340 mμ in a Beckman DB spectrophotometer against a blank which contained all the reagents except ferredoxin. The absorbancy of the ferredoxin at 340 mμ was subtracted from the observed values to get the absorbancy due to NADPH. Protein concentration of the ferredoxin solution was determined using Folin-Ciocalteu reagent according to Sutherland et al. (41). The standard used was a freshly prepared solution of bovine serum albumin. The weight of ferredoxin obtained by this method was higher than the actual dry weight of the protein and a correction factor was determined, after the purification of the protein, for calculating the weight of protein from the value obtained by the Folin-Ciocalteu assay.

Absorption Spectra:

Pure ferredoxin has characteristic absorption peaks in

the visible region of the spectrum. So, the purity of the effluents during chromatographic purification of ferredoxin was checked by recording the absorption spectra of the samples in a Cary model 14 spectrophotometer.

The effect of reagents like sodium dithionite, urea, mersalyl, and CMB on ferredoxin was also studied by recording the absorption spectra of the protein after incubation with the respective reagents. Some of these reactions were carried out in the absence of air. The reactants were maintained in anaerobic condition in a special type of absorption cell supplied by Quaracell Products, New York. This cell had a long glass stem, 9 1/2 cm long, fused over the conventional 3 ml absorption cell. The mouth of the cell was closed with a serum stopper through which a syringe needle was inserted. The needle was connected to a specially constructed vacuum manifold and the contents of the cell degassed. The cell was then alternately flushed with hydrogen and evacuated, several times, to insure the complete removal of air. Finally the space above the reaction mixture was filled with hydrogen. Reagents were added into the vessel, through the serum stopper, by means of a syringe.

Determination of dry weight:

The protein concentration of a sample of freshly prepared ferredoxin solution was determined according to Sutherland et al. (41). The absorbancy of the solution at 277 and 420 μ was also measured. Two ml of the same solution was dialyzed

in 8 mm dialysis tubing against several changes of deionized water for 24 hours, the water being changed every 8 hr. The dialysis tube was cut and the contents transferred to a previously weighed platinum crucible. The tube was washed with a few drops of water and the washings were added to the main dialyzate. The crucible was partially covered with a platinum lid and heated in an evacuated oven at 60° for 12 hours. The crucible was then cooled in a desiccator over phosphorus pentoxide and weighed. The residue was heated to 60° , cooled, and weighed, repeatedly, till there was no further change in weight. The crucible was then heated to 600° for 24 hours in a muffle furnace, cooled and weighed. The ash obtained was saved for determination of iron content.

Determination of total nitrogen:

The total nitrogen in the protein was determined by conversion of the protein nitrogen to ammonium sulfate by the Kjeldahl method, and estimating the ammonium content with Nessler's reagent. The ferredoxin sample used was the same as that which was used for the dry weight determination. Two-tenths milli liter of the protein solution was heated with 0.2 ml of concentrated sulfuric acid for 30 minutes in a 25 ml Kjeldahl flask. The flask was cooled, two drops of 30% hydrogen peroxide was added to it, and the flask heated again for 5 hours. The digested protein was cooled in ice, neutralized with 0.4 N sodium hydroxide, and diluted to 25 ml with water. Nessler's reagent, prepared according to

Seely and Vandemark (42) was added to various fractions of the diluted digest and the absorbancy of the resulting colored solution was measured, after 10 minutes, at 420 m μ , in a Bausch and Lomb Spectronic 20 spectrophotometer. The weight of the nitrogen in the sample was calculated by comparing the absorbancy values with that of a standard curve obtained from ammonium chloride and Nessler's reagent.

Determination of inorganic sulfide:

Inorganic or labile sulfide in a non-heme iron protein is sulfide that is liberated from the protein by the action of dilute acids. The inorganic sulfide content of taro ferredoxin was determined by conversion to methylene blue according to Fogo and Popowski (43) as modified by Lovenberg et al. (44). One-half milli liter of a mixture of ferredoxin solution and water was taken in tubes, 10 x 75 mm, and 1.3 ml of 1% zinc acetate and 0.05 ml of 12% sodium hydroxide were added. The tubes were stoppered and 0.25 ml of 0.5% N,N-dimethylphenylenediamine hydrochloride (prepared by dissolving N,N-dimethyl-p-phenylenediamine sulfate in 5.5NHCl), and 0.05 ml of 0.23M ferric chloride were added to each tube, the stopper being replaced after each addition. After 20 minutes, 0.85 ml of water was added to each tube and the absorbancy of the methylene blue formed was measured at 670 m μ against a blank which contained all reagents except ferredoxin. A solution of sodium sulfide which had been standardized iodimetrically according to Vogel (45) was used

as standard for a calibration curve.

Sodium sulfide was standardized by the following procedure. A standard solution of sodium arsenite was prepared by dissolving 1.25 g of pure arsenious oxide in 2.5 N sodium hydroxide, neutralizing the solution with 1 N hydrochloric acid, and diluting the mixture to 250 ml. The normality of the solution was calculated. An approximately decinormal solution of iodine was prepared by dissolving about 12.7 g of iodine crystals in potassium iodine solution and diluting to one liter with water. The iodine solution was standardized by titration against the sodium arsenite, in the presence of sodium bicarbonate, using starch as an indicator. The sodium sulfide solution was treated with excess of sodium arsenite and dilute hydrochloric acid when the sulfide was precipitated as arsenious sulfide. The precipitate was filtered off quantitatively and the unused arsenite in the filtrate was estimated by titration against the iodine solution. The normality of the sodium sulfide solution was calculated from the amount of sodium arsenite consumed by the sulfide.

Determination of iron content.

The iron content of the protein was determined using 4,7-diphenyl-1-10-phenanthroline (bathophenanthroline) according to the method of Diehl and Smith (46). In this method, an acidic solution of the protein is treated with hydroxylamine to reduce any ferric iron to the ferrous state and the ferrous iron is complexed with bathophenanthroline to form a

colored compound which is estimated spectrophotometrically.

About 0.3 mg of ferredoxin was heated with 3 ml of 1% HCl, in a 15 ml centrifuge tube, at 80° for 10 minutes. The mixture was centrifuged and the supernatant was transferred to a 10 ml volumetric flask. The sediment was washed with deionized water, centrifuged, and this supernatant was also poured into the flask. The process was repeated twice. The solution in the flask was diluted to 10 ml. Various fractions of this solution were used for iron estimation. The iron content of the dry ash, obtained from ferredoxin, was also estimated after dissolving the ash in warm dilute hydrochloric acid. The reaction mixture consisted of:

Ferredoxin solution + water	1.1 ml
Hydroxylamine hydrochloride, 10%	0.2 ml
Sodium acetate, 10%	0.8 ml
Bathophenanthroline, 0.001M	0.4 ml
Isoamyl alcohol	1.5 ml

The mixture, taken in a 13 x 100 mm tube, was shaken well and allowed to settle. About 1 ml of the colored complex was removed from the isoamyl alcohol layer and its absorbancy was measured at 533 mμ in a 1 ml absorption cell. The concentration of the iron in the solution was calculated by reference to a calibration curve prepared with standard iron solution.

Disc electrophoresis on acrylamide gels.

Poly acrylamide gels of 7.5% and 30% concentration were prepared and run in 0.038M Tris-glycine buffer, pH 8.3, according to Ornstein and Davis (47). About 100 to 200 microgram of ferredoxin (prepared from taro or spinach) was subjected to electrophoresis in a standard 7.5% gel, in 6 x 60 mm columns, at a current strength of 2.5 ma per column. Bromophenol blue was used as the marker dye. Electrophoresis was over in two hours. After observing the colored bands and their positions with respect to the marker dye, the gels were removed from the glass tubes. They were then cut at the position of the marker dye and stained with 1% solution of amido black in 7.5% acetic acid, to detect colorless proteins. The stained gels were washed with 7.5% acetic acid (sometimes destaining was done by electrophoresis in acetic acid). The relative intensities of the stained bands were traced in a Photovolt Corporation Densicord. Electrophoresis was carried out in small pore (30%) gels also, in glass tubes. These gels are very difficult to remove from the tubes intact, and so the gels were not stained after electrophoresis.

Moving boundary electrophoresis:

Free boundary electrophoresis of ferredoxin was carried out in a Perkin-Elmer Model 38 Electrophoresis apparatus provided with Schlieren optical assembly. A freshly prepared solution of ferredoxin (4 mg per ml) was dialyzed against pH

6.5 sodium phosphate-sodium chloride buffer of ionic strength 0.1 for 24 hours. The buffer was saved for electrophoresis. The ferredoxin was then taken in a standard 2 ml Tiselius cell and the apparatus was assembled as suggested in the instruction manual (Instruction Manual: Model 38 Tiselius Electrophoresis Apparatus The Perkin Elmer Corporation, Norwalk, Conn.). The cell and surroundings were cooled to 2° and allowed to attain equilibrium. When boundaries began to appear, the Schlieren assembly was turned on and a current of 14 ma passed through the assembly at an EMF of 135 volts. Photographs of the ascending and descending boundaries were taken at definite intervals using a Polaroid Land camera fitted to the apparatus.

Starch gel electrophoresis:

Starch gel electrophoresis was conducted in the apparatus described by Ashton (48) using a discontinuous buffer system. The electrolyte solution consisted of 1.35 g of lithium hydroxide monohydrate and 11.8 g of boric acid per liter giving a pH of 7.8. The gel buffer contained 1.6 g of citric acid monohydrate and 4.8 g of Tris per liter giving a pH of 8.0. Gels were prepared from Connaught hydrolyzed starch (Connaught Laboratories, Toronto, Canada) using a mixture of the electrolyte and gel buffer in the ratio 1:9 (v/v). Ferredoxin samples were absorbed on to Whatman 3 MM filter paper strips and were positioned into the gel at the anode end. Electrophoresis was run in a refrigerated compartment at 400-

500 with an initial current of 4 ma per cm width of the gel and was completed in about 3 hr. The gel was removed and stained with 0.05% solution of nigrosine black in methanol acetic acid-water (5:1:5 by volume).

Molecular weight determination.

The molecular weight of ferredoxin was determined by gel filtration and density gradient centrifugation methods.

Gel filtration.

Gel filtration was performed in Sephadex G-100 columns, prepared and run according to the procedure of Andrews (49) and of Whitaker (50). About 5 mg of ferredoxin, dissolved in 1 ml of Tris-HCl buffer, was layered on top of a column of Sephadex G-100, 1.6 x 113 cm, kept at 4° and equilibrated with 0.22M Tris-HCl buffer, pH 7.5. The protein was eluted with the same buffer, stored in a reservoir, at a height of 15 cm from the bottom of the column. Effluent fractions of approximately 3 ml volume were collected every 20 minutes in tubes loaded on a G.M. fraction collector. The concentration of the ferredoxin in the fractions was determined by measuring the absorbancy at 280 mu against a blank, which was a fraction eluted just before the ferredoxin. The column was standardized by running through it, pure specimens of beef heart cytochrome c, trypsin, beef heart lactic dehydrogenase and bovine serum albumin. The void volume of the column was determined using Blue Dextran 2000.

Sucrose gradient centrifugation.

The sedimentation coefficient and molecular weight of ferredoxin were determined by sucrose density gradient centrifugation by the method of Martin and Ames (51). Five per cent and 20% solutions of sucrose were prepared in 0.1M phosphate buffer, pH 6.8. Eighteen milliliters of 5% sucrose and 16.5 ml of 20% sucrose were poured into the left and right limbs respectively of a triple outlet Density Gradient Mixer (Buchler Instruments, New Jersey). Sucrose gradients of 11.5 ml volume each were collected in three Beckman ultracentrifuge tubes, 9/16 x 3 1/2 inches, and stored at 4° for 6 hours. About 0.5 ml of 5% ferredoxin solution was then layered on top of the gradient in one of the tubes and the same volumes of horse heart cytochrome c and trypsin were layered in the other tubes. A drop of mineral oil was layered on top of the proteins. The tubes were then balanced and loaded into a pre-cooled swinging bucket rotor (Beckman Spinco Model L 2-65 Ultra Centrifuge, maintained at 4°, at 41,000 RPM, for 64 hours. The tubes were then pierced at the bottom and fractions of 25 drops were collected. Each fraction was diluted with 2 ml of water and its absorbancy was measured at 280 mμ against a suitable blank. The sedimentation coefficient and the molecular weight of ferredoxin were calculated from the rate of migration of protein in the gradient with reference to the standards using the formula given by Martin and Ames (51).

Phosphoroclastic assay.

The capacity of taro ferredoxin to substitute for bacterial ferredoxin in the formation of acetyl phosphate, from pyruvate and inorganic phosphate was measured by the method of Lovenberg *et al.* (44). Bacterial ferredoxin and ferredoxin-free bacterial extract (clastic system) were prepared from dry cells of Clostridium pasteurianum according to Mortenson (52). The protein concentration of the clastic system was determined by the biuret method and that of bacterial ferredoxin from its absorbancy at 390 mμ $E_{1\text{cm}}^{1\%} = 33.2$, (53). The protein concentration of taro ferredoxin was determined using Folin-Ciocalteu reagent. A reaction mixture, consisting of:

Sodium pyruvate	1M	0.1 ml
Coenzyme A	0.001M	0.1 ml
Clastic system (40 mg per ml)		0.2 ml
Potassium phosphate	0.25M, pH 6.8	0.1 ml and
Ferredoxin + 0.1M acetate, pH 5.8		0.5 ml

was incubated at 30° for 15 minutes. The acetyl phosphate formed was estimated by the method of Lipmann and Tuttle (54). The reaction mixture containing acetyl phosphate was incubated for 10 minutes with 28% hydroxylamine hydrochloride. Three milliliters of ferric chloride were then added and the mixture was centrifuged. The absorbancy of the red supernatant containing acidic ferric hydroxamate was measured in a Klett-Summerson photoelectric colorimeter, with a green

filter, against a blank which contained all reagents except ferredoxin.

Determination of SH content.

Spectrophotometric titrations with three reagents were carried out to determine the number and nature of cysteine groups in the protein.

1. Titration with CMB. A standard solution of CMB in phosphate buffer was added, in aliquots, to a solution of ferredoxin in 0.05M phosphate, pH 6.5 and the increase in absorbancy at 255 mu was measured, in a Cary 14 Spectrophotometer, as described by Boyer (55). In a preliminary experiment, a known amount of ferredoxin was treated with excess of CMB reagent, and the absorbancy of the mixture at 255 mu was measured at different intervals of time. The reaction was complete in 20 minutes. In all later titrations, the ferredoxin-CMB mixture was incubated at least for 20 minutes before measuring the absorbancy. The titrations were carried out, in the presence and absence of air, with native ferredoxin and ferredoxin dissolved in 8M urea.

CMB reagent was prepared by dissolving the sodium salt of p-chloromercuribenzoic acid in 0.05 M sodium pyrophosphate, adjusting the pH to 6.5 with 0.05 M NaH_2PO_4 , and then diluting to the required strength by the addition of 0.05 M phosphate buffer, pH 6.5. The concentration of the solution was calculated from its absorbancy at 232 mu (55). Standard solutions of sodium sulfide (45) and glutathione were used

as references.

To study the effect of CMB titration on the absorption maxima of ferredoxin, the absorbancy changes at 277, 330, 420 and 465 μ were also recorded during the titration.

2. Titration with mersalyl. Mersalyl titration was carried out by the method described by Klotz and Carver (56). A millimolar solution of the reagent was prepared by dissolving 25.3 mg of the sodium salt of mersalyl acid and 15 mg of sodium chloride in 50 ml of 0.1 M sodium acetate buffer, pH 5.8. A 2×10^{-4} M solution of the dye, pyridine 2-azo-p-dimethylaniline in acetate buffer was used as an internal indicator. When the reaction with the protein is complete, the next drop of mersalyl added will react with the dye giving a pink color with a high absorption at 550 μ . This is the end point of the titration.

Aliquots of standard mersalyl reagent were added to reaction mixtures containing 0.8 ml of the dye and about 0.1 micromole of ferredoxin in a total volume of 2.5 ml acetate buffer, pH 5.8. After 20 minutes incubation the absorbancy of the mixture at 550 μ was measured against a blank containing the acetate buffer. A standard solution of reduced glutathione was used as reference.

3. Titration with DTNB. To study the effect of guanidine hydrochloride on the SH groups, ferredoxin was titrated against a solution of DTNB by the procedure described by Ellman (57). A millimolar solution of DTNB reagent was pre-

pared in 0.1M phosphate buffer, pH 8.0. A solution of ferredoxin in pH 8.0 phosphate buffer was mixed with a 10 to 15 molar excess of DTNB reagent and the absorbancy of the mixture was read at 412 m μ against a blank to which the reagent was not added. A correction was made for the absorbancy of the unused reagent at 412 m μ . The number of SH groups titrated was calculated from the maximum absorption recorded at 412 m μ using a molar extinction coefficient of 13,600 for the thioenol formed at this wavelength. Titrations were also carried out using solutions of ferredoxin in 4M guanidine hydrochloride, pH 7.0, with or without EDTA. In guanidine hydrochloride titrations the blank contained guanidine and DTNB reagent, but no ferredoxin. Cysteine hydrochloride and standard sodium sulfide were used as references.

Amino acid analysis. The number of cysteine residues in the protein was also determined by amino acid analysis of the carboxymethylated protein.

Determination of mercury bound to ferredoxin.

A known amount of ferredoxin (ca 3 mg) was mixed with varying volumes of CMB, in 0.05 M phosphate buffer, pH 6.5, and the mixtures were shaken at room temperature for 30 minutes. They were then dialyzed against repeated changes of distilled water for two days. (The dialysis tubing was previously treated with CMB to remove any sulfide, and then washed in a continuous stream of distilled water to remove the CMB). After dialysis, the tubes were cut and the contents

were quantitatively transferred to graduated cylinders. The mercury present in the dialyzates was estimated by atomic absorption spectrophotometry.

Measurements of atomic absorption were carried out essentially by the procedure of Fuwa et al. (58), using the apparatus assembled by Dr. R. H. McKay of this department. A Westinghouse WL 22847 hollow cathode discharge tube, operated at a current of 10 ma, was the emission source, and a Beckman atomizer burner was used to spray the sample into the 1.3 x 25 cm alumina absorption cell. The fuel used consisted of a mixture of hydrogen, at a pressure of 2.5 pounds per square inch, and oxygen, at a pressure of 14 pounds per square inch. The flow rate of liquid through the burner was approximately 2 ml per minute. The absorption was measured at 2537 Å in a Carl Zeiss PMQ II Spectrophotometer, operated at maximum sensitivity and a slit width of less than 0.1 mm. From the absorbancy values, the concentration of mercury in the samples was calculated by reference to curves constructed with standard mercuric chloride or CMB solution. The water, that was present, outside the dialysis tubing, in the final dialysis, served as a blank.

Preparation of derivatives of ferredoxin.

For amino acid analysis and determination of terminal amino acid residues, two derivatives of ferredoxin were prepared.

1. Oxidized, iron and sulfur free ferredoxin.

Iron and inorganic sulfide were removed from the protein by the method of Tanaka et al. (59). To a solution of 100 mg of ferredoxin (in 6 ml of water), cooled in ice, was added, in drops, 2 ml of 20% trichloroacetic acid. The ferredoxin was immediately decolorized and a white precipitate appeared. The mixture was let stand for one hour in the cold and then centrifuged. The sediment was washed three times with 5 ml volumes of a mixture of ether and 95% ethanol, and finally dried in vacuo.

The cysteine residues in the iron and sulfur free ferredoxin were oxidized to cysteic acid with performic acid as described by Moore (60). Nine milliliters of 88% formic acid was added to 1 ml of 30% hydrogen peroxide, the mixture let stand for one hour at room temperature and then cooled to 0°. Four milliliters of the resulting performic acid was added to 40 mg of trichloroacetic acid treated ferredoxin. A precipitate was formed. The reaction mixture was left in the cold room overnight. Then it was diluted five fold with water and lyophilized.

2. S-Carboxymethyl ferredoxin:

Carboxymethylated ferredoxin was prepared as described by Crestfield et al. (61). The reaction was carried out in 25 ml plastic bottles provided with screw caps. Two pieces of nalgene tubes were inserted through the cap to serve as inlet and outlet for nitrogen gas which was passed through

the bottle throughout the reaction. Two milliliters of ferredoxin (10 mg) solution were taken in the bottle and to it was added 3.6 g of recrystallized urea, 0.3 ml of 5% EDTA solution, 3 ml of Tris-HCl buffer, pH 8.6, and 0.1 ml of mercaptoethanol. The mixture was covered with 10 ml of 8 M urea solution. Nitrogen was passed through the reaction mixture, at room temperature, for 4 hours. The reaction mixture which was reddish brown in the beginning became colorless by this time. The contents of the bottle were then transferred, in the dark, to a beaker, and a solution of 0.27 gm of recrystallized iodoacetic acid in 1 ml of 1N NaOH was added. Nitrogen was passed through the mixture for 10 minutes. Then it was poured on top of a 4 x 40 cm column of Sephadex G-75 equilibrated with 0.02M ammonium acetate and wrapped in aluminum foil. The protein was eluted from the column with 0.02M ammonium acetate solution as suggested by Kresztes-Nagy and Margoliash (62). Since the column had a good flow rate (40 ml per hour), no air pressure was used in elution. Fractions of 10 ml were collected and the carboxymethylated protein was located in the eluate fractions by measuring the absorbancy at 280 m μ . Fractions containing the protein were pooled and evaporated in a flash evaporator. The residue was dissolved in 5 ml of water and evaporated to dryness under nitrogen.

Determination of amino acid composition:

The amino acid composition of the performic acid-oxidized

ferredoxin was determined quantitatively using a Beckman Spinco Model 120 amino acid analyzer according to the instructions given by the manufacturer (Spinco Model 120-Instruction Manual and Hand Books). A solution containing about 0.05 micromole of ferredoxin was taken in a 16 x 150 mm pyrex tube and evaporated in nitrogen. A small crystal of phenol and 1 ml of 6N HCl were added to the tube which was then evacuated and sealed. The tube was heated at 110° for 24 hours. The tube was then cooled, cut open and the HCl was removed by evaporation under a stream of nitrogen. The dried hydrolyzate was then dissolved in pH 2.2 sodium citrate buffer and aliquots of the solution were run in the long and short columns of the amino acid analyzer. From the chromatograms obtained, the concentration of each amino acid was calculated by reference to standard chromatograms from runs with standard amino acid mixtures.

The amino acid composition of the S-Carboxymethylated ferredoxin was also determined by the same procedure, after 24 hour hydrolysis.

Determination of the amino terminal amino acid.

The amino terminal amino acid of the protein was identified by two methods.

1. By using FDNB. The dinitrophenyl (DNP) derivative of the protein was prepared according to Fraenkel-Conrat et al. (63), the DNP protein was hydrolyzed, and the amino terminal amino acid was separated as the DNP derivative.

Two drops of FDNB reagent and 0.1 ml of 95% ethanol were added to a solution containing 0.2 micromole of ferredoxin in 1 ml of 1% aqueous sodium bicarbonate. The mixture was shaken for 4 hours to complete the reaction and then the excess of FDNB was removed by extraction with ether. The residue was treated with 1 ml of 6N HCl and let stand overnight at 4°. The mixture was then centrifuged and the supernatant was discarded. The sediment was mixed with 1 ml of 6N HCl in a pyrex tube. The tube was sealed under vacuum and then heated for 16 hours at 110°. After hydrolysis, the reaction mixture was diluted with water and the aqueous solution was shaken with ether to separate the ether-soluble DNP amino acids. The yellow ethereal extract was dried, dissolved in acetone and then chromatographed on a thin layer of silica gel-G using a mixture of chloroform, benzyl alcohol and acetic acid (7:3:3 by volume) as the solvent. Standard DNP amino acids were used as reference. To detect the presence of any water-soluble DNP amino acid in the hydrolyzate, the aqueous phase of the hydrolyzate was separated by thin layer chromatography using n-propanol-34% aqueous ammonia (7:3 by volume) as the solvent system.

A dinitrophenyl derivative was also prepared from oxidized ferredoxin. After hydrolysis of the DNP protein and ether extraction of the hydrolyzate, the ether-soluble DNP amino acids were chromatographed on a Whatman No. 1 paper using 3% aqueous ammonia-tertiary amyl alcohol (1:1 by

volume) in one direction and 1.5 M phosphate buffer, pH 6, in the second direction. Standard DNP amino acids were also spotted on the paper for chromatography in the second direction. The aqueous phase was separated by thin layer chromatography as before.

2. By using dansyl chloride. The protein was treated with dansyl chloride by the procedure of Gray and Hartley (64), the dansylated protein was hydrolyzed and the dansyl amino acid at the amino terminal was identified.

About 0.2 micromole of oxidized ferredoxin, in 1 ml of 0.01 M sodium bicarbonate solution, was taken in a 18 x 150 mm pyrex tube wrapped in aluminum foil. One tenth of a milliliter of dansyl chloride (3 mg in 1 ml acetone) was added to the protein and the mixture was shaken for 3 hours at room temperature. It was then dried under a stream of nitrogen, mixed with 1 ml of 6N HCl and sealed under vacuum. Hydrolysis was effected by heating at 110° for 12 hours. The hydrolyzate was dried in a stream of nitrogen, dissolved in 2 drops of acetone-acetic acid mixture and the solution was spotted on a glass plate coated with a thin layer of silica gel-G. Standard dansyl amino acids were also spotted on the plate which was then heated for 20 minutes at 110°. The chromatogram was developed using the solvent system of Nedkov and Genov (65), viz, chloroform-methyl acetate-methanol-acetic acid (90:150:45:2 by volume). The dansyl amino acids were detected on the plate by their fluorescence under a

U.V. lamp.

Determination of carboxyterminal amino acid.

The carboxyterminal amino acid was identified by hydrazinolysis, and by the action of carboxypeptidase enzymes on the protein.

1. Hydrazinolysis. Hydrazinolysis of the protein was performed by the procedure of Bradbury (66) with slight modifications. About 0.1 micromole of dry, native, ferredoxin was mixed with 25 mg of hydrazine sulfate and 0.2 ml hydrazine (95%+), in a pyrex tube. The tube was sealed under reduced pressure and then heated at 60° for 16 hours. After cooling, the tube was cut and the contents were dried in a jet of nitrogen. The residue was treated with 1 ml of 1 M acetic acid and evaporated under nitrogen. The resultant mass was treated with 1 ml of acetone and dried under nitrogen. The dry residue was dissolved in 1 ml of 0.2N sodium citrate buffer, pH 2.2, and the pH of the solution was adjusted to 2.2 by adding 1% HCl. Aliquots of the solution were analyzed in the long and short columns of the Beckman Spinco amino acid analyzer.

2. Digestion with carboxypeptidases. Carboxypeptidase digestion was carried out by a modification of the procedure of Fraenkel-Conrat et al. (63). DFP carboxypeptidase, purchased commercially, was suspended in water and centrifuged. The sediment was suspended in 1% aqueous sodium bicarbonate, and 0.1N NaOH was added to the suspension till the enzyme was

completely solubilized. The pH of the enzyme solution was immediately reduced to 8.0 with 0.3 M acetic acid. The concentration of the enzyme in the solution was determined by measuring the absorbancy at 278 mu ($E_{278 \text{ mu}}^{1\%} = 19.6$).

A solution of native ferredoxin, containing about 0.1 micromole of protein, was evaporated to dryness in a current of nitrogen. The dry protein was dissolved in 2 ml of 0.1% sodium bicarbonate solution. Solubilized DFP carboxypeptidase A was added to the protein solution taken in a tube, to give a ferredoxin to enzyme ratio of 20:1. The reaction mixture was incubated, with shaking, at 40°, for 24 hours. Enzymic digestion was then stopped by adding 0.3 M acetic acid to pH 3.0 and the contents of the tube were evaporated under nitrogen. Samples of ferredoxin solution and enzyme solution were also evaporated to be used as controls. The dried carboxypeptidase digest was dissolved in water and the solution was divided into two parts, one part of the solution was dried again, the dried mass was dissolved in acetone and chromatographed on a Whatman No. 1 paper, in the descending direction, using the upper phase of a butanol-acetic acid-water mixture (4:1:5 by volume) as the solvent. Standard amino acids were also spotted on the paper as references. The second part was evaporated under nitrogen, the residue was dissolved in pH 2.2 sodium citrate buffer and the solution was used for the quantitative analysis of amino acids in the Beckman-Spinco analyzer. The control samples of oxidized

ferredoxin and carboxypeptidase A were also run in the amino acid analyzer.

Oxidized ferredoxin, and carboxymethyl cysteinyl ferredoxin were also digested with carboxypeptidase A, for various intervals of time, and the amino acids liberated, in each case, were determined quantitatively using the amino acid analyzer.

Enzymic digestion of the protein was repeated using carboxypeptidase B, instead of carboxypeptidase A, to detect the presence of basic amino acids at the carboxyterminal.

Determination of Tryptophan content.

Tryptophan was estimated spectrophotometrically. Due to the presence of iron and sulfide in the molecule of ferredoxin, the ultraviolet absorbance of the native ferredoxin in the 280 to 300 mu region is much higher than the combined absorbance of the aromatic amino acids in the molecule. Therefore, tryptophan estimations were carried out with the native protein, acid precipitated protein, and with oxidized protein. Three methods were employed.

1. By action of alkali. Ferredoxin was dissolved in 0.1N NaOH and the absorbancy of the solution at 280 and 294.4 mu was measured. From the absorbancy values, the molar ratio of tyrosine to tryptophan was calculated using the Goodwin and Morton formula (67).

2. By the action of N-bromosuccinimide in urea. In this method due to Funatsu *et al.* (68), a solution of ferredoxin in pH 4.6, acetate buffer, 0.2 M, was treated with a millimolar solution of N-bromosuccinimide in 8 M urea. The decrease in absorbancy of the protein at 280 mμ, due to the oxidation of tryptophyl residues, was measured and the extinction due to tryptophan was calculated using the empirical factor given by Patchornik *et al.* (69).

3. By the action of 6 M guanidine hydrochloride. The protein was dissolved in a 6 M solution of guanidine hydrochloride in 0.2 M phosphate buffer, pH 6.5. The absorbancy of the protein solution at 280 and 288 mμ was measured and from the absorbancy values, the tyrosine and tryptophan content were calculated according to Edelhoch's formula (70).

Basic hydrolysis. An attempt was made to estimate the tryptophan content of native ferredoxin, chemically, according to the procedure of Noltman *et al.* (71). About 6 mg of native ferredoxin, in a 18 x 150 mm Vycor tube (No. 19800, Corning Glass Works, Corning, New York), was mixed with 0.75 g of $\text{Ba(OH)}_2 \cdot 8 \text{H}_2\text{O}$ and 0.6 ml of water. The tube was cooled to 0°, evacuated, and sealed. Hydrolysis was carried out by heating the tube at 110° for 72 hours. After cooling, the tube was cut and the contents were transferred, by shaking with hot water, into a 50 ml plastic centrifuge tube. The barium ion in the mixture was precipitated by bubbling carbon dioxide, generated from dry ice and water, through it. The

precipitate was removed by centrifugation and the supernatant was evaporated under reduced pressure to 1 ml volume. This liquid was filtered through a millipore filter and the filtrate was lyophilized. The residue was dissolved in pH 2.2 sodium citrate buffer and aliquots of the solution were used in the short and long columns of the amino acid analyzer. The tryptophan content of the protein was calculated from the leucine recoveries and from the known leucine content of ferredoxin assuming equal destruction of these two amino acids during alkaline hydrolysis.

Finger print analysis of taro and spinach ferredoxins.

For a general comparison of the amino acid residues in taro and spinach ferredoxins, the two proteins were hydrolyzed with chymotrypsin and the peptides liberated were separated either by electrophoresis followed by chromatography, or by two dimensional paper chromatography. The number and R_f values of the peptides were then determined by staining with ninhydrin. The chymotrypsin was freed of any trypsin activity by prior treatment with tosyl lysyl chloromethyl ketone (TLCK chymotrypsin) as suggested by Mares-Guia and Shaw (72).

About 0.01 micromole of TLCK chymotrypsin was added to a solution of 0.5 micromole of a carboxymethyl cysteinyl ferredoxin in 3 ml of phosphate buffer, pH 6.8. The mixture was continuously stirred at 35° and the pH was adjusted and then maintained at 8.0 by the addition of 0.1N NaOH. About 0.005 micromole more of the enzyme was added after 2 hours.

After 8 hours of digestion, the reaction was terminated by adding 1M acetic acid till the pH dropped to 5.0. The solution was evaporated to dryness and the dry residue was used for electrophoresis and chromatography.

High voltage paper electrophoresis was conducted in the apparatus supplied by Enso, Salt Lake City. A portion of the chymotryptic digest was dissolved in pyridine-acetic acid-water (100:4:900) buffer, pH 6.4, and the solution was applied to a Whatman 3MM paper as described by Bailey (73). Electrophoresis was run in the same buffer, for 2 hours, at 500 volts. The paper was then dried, and submitted to chromatography in the descending direction in butanol-pyridine-acetic acid-water (30:20:6:2 V/V). After drying the paper, in a current of air, it was sprayed with 0.02% ninhydrin in acetone. The peptide spots appeared on warming for a few minutes at 60°.

Two dimensional paper chromatography of the chymotryptic digest was carried out according to Tsuru et al. (74). The sample spotted on a Whatman No. 3 paper was subjected to descending chromatography in n-butanol-acetic acid-water (4:1:2 V/V) in the first direction and in n-butanol-pyridine-water (1:1:1 by volume) in the second direction. The paper was then dried and sprayed with 0.2% solution of ninhydrin in butanol, saturated with water. Purple spots appeared on heating the paper at 100° for a few minutes. After marking the ninhydrin positive spots, Ehrlich reagent was sprayed

over the spots to detect peptides containing tryptophan.

EPR studies.

The electron paramagnetic resonance spectra of native and reduced taro ferredoxins were observed and recorded in a Varian V-4500-10 A EPR spectrometer with 100kcs field modulations. Measurements were made at ambient (25°) and liquid nitrogen (-195°) temperatures. The instrument was tuned for operation by following the directions given in 'Operating Instructions' (Publication No. 87-114-200, Varian Associates, Palo Alto, California). The Klystron oscillator was operated at a frequency of 9.5 kMc and the attenuator dial of the x-Band Micro Wave Bridge was set at 10 db throughout the experiment. Ferredoxin solution (0.2 ml containing 3 mg of protein) in pH 7.0 phosphate buffer was taken in a quartz EPR sample holder and placed in the sample cavity.

After adjusting the signal through the oscilloscope, the instrument was scanned for the detection of EPR signals in the field range of 1250 to 3750 gauss and the spectra of the signal was recorded. The sample was then mixed with 0.2 ml of 0.1 M sodium dithionite (prepared in pH 7.0 phosphate and stored in a helium atmosphere) and the spectra of the mixture was recorded in the same magnetic field range.

EPR measurements were repeated at liquid nitrogen temperature. The ferredoxin solution was taken in a cylindrical quartz tube (3 x 250 mm). The tube was closed with a serum stopper and the air above the sample was replaced with hydro-

gen gas. After freezing the sample in liquid nitrogen, the tube was inserted in a specially constructed Nitrogen Dewar placed in the EPR cavity. Gaseous nitrogen was passed through the cavity to insure that no water vapour condensed in the cavity during the low temperature operation.

The signals from the sample were recorded in the range of magnetic field from 1250 to 3750 gauss. The tube was then removed from the Dewar and immediately dipped in cold water to thaw the sample. Sodium dithionite solution was added anerobically into the tube, the mixture was frozen and the EPR spectra of the frozen mixture was recorded in the usual magnetic field range.

The EPR spectra of spinach ferredoxin was also recorded in the oxidized and reduced states at ambient and liquid nitrogen temperatures. A separate measurement of the signal generated from sodium dithionite was also made.

RESULTS

Purification of ferredoxin.

A summary of the yields and purifications obtained in each step of isolation, starting with 1.1 kg of leaves is given in Table I. The average yield was about 25 mg of pure protein from 1 kg of leaves. This yield compares favorably with the yield of ferredoxin from alfalfa (62) and spinach (75). The yield was the same whether the leaves were used fresh or after a week's storage in the freezer. However, a decrease in ferredoxin content was noticed when the leaves were harvested in the evening rather than in the morning. Tris-HCl buffer, pH 7.5, was used in the homogenization of the leaves, since low yields were obtained in some of the earlier studies where distilled water was used for homogenization. Dialysis was avoided to reduce the time required for purification. The purified protein was stored under hydrogen to prevent oxidation and consequent deactivation, by air. For most of the work, it was convenient to store the protein as a concentrated solution in the buffer, instead of as a lyophilized solid. The protein samples were usually used within a month after their preparation.

Electron transfer activity of ferredoxin.

Fig. 1 gives the relation between the amount of NADPH formed and the concentration of protein added, with different preparations of taro ferredoxin, using the photoreduction

assay of San Pietro (40). In this assay, one unit of ferredoxin activity is defined as "the amount which produces a change in optical density of 1.0 in 10 minutes at 340 m μ when the reaction mixture contains 0.1 mg of chlorophyll per 3 ml". When absorbancy measurements were made without centrifuging the reaction mixture, tiny chloroplast particles floated in the solution and interfered with the measurements. Therefore, the reaction mixture was centrifuged to sediment the particles and the supernatant was used. The maximum activity observed was 29 units per mg (Fig. 1, curve A) with a sample of taro ferredoxin which had a 420 to 277 m μ absorbancy ratio of 0.43. This corresponds to the reduction of 139 micromoles of NADP per mg of ferredoxin, per mg of chlorophyll in 10 minutes. The specific activity of spinach ferredoxin determined with Swiss chard chloroplasts was comparable to that of the taro protein.

The activity of ferredoxin decreases with aging. Curve B represents the NADP photoreduction by an aged preparation of ferredoxin with a 420 m μ to 277 m μ ratio of 0.38. The activity of the sample was 15.3 units per mg. The specific activity of a sample with a 420 m μ to 277 m μ absorbancy of 0.40 was 22.5. Curve C represents the photoreduction activity of the supernatant obtained, after ammonium sulfate fractionation, in the course of purification of ferredoxin. Curve D was obtained with one of the earlier fractions eluted out during the DEAE-cellulose column chromatographic purifi-

cation of ferredoxin. The low activity with these fractions is due to the presence of other proteins as contaminants.

Absorption spectra. Pure ferredoxin is red in color and the spectrum of the protein shows absorption maxima at 465, 420, 330, and 277 mu. During the isolation of ferredoxin, the purification achieved in each step can be followed by recording the absorption spectra of the respective preparations. The spectra of crude ferredoxin preparations show an absorption maximum near 260 mu, but no absorption peaks in the visible spectral region. As the protein preparation gets more and more purified, absorption maxima appear in the visible region and the 260 mu absorption peak in the ultraviolet region is shifted toward 277 mu. Fig. 2 gives the absorption spectrum of pure taro ferredoxin and of a sample of spinach ferredoxin prepared in our laboratory and Fig. 3, the absorption spectra of three ferredoxin fractions in different stages of purification.

Table II gives the ratios of absorbancy of taro ferredoxin in the visible and near ultraviolet region to the absorbancy in the ultraviolet region. For comparison, the corresponding ratios of some other plant ferredoxins are also included in the table. It has been suggested that the higher ratio of absorbance in the visible region to that in the ultraviolet region of parsley and brassica ferredoxins is due to the absence of tryptophan residues in these ferredoxins (27).

Electrophoresis.

Starch gel electrophoresis was run to detect the presence of aggregated molecules of ferredoxin, and other impurities in the preparation. The presence of polymers has been reported in the spinach protein at pH 2.2 by Appella and San Pietro (29), and in alfalfa ferredoxin (free from labile sulfide) by Keresztes-Nagy and Margoliash (62). Electrophoresis on starch gel with a fresh preparation of taro ferredoxin (420:277 μ absorbancy = 0.43) revealed only a single protein band (Fig. 4a). The band appeared dark on a blue background on staining and had migrated about 5 cm in the 7 cm gel strip in three hours of electrolysis. This indicates the protein is free from polymers. (The protein was shown to be the monomer by molecular weight determination of the sample). However, when the electrophoresis was repeated using an aged preparation (stored aerobically) with a 420:277 μ absorbancy ratio of 0.38, a very light, slow moving band was seen along with the main band (Fig. 4b). When the band had moved 68 mm from the origin, the minor component had moved 65 mm. This slow moving band may be due to some aggregated molecules of ferredoxin formed during storage.

Free boundary electrophoresis was performed mainly during the earlier stages of the work to test the homogeneity of the ferredoxin preparations isolated from taro leaves. Whenever more than one peak appeared in the electrophoretic runs, such samples were either rejected or rechromatographed. The chief

contaminants in these samples were proteins of high mobility than ferredoxin. The ferredoxin peaks were not well defined due to the high color of the preparations used. Single peaks moving with a fairly high velocity were obtained with samples purified by Sephadex G-75 chromatography. The mobility of the pure protein at 0°, at pH 7.0 in phosphate buffer of ionic strength 0.1, was $10.0 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \text{ volt}^{-1}$. The mobility of native alfalfa ferredoxin in phosphate buffer of ionic strength 0.1 at pH 7.2, is $15.6 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \text{ volt}^{-1}$ (Reference 62), and that of spinach ferredoxin is $7.57 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \text{ volt}^{-1}$, in phosphate buffer of ionic strength 0.1 and pH 7.0, at 4° (Reference 29).

Polyacrylamide gel electrophoresis.

Electrophoresis on polyacrylamide gels was very convenient for a rapid analysis of the protein fractions eluted during the chromatographic purification of ferredoxin. The fractions were first run through the standard 7.5% gel which separated proteins in the molecular weight range 400,000 - 10,000. The colored bands formed on electrophoresis were observed directly and the colorless proteins were detected after staining the gel. Preparations which were found homogeneous in the standard gel were further examined by electrophoresis in the small pore 30% gel which resolved compounds in the molecular weight range of 10,000 - 3,000.

Fig. 5 shows the bands obtained on electrophoresis of taro and spinach ferredoxin samples purified by Sephadex G-75

chromatography. Electrophoresis in the standard gel revealed only a single red band moving along with the marker dye. But, when the gels were stained with amido black, three faint bands lying close together were seen midway between the origin and the position of the major band (Fig. 5a). When traced in a densitometer, the total absorbance due to these three bands was less than 1% of the absorbance of the major band. The interesting observation was that the band patterns were identical in spinach and taro ferredoxins. The colorless components responsible for the minor bands may be due to decomposition products of ferredoxin or artifacts of isolation procedure. Garbett et al. (76) have reported the presence of traces of impurities, probably poly-phenolic, in spinach and parsley ferredoxins.

Electrophoresis in 30% acrylamide gel (Fig. 5b) showed only a single red band with an R_f value (ratio of distance moved in the gel by the protein to the distance moved by the marker dye) of 0.75. Margoliash has observed (personal communication to Dr. H. F. Mower) different genetic variants of ferredoxin in the protein isolated from alfalfa. If the taro preparation contained such variant forms of ferredoxin, they would have shown up as separate bands during electrophoresis in 30% gel provided they possessed different molecular weights or net charge. However, the absence of multiple bands does not prove that the ferredoxin consists of homogeneous molecules with identical amino acid sequences.

Chemical composition of ferredoxin.

A freshly prepared solution of ferredoxin, found homogeneous by electrophoresis, and with a 420 to 277 mμ absorbance ratio of 0.43 was used for the following determinations as described under methods.

1. Determination of dry weight and ash content.
2. Determination of nitrogen content.
3. Estimation of iron and sulfide.
4. Determination of extinction coefficients at wavelengths of maximum absorption.
5. Determination of protein using Folin-Ciocalteu reagent.

The weight of dry protein obtained from 2 ml of ferredoxin solution after extensive dialysis against distilled water, evaporation, and drying at 60°, was 15.6 ± 0.3 mg. The ash content of the dry residue after ignition to constant weight at 600°, was 3.6%. The ash contained 26% iron which corresponds to 37% Fe_2O_3 . The amount of ash in the protein, not accountable as Fe_2O_3 is, therefore, 2.3%.

The nitrogen content, from duplicate Kjeldahl determination, was 14.4%.

Iron analysis was performed, on the dry ash after digestion with acid and on the native protein after treatment with dilute acid. The amount of iron in ferredoxin, estimated from analysis of the ash, was $0.90 \pm 0.03\%$. Direct analysis of native ferredoxin gave a value for the iron content of

$0.93 \pm 0.03\%$. In the assay system used, the absorbancy of the ferrous-bathophenanthroline complex at 533 mu, when the reaction mixture contained one micromole of iron, was 0.275. This corresponds to a molar extinction coefficient of 23,000 at 533 mu for the iron-dye complex in isoamyl alcohol. With the standard iron solution, results of duplicate determinations agreed within $\pm 1\%$. The ferredoxin solution used for iron analysis contained 3 to 5 microgram of iron per milliliter.

The inorganic sulfide content of the ferredoxin preparation was 0.53% or 2 atoms of sulfur per 12,000 g of protein. In this assay system, the methylene blue formed from one micromole of sodium sulfide gave an absorbancy of 8.0 at 670 mu. This extinction value is 15% lower than that reported by Lovenberg et al. (44). This may be due to slight differences in the temperature at which the reaction was carried out and in the pH of the reagents used, as mentioned by Fogo and Popowski (43). High concentrations of sulfide inhibit the formation of methylene blue color and, therefore, sulfide determinations were carried out using ferredoxin solutions which gave a linear relationship between the absorbancy of the methylene blue formed and the concentration of protein added.

Protein concentration of ferredoxin preparations was determined in many cases, using the Folin-Ciocalteu reagent with bovine serum albumin as reference standard. It was

found that the dry weight of the protein was $74 \pm 3\%$ of that determined by colorimetric method. This conversion factor (0.74 g per g) was therefore used for calculating the true weight of ferredoxin from the value obtained with the Folin-Ciocalteu reagent. Our spinach ferredoxin preparation also gave a higher color yield in the assay, comparable to that of the taro protein.

Chou and Goldstein (77) have attributed the large color increments given by some proteins in the alkaline copper reaction to the presence of sequence of amino acids containing functional chains, and to arginine, histidine and glutamic acid residues which are highly chromogenic in peptide linkages. One mole of ferredoxin (ca 11,000 g) contains one histidine, one arginine and 16-17 glutamic acid (or glutamine) residues. The same weight of bovine serum albumin contains approximately three histidine, four arginine and 13 glutamic acid (or glutamine) residues according to the data given by Putnam (78). These amino acids by themselves are not, therefore, responsible for the enhanced color yield with ferredoxin with respect to bovine serum albumin as standard. The enhanced yield may therefore be due to certain peptide sequences, a relatively higher serine, tryptophan and valine content, and the presence of iron and inorganic sulfide in ferredoxin. It may be mentioned in this context that the amino acid sequence of spinach ferredoxin worked out by Matsubara et al. (75) shows two tripeptides with the Glu-Glu-

Glu sequence.

Lovenberg et al. (44) have reported that the dry weight of salt-free C. pasteurianum ferredoxin was 70% of that determined by the colorimetric protein method. The bacterial and plant ferredoxins therefore seem to have some common peptide sequences which are responsible for the enhanced color yield.

The ratio of absorbancy of ferredoxin at wavelengths of maximum absorption is given in Table II. The molar extinction coefficients of ferredoxin at difference wavelengths calculated from the absorbancy value and the iron content, assuming that a molecule of ferredoxin contains two atoms of iron, is given in Table III.

The absorbancy of the protein solution was measured also at 280 and 260 mu. The ratio of absorbancy at 280 to 260 mu was 1.12. The protein concentration of the solution was calculated from this data using the formula of Warburg and Christian quoted by Layne (79). The dry weight of the protein was $65 \pm 4\%$ of that determined by the spectrophotometric method. The result was the same with spinach ferredoxin also. The spectrophotometric method is based on the assumption that the ultraviolet absorption of a protein at 280 mu is mainly due to the tyrosine and tryptophan residues, but a large fraction of the 280 mu absorption of ferredoxins is not accounted by the constituent aromatic amino acids. Therefore, we can expect a discrepancy between the true weight of

ferredoxin and the weight calculated from the ultraviolet absorption by the method of Warburg and Christian.

Molecular Weight Determination.

Gel filtration. The relation between the log of molecular weights of proteins and the ratio of their elution volume to void volume (V/V_0) during gel filtration is illustrated in Fig. 6. Taro ferredoxin was eluted from the gel column just before cytochrome c. The molecular weight of ferredoxin with reference to the standard curve is approximately 13,000. Duplicate runs were made with the standards and ferredoxin and no difference in elution volumes were observed.

Sedimentation Analysis. The absorbancy at 280 mu of the protein fractions sedimented in the sucrose gradient is given in Fig. 7. Measurement of the 420 to 277 mu absorbancy ratio of the ferredoxin in the gradient showed that the protein did not undergo any deterioration during the centrifugal run. Ferredoxin and cytochrome c sedimented to the same extent. The ratio of the distances traveled from the meniscus, R , (51) by ferredoxin and cytochrome c, was 1.0. The R value of ferredoxin with respect to trypsin was 0.60. The sedimentation constant of ferredoxin, calculated on the basis of an $s_{20,w} = 1.83S$ for cytochrome c (80), is 1.8S and with reference to an $s_{20,w} = 2.48S$ for trypsin (81), is 1.5S. The sedimentation constant determined by direct ultracentrifugal analysis was 1.36S for spinach PPNR (29), 1.65S for

alfalfa ferredoxin (62) and 1.55S for Euglena gracilis PPNR (82). It is seen that the S value of taro ferredoxin is close to that of other plant ferredoxins.

Martin and Ames (51) have calculated approximate molecular weights of proteins from their sedimentation ratios using the relation $\frac{s_1}{s_2} = \left(\frac{MW_1}{MW_2}\right)^{2/3}$. On this basis, ferredoxin has a molecular weight of approximately $12,000 \pm 1,000$ with reference to a molecular weight value of 12,300 for the cytochrome c (Sigma Chemicals) and 24,000 for the trypsin used in the experiment.

The molecular weight of ferredoxin calculated from its amino acid composition and iron and sulfide content is between 10,700 and 11,000.

Action of sodium dithionite on the absorption spectra.

Sodium dithionite solution (5 mg per ml of Tris buffer, pH 7.8) was added in small aliquots to a solution of ferredoxin (ca 1.5 mg) in a 3 ml absorption cell. The contents of the cell were mixed immediately and the absorption measured at 420 mu. The maximum loss in absorbancy at 420 mu, after correcting for dilution, was 51%. When the experiment was repeated under anaerobic conditions, the absorbancy decrease was 49%. In both cases, the final mixture contained more than hundred-fold molar excess of dithionite. These results are in agreement with those of Whatley et al. (83) and Fry et al. (84) where a 50% loss of 420 mu absorption was observed with spinach ferredoxin reduced with dithionite. Sobel and Loven-

berg (85) have observed a 54% loss of absorption at 415 mu with dithionite-treated C. pasteurianum ferredoxin and Malkin and Rabinowitz (22) have reported a 51% decrease in absorption with C. acidi-urici ferredoxin when treated with dithionite. Though the chemical changes accompanying the dithionite reduction were not investigated in detail, it is interesting to note that the behaviour of bacterial and plant ferredoxin toward the reagent was similar.

The ferredoxin samples treated with sodium dithionite were examined after storage at 4°, overnight. The aerobically maintained mixture lost 83% of its absorption (prior to storage) at 420 mu while the anaerobic sample lost only 33% of its prestorage absorption. The absorption spectra of dithionite-treated ferredoxin, before and after incubation at 4°, are shown in Fig. 8. When a large excess of dithionite (ca 500 molar) was added to ferredoxin, in air, at room temperature, the protein was irreversibly bleached within an hour. The spectrum of the protein after removal of the dithionite by dialysis against water showed no visible absorption, but the 277 mu peak in the ultraviolet was shifted to 267 mu (Fig. 9). A similar shift in the U.V. peak was observed by Tagawa and Arnon (15) with spinach ferredoxin after reduction with hydrogen and C. pasteurianum hydrogenase. This shift cannot be due to pH reduction occurring in the mixture since even trichloroacetic acid-treated ferredoxin retained the peak near 277 mu. It is possible that treatment

with excess of sodium dithionite resulted in the loss of iron and labile sulfide from the protein.

Action of 8 M urea on the absorption spectra.

The absorption spectra of taro ferredoxin (ca 1.4 mg in 3 ml) in 8 M urea (pH > 6) was recorded following five minutes of aerobic and anaerobic incubation at room temperature. The spectra in both cases showed a peak around 455 μ in place of the usual 465 μ peak observed in native ferredoxin. Since there was no previous mention of this type of absorbancy shift in the literature, the experiment was repeated with spinach ferredoxin. The spectral shift in the visible region was observed with spinach ferredoxin also. This clearly suggests that some conformational change occurs in the plant ferredoxin molecule on treatment with 8 M urea. Garbett et al. (76) have reported a change in the circular dichroism of spinach ferredoxin on treatment with 8 M urea. These authors suggest that "the effect of urea is presumably to alter the helical nature of the protein around the non-heme iron chromophore".

The urea-Fd mixtures were examined again after incubation at 4° for 40 hours. The spectra of the samples are given in Fig. 10. The ferredoxin kept exposed to air lost most of its visible absorption, while the anaerobically kept specimen lost about half of the visible absorption. A solution of ferredoxin in phosphate buffer, kept as a control, retained most of its visible absorption under identical condi-

tions (Fig. 10). The absorbancy at 277 mu was not altered in all cases as is illustrated in Table IV.

Malkin and Rabinowitz (86) have reported recently that a decrease in 415 mu absorption occurs in C. acidi-urici ferredoxin when it is incubated for 60 minutes with 4 M guanidine hydrochloride or 6.4 M urea, the decrease being greater under aerobic than anaerobic conditions. The iron of this ferredoxin was more readily available for chelating agents in the presence of denaturing agents than it was with the native protein. Brumby et al. (87) have observed the progressive loss of iron and visible absorption with xanthine oxidase when the protein was incubated with urea. A similar loss of iron from the molecule of taro ferredoxin could have occurred on prolonged incubation of the protein in 8 M urea.

Phosphoroclastic assay.

Fig. 11 shows the relation between the amount of acetyl hydroxamate formed and the concentration of ferredoxin used in the phosphoroclastic assay. The 390 to 280 mu absorbance ratio of the bacterial protein used was 0.73, and the 420 to 277 mu absorbance ratio of the taro protein, was 0.38.

Lovenberg et al. have defined a unit of ferredoxin activity in this assay as "the amount needed to effect a change of 1.0 in the absorbance of 540 mu" and is equivalent to the amount of ferredoxin which produces 6 micromoles of acid hydroxamate per 10 minutes. The activity of our bacterial ferredoxin was 11 units per mg. If we assume the molecular

weight of the plant protein to be double that of the bacterial protein, then bacterial ferredoxin is four times as active as plant ferredoxin on a molar basis. Valentine et al. (88) have observed that C. pasteurianum ferredoxin is four times as active as spinach PPNR in the cleavage of pyruvate by clastic extracts on a weight basis. It should be emphasized here that the bacterial and plant ferredoxins used were deteriorated samples and the values obtained by the assay have only qualitative significance in proving that taro ferredoxin can substitute in the electron transfer reaction of bacterial extracts.

Titration with mercurials.

It is difficult to determine the exact number of sulfide and sulfhydryl groups in ferredoxin by spectrophotometric titration. This is in part due to the sensitivity of ferredoxin to exposure to air. We have found a decrease in sulfide content with taro ferredoxin samples of low 420 to 277 mμ absorbancy ratio (< 0.43). To minimize the oxidation of the protein, the titrations were carried out in a hydrogen atmosphere, as described under methods.

The standards used, reduced glutathione and sodium sulfide, combined with stoichiometric amounts of CMB and mersalyl. The molar extinction coefficient of the CMB-SH complex in phosphate buffer, pH 6.5, was approximately 7×10^3 with reduced glutathione. The results of CMB and mersalyl titrations with different ferredoxin preparations are given in

Table V. The sulfide content, determined by the Fogo and Popowski method (43), and the 420 to 277 mu absorbancy ratio of some of the preparations are also included in the table. Figs. 12 and 13 show the relation between the absorbancy of the mercury-SH complex and the concentration of mercurials added.

It is evident from the results that: (1) both CMB and mersalyl react to the same extent with ferredoxin; (2) urea does not expose any buried SH groups since there was no appreciable change in the titre in the presence of 8 M urea, and (3) the number of moles of mercurial reacting with a mole of ferredoxin decreases with decrease in relative visible absorption of the protein. The maximum number of moles of mercurials reacting with a mole of ferredoxin is eight with taro, and nine with spinach.

The correlation between the loss in visible and near U.V. absorbancy of taro ferredoxin and the amount of mercurial reacted with the protein is illustrated in Fig. 14. The decrease in visible absorbancy paralleled the increase in absorbancy of the protein-mercurial complex at 255 mu. The visible absorbancy of the protein was not completely lost when the sulfide and sulfhydryl groups were completely titrated. However, the spectrum of the protein after dialysis of the reaction mixture against distilled water showed no absorbancy in the visible region. Analysis of this protein solution showed the absence of both iron and labile sulfide

in it. Thus mercurials displace both labile sulfide and iron from ferredoxin. The residual absorbancy of ferredoxin-mercurial mixture in the visible region may be due to the presence of iron (not bound to the protein) in the reaction mixture.

Estimation of bound mercury.

Malkin and Rabinowitz (89) have prepared bacterial 'apo-ferredoxin', containing no iron and labile sulfide, by passing mersalyl-treated C. acidi-urici ferredoxin through ion exchange columns and separating the products. Apoferredoxin bound to CMB was similarly prepared from taro ferredoxin by incubating the protein with CMB and then separating the protein from unreacted CMB, CMB-labile sulfide complex, and iron displaced from the protein, by extensive dialysis against water. Determination of the mercury content of the apoferredoxin would give the number of atoms of mercury that are bound to the cysteine groups of the protein.

Table VI gives the results of mercury analysis of CMB-ferredoxin. The liquid outside the dialysis bag which was used as a blank contained no mercury. Under the conditions of assay, a standard solution of mercuric chloride containing 100 ppm of mercury gave an absorbancy of 0.60 units at 253.7 μ , while a CMB solution with the same mercury concentration had an absorbancy of 0.62 units. All calculations of mercury content were made with reference to the mercuric chloride absorbancy standard.

From the table, it is clear that the maximum number of mercury atoms bound to the apoferredoxin is four. The fact that the apoferredoxin contained bound mercury even when the native protein was incubated at a CMB:Fd ratio of 4:1, shows that both the sulfide and sulfhydryl groups react simultaneously with the mercurial.

The CMB-ferredoxin reaction was allowed to proceed only for 30 minutes. If the protein is incubated for a long period, there is a possibility of amino acids other than cysteine combining slowly with the mercurial, especially when there is excess of reagent present (55).

Titration with DTNB.

The effect of 4 M guanidine hydrochloride on the protein was studied by titration of the protein against DTNB in the presence of the denaturing agent. The results, shown in Table VII are only of qualitative significance. Both standards used, cysteine and sodium sulfide, reduced the reagent rapidly and stoichiometrically under all conditions of titration. But, the color of the reduced reagent began to fade gradually after about 30 minutes, as judged by the loss of absorbancy at 412 mμ. The reaction between ferredoxin and DTNB in the presence of 4 M guanidine hydrochloride was also very fast, though all cysteines and inorganic sulfides were not titrated. The color of the DTNB-Fd mixture also faded after 30 minutes, as illustrated, in Fig. 15. The reaction between the native protein and DTNB was very

slow and maximum absorbancy at 412 mμ was obtained only after 42 hours. The amount of thioenol formed was still less, when the native protein was titrated in the presence of EDTA. The anaerobic titration in the presence of 4 M guanidine chloride was not affected by EDTA, but the number of DTNB equivalents reduced was lower in the aerobic titration in the presence of EDTA.

Though the results are difficult to interpret, it is clear that the rate and extent of DTNB reaction is enhanced by treating the ferredoxin with guanidine hydrochloride. This should be the result of some conformational change in the protein molecule which results in the exposure of DTNB-sensitive groups. Control experiments with cysteine hydrochloride showed that neither guanidine hydrochloride nor EDTA affected the DTNB titre of the amino acid. Since addition of EDTA to the denatured protein did not increase the number of DTNB equivalents combining with the protein, it may be inferred that free SH groups are not liberated by the chelation of iron with EDTA. Addition of EDTA to the native protein decreased its activity toward DTNB. Possibly, during the removal of iron from the native protein, the SH groups are oxidized and the shape of the protein molecule itself is altered, making it less active. In similar studies with C. acidu-urici ferredoxin, it was found (Reference 86) that in the presence of 4 M guanidine hydrochloride, DTNB reacted exclusively with the inorganic sulfide in the protein,

but in the presence of EDTA and guanidine hydrochloride, both the cysteine and inorganic sulfide reacted. This suggests that the iron-cysteine bonding, if there is any, is not entirely identical in bacterial and plant ferredoxins.

Tryptophan determination.

Results of tryptophan estimation in the protein by three spectrophotometric methods are given in Table VIII. The absorption of native ferredoxin measured at 280 mμ is about twice that calculated from the aromatic amino acid content of the protein. It has been suggested that the aromatic residues, the labile sulfide, and the spectral contribution of the two iron atoms account for the entire absorption of alfalfa ferredoxin at 277 mμ (62). The extinction coefficient of native ferredoxin at 280 mμ in 6 M guanidine hydrochloride, pH 6.5, is 23,300, while that of oxidized ferredoxin is 10,400 in the same solvent. Since the latter value almost agrees with the total tyrosine and tryptophan absorption of the protein, it may be presumed that the performic acid treated protein is completely free from iron and from any structure which enhances the U.V. absorption of the native protein.

The absorption spectra of native and oxidized ferredoxin in alkali is given in Fig. 16. The shape of the absorption curve of oxidized ferredoxin in 0.1N NaOH (pH > 12) resembles the U.V. spectra of tyrosine-tryptophan mixtures with a molar ratio of Tyr:Trp ≥ 3 (90).

Basic hydrolysis.

The tryptophan and leucine content of the protein after 72 hour basic hydrolysis, were respectively 0.33 and 4.2 moles. Since amino acid analysis of acid hydrolysates of ferredoxin show the presence of 6 leucine residues per mole of protein, the leucine recovery on basic hydrolysis is 70%. Assuming equal destruction for both amino acids, the number of tryptophan residues in the molecule is 0.47. Though it is difficult to tell the exact number of tryptophan residues due to the low yield of the amino acid in basic hydrolysis, it is probable that the protein contains one tryptophan residue per mole.

When the finger print obtained from the chymotryptic digest of the protein was sprayed with Ehrlich reagent, one tryptophan positive spot was seen. A corresponding spot was observed in the finger print of spinach ferredoxin digest which contains one molecule of tryptophan. The extinction coefficients at 277 μ of taro and spinach ferredoxins are similar. Spinach ferredoxin contains four tyrosine, one tryptophan, two iron, and two sulfide molecules per molecule of protein. Taro ferredoxin has been shown to contain four tyrosine residues and two atoms of iron and inorganic sulfide in one molecule and so probably should contain one molecule of tryptophan, to account for the similarity with the spinach protein in U.V. absorption. We cannot attach much importance to the tryptophan values obtained by the

spectrophotometric titration, since the U.V. extinction of the native protein is raised by the presence of iron, and that of the oxidized protein may be lowered due to the loss of some tryptophan.

Amino acid composition.

The amino acid composition of taro ferredoxin, calculated from the direct analysis of acid hydrolysates of the carboxymethylated and oxidized proteins is given in Table IX. Table X gives a comparison of the amino acid composition of the taro protein with those of spinach and alfalfa, the only other plant ferredoxins whose compositions are known. Spinach ferredoxin was shown to contain 97 amino acid residues only after the determination of the complete amino acid sequence (75). Direct analysis indicated the presence of 99-100 (75), and 97-101 (27) amino acids in the protein. It is therefore possible that when the complete sequence of taro ferredoxin is worked out, the total number of amino acid residues in the molecule may turn out to be the same as that of spinach ferredoxin.

All the three ferredoxins contain the same number of arginine, tryptophan, isoleucine, tyrosine, and phenylalanine residues. Methionine is absent in the ferredoxin compositions given in Table X. However, Fry and San Pietro (27) have reported the presence of a methionine residue in their spinach ferredoxin preparation, and Matsubara et al. (75) have indicated the possible presence of a methionine containing

variant of ferredoxin in the spinach protein used for sequence determination. The major differences in the amino acid composition are 1) a higher glycine and a lower alanine content in taro ferredoxin; 2) a high proportion of aspartic acid+asparagine and a lower proportion of glutamic acid+glutamine in spinach ferredoxin; and 3) the presence of an additional residue of histidine and cysteine and one residue less of proline in alfalfa ferredoxin. The degree of these differences in composition is minimized if we group together, as in Table XII, those amino acids which are chemically very close and whose codons differ only by a single base. It is interesting to note that the content of total basic, neutral, and acidic amino acids is almost identical in taro and spinach ferredoxins.

NH₂ terminal amino acid determination.

Fig. 17 gives a trace of the thin layer chromatogram obtained from the ethereal extract of DNP-ferredoxin hydrolysate. There was only a single yellow spot in the chromatogram which originated from the sample. The R_f of this spot corresponded with that of DNP alanine which was one of the standard DNP amino acids spotted on the thin layer. The aqueous layer of the DNP hydrolysate was chromatographed on silica gel along with standard DNP cysteine, DNP arginine E-DNP lysine, O-DNP tyrosine, and Im-DNP histidine. The chromatogram of the sample contained a faint yellow spot with an R_f value equal to that of Im-DNP histidine. When thin layer

chromatography was repeated with the aqueous extracts obtained from the acid hydrolysates of FDNB treated oxidized ferredoxin, two yellow spots were obtained. The Rf values of these were the same as O-DNP tyrosine and Im-DNP histidine respectively. These derivatives are formed by condensation of FDNB with the -OH group of tyrosine and imidazole group of histidine and not necessarily from the NH₂ terminal amino acid.

Two dimensional paper chromatography of the ether extract from oxidized ferredoxin revealed two yellow spots with Rf values equal to those of DNP alanine and DNP glycine (lower Rf) respectively. These spots were eluted with 4% aqueous sodium bicarbonate and the ratios of absorbancy at 390 to 360 mμ of the eluates were determined. The ratio was 0.59 for the eluate corresponding with DNP alanine and 0.79 for the other. The 390 to 360 mμ ratio for solution of standard DNP alanine, DNP glycine, and dinitrophenol were respectively 0.60, 0.62, and 0.81. So, the yellow spot with an Rf value similar to DNP glycine should be due to dinitrophenol. Thus the only aminoterminal amino acid of ferredoxin detectable by the FDNB method is alanine.

The result of thin layer chromatography using acid hydrolysates of the dansyl derivative of ferredoxin is illustrated in Fig. 18. The spots marked on the chromatogram were detected by their fluorescence under a U.V. lamp. There were three fluorescent spots originating from the sample. The Rf

of the central spot was the same as that of dansyl alanine. The compound moving ahead of the central spot was found to be dansyl amine and the third spot was found to be dansyl hydroxide, by comparison with standards run under identical conditions (65). Alanine is, therefore, the only aminoterminal amino acid in the protein.

Carboxy terminal amino acid.

The amino acids released from ferredoxin as a result of hydrazinolysis are given in Table XII. The results indicate alanine to be the C-terminal amino acid. Low yields of C-terminal amino acids have been reported with many proteins. Bradbury (66) has observed that hydrazinolysis of insulin liberated 37% of C-terminal alanine and about 10% each of non-C-terminal glycine, serine and glutamic acid. Recently, Shore and Shore (91) obtained 30% of C-terminal threonine and 15% each of non-C-terminal glycine and serine after hydrazinolysis of a protein moiety from human serum. Only 56% of free alanine added to insulin was recovered after hydrazinolysis (66) showing that this amino acid was decomposed to a large extent during the reaction. The non-C-terminal amino acids are produced probably as a result of hydrolysis of their hydrazides during or after hydrazinolysis (66). The high glycine content in the hydrazinolysis product from ferredoxin may be due to the presence of a high proportion of glycine in the native protein and the ease of hydrolysis of the glycine hydrazide.

Carboxy peptidase digestion of taro ferredoxin in the native and carboxymethylated state released mainly alanine, threonine, leucine, and serine. The amounts of these amino acids liberated under various experimental conditions is given in Table XIII. In addition to the amino acids mentioned in the table, approximately 0.5 mole per mole of protein, of glutamic acid, aspartic acid and glycine were also liberated from the oxidized ferredoxin preparation. It is possible that during the preparation of this sample of ferredoxin, the molecule was cleaved either by trichloroacetic acid or by performic acid. Serine may be an artifact, or the fourth amino acid from the C-terminal. It is evident from the results that alanine is the C-terminal amino acid of the protein. The penultimate amino acid may either be threonine or leucine. Spinach ferredoxin has the C-terminal sequence Leu-Thr-Ala (75) and it is quite possible that this sequence is identical for taro and spinach ferredoxins. Paper chromatography of the 24 hr. carboxypeptidase A digest (Fig. 19) of native protein did not reveal any spots corresponding to asparagine or glutamine. Therefore, the threonine and serine peaks obtained during amino acid analysis of the enzyme digest are not contaminated with asparagine or glutamine, two amino acids which are eluted along with threonine and serine in the analyzer.

Paper chromatography and amino acid analysis of the 24 hr. digest of ferredoxin with carboxypeptidase B, did not show

the presence of any free amino acid in the digest. This proves the absence of any basic amino acid at the C-terminal of ferredoxin. The carboxypeptidase B used for the digestion of ferredoxin was found to be active in a separate reaction carried out with peptides.

Finger prints of taro and spinach ferredoxins.

Fig. 20 shows the separation by electrophoresis and chromatography of the peptide fragments released from spinach and taro ferredoxins by chymotryptic digestion. Very poor separation was effected by electrophoresis since the mobility of the peptides was very low. The relative positions of the peptides in the chromatogram are the same for both proteins, though the Rf values of the spinach fragments were slightly higher. Peptide no. 8 in the spinach chromatogram was absent in that of taro ferredoxin. Peptide no. 1 in the taro digest was Ehrlich reagent positive and so should contain tryptophan. Presence of tryptophan was not tested in the corresponding spinach ferredoxin digest.

A better separation of the peptides was achieved by two dimensional paper chromatography (Fig. 21). Ten fragments were obtained from spinach and eight from taro ferredoxin. Matsubara et al. (75) have also obtained ten peptides from spinach ferredoxin after chymotryptic digestion. It is possible that the number of peptides released by chymotrypsin is less in taro ferredoxin because of its lower content of leucine compared to the spinach protein. Most of the peptides

occupy similar positions in both chromatograms. Peptide no. 1 in both chromatograms was shown to contain tryptophan by spraying with Ehrlich reagent.

Finger print analysis shows many similarities in the composition of spinach and taro ferredoxins and also proves that the composition is not identical for the two proteins.

EPR studies:

Preliminary investigations using an aqueous solution of taro ferredoxin (15 mg/ml) indicate that the native and reduced protein do not exhibit any EPR signal at room temperature. When cooled to liquid nitrogen temperature, the solution of the native protein gives a resonance signal with an apparent g value of 4.45 (Fig. 22a). A similar signal at $g = 4.27$ was observed by Hall et al. (92) in spinach ferredoxin purified by three different methods. However, these authors in a later communication (93) attributed the signal to the presence of an impurity in the ferredoxin preparation. Beinert and Palmer (94) also mention a generally observed resonance of iron (III) in high-spin state in biological materials which occurs at $g = 4.2-4.3$ and is attributed to impurities. Further work is necessary to ascertain whether the resonance signal observed with taro ferredoxin is due to the presence of iron or to the impurity. It may be mentioned that a $g = 4.432$ signal is exhibited by Micrococcus aerogenes rubredoxin in the oxidized state and is considered to arise from high-spin Fe (III) in rhombic field (95).

Fig. 22b shows the EPR spectrum of taro ferredoxin reduced with sodium dithionite and is similar in appearance to the spectra of reduced non-heme iron proteins with the characteristic $g = 1.94$ signal. The shape of the spectrum resembles very closely the EPR spectra of reduced spinach ferredoxin (96). The apparent g values of taro ferredoxin ($g_x = 1.96$, $g_y = 2.02$, and $g_z = 2.12$) are slightly higher than those of reduced spinach ferredoxin but these are only approximate values.

EPR signals were not observed from an aqueous solution of spinach ferredoxin (11 mg/ml), either in the native or in the reduced state, even at liquid nitrogen temperature. There are indications that the spinach ferredoxin signal is much harder to observe, requiring a higher concentration of protein and a lower temperature. Aqueous sodium dithionite gave the characteristic signal of the free electron at ordinary and liquid nitrogen temperatures.

DISCUSSION AND CONCLUSION

The primary object of the present research was to isolate pure ferredoxin from a plant available at all seasons in Hawaii and to establish its identity as a ferredoxin by studying its various physical parameters and chemical properties, and amino acid composition. This would guarantee a ready supply of plant ferredoxin for future work in this laboratory. It was felt that it would be advantageous for a comparative study to select a different class of plant from spinach and parsley for the extraction of the protein. The final choice was taro (Colocasia esculanta), a tropical monocotyledonous plant. Taro ferredoxin is the first example of a ferredoxin isolated from a monocotyledon. It was our aim to look for structural variations, if any, in the taro protein to establish in terms of measurable changes the differences between taro ferredoxin and the other two plant ferredoxins isolated from dicotyledonous plants. Some observations have been made during these investigations of the properties of taro ferredoxin which may lead to the understanding of the structure-function relationship of the protein. The determination of the amino acid sequence of taro ferredoxin, and the structure-function relationship are planned as part of the future research on this protein.

The modified isolation procedure devised for taro ferredoxin is superior to prepare pure ferredoxins in good yield

from plants compared to the methods of San Pietro (40) and Tagawa and Arnon (36). The method devised does not involve the use of large excess of acetone or extensive dialysis required in the original method. The use of pH 7.5 Tris buffer for homogenization of the leaves prevents the decomposition of the protein due to lowering of the pH, which would occur if water is used in homogenization.

One molecule of taro ferredoxin contains two atoms of iron and two atoms of 'inorganic' sulfide. The smell of hydrogen sulfide is noticeable when the protein is treated with dilute hydrochloric acid or trichloroacetic acid. All plant ferredoxins, so far isolated, contain two atoms of iron and labile sulfur per mole.

In alfalfa (personal communication to Dr. Mower from Dr. Margoliash) and spinach ferredoxins (75), presence of more than one type of ferredoxin has been observed in the course of amino acid sequence determination. There is a strong possibility for the presence of protein variants in the Koa ferredoxin, the sequence of which is now worked out at the University of Hawaii (Miss A. Benson, personal communication). These plants, therefore, may contain more than one type of gene directing the synthesis of their ferredoxins. Electrophoreses did not indicate the presence of variants in taro ferredoxin. Electrophoretic studies alone are not sufficient to detect protein variants unless amino acid substitutions produce appreciable changes in the net charge or

molecular weights of these protein variants. Our source of taro ferredoxin was plants grown in the same area for generations. Taro plants are propagated by vegetative reproduction, while alfalfa and spinach are propagated by sexual reproduction. Hence, the probability of hybridization is less in taro than in the other two plants. Determination of the amino acid sequence of taro ferredoxin will help to establish the genetic homogeneity of the protein.

The molecular weight values of taro ferredoxin are $12,000 \pm 600$ from iron analysis, $12,500 \pm 1200$ from gel filtration and sedimentation analysis, and $11,000 \pm 300$ from amino acid composition. These values agree closely with the molecular weights determined for spinach (75) and alfalfa (62) ferredoxins.

The absorption spectrum of taro ferredoxin is similar to those of other plant ferredoxins, with characteristic peaks in the visible and ultraviolet regions. The values of the extinction coefficients of taro and spinach ferredoxins at 277 m μ and 420 m μ agree closely.

The color and visible absorption of taro ferredoxin decrease on storage of the protein in air. The decrease in the visible absorption of taro ferredoxin on exposure to air is accompanied by a decrease in the labile sulfide content of the protein (Table V). Though no correlation studies were made between the loss in visible absorption and the iron content of taro ferredoxin, the amount of iron present in two

samples of ferredoxin with $A_{420 \text{ m}\mu}$ to $A_{277 \text{ m}\mu}$ ratios of 0.43 and 0.38 were identical. Thus the presence of two moles of inorganic sulfide is essential to maintain maximum visible absorption in taro ferredoxin.

The visible absorption of the protein is partially or completely lost by treatment of ferredoxin with reagents like sodium dithionite, 8 M urea, and organic mercurials. It has been observed with spinach ferredoxin, that sodium dithionite reduces one of the two ferric ions in the protein to the ferrous state (108). The complete bleaching of taro ferredoxin color on addition of excess of sodium dithionite, in air, may be due to the displacement of iron bound to the protein. The reactivity of iron chelating agents toward spinach ferredoxin is enhanced very much on incubation of the protein with sodium dithionite as observed by Bayer (109). The spectral shifts observed on treatment of taro ferredoxin with 8 M urea, soon after the addition of urea may be due to conformational changes occurring around the iron-sulfur chromophoric group. It is possible that on prolonged incubation in urea, both the iron and inorganic sulfide are displaced from the protein. Loss of iron on urea-treatment has been observed with xanthine oxidase (87), another non-heme iron protein of the ferredoxin type. The loss in visible absorption occurring on mercurial treatment of the protein is due to the removal of labile sulfide and iron from it since analysis of the mercurial-bound ferredoxin showed the absence of these

elements in the apoprotein. Thus the visible absorbancy of taro ferredoxin is dependent on the presence of iron and inorganic sulfide in a particular type of configuration. The absorbancy in the ultraviolet region (277 mu) is due to the aromatic amino acids and iron atoms in the protein.

The group of non-heme iron electron transfer proteins includes the bacterial and chloroplast ferredoxins, mammalian mitochondrial proteins like succinic dehydrogenase, xanthine oxidase, adrenodoxin, etc. All these non-heme iron proteins contain iron and labile sulfide in a 1:1 ratio. One of the chief physical properties which distinguish these non-heme iron proteins from heme proteins like cytochromes, catalase etc. is their characteristic absorption spectra. The spectra of non-heme iron proteins, in the visible region, show broad absorption peaks between 380 and 470 mu. The intensities of these absorptions decrease on reduction of the protein chemically or enzymatically.

The shape of the visible absorption spectra of hemoproteins is quite different and is made up of the absorption due to the porphyrin and the absorption arising out of the iron-ligand interaction in the heme moiety. Low spin ferrous complexes show a three-banded spectrum with a sharp Soret peak at 400 to 420 mu and two other peaks with less intensities in the regions 520 to 535 and 555 to 565 mu. High-spin ferrous complexes have spectra with the Soret peak and another band around 550 mu. The Soret peak of the hemoproteins is con-

tributed by the porphyrins. There is no decrease in the intensities of the absorption peaks when the heme proteins are reduced by sodium dithionite (110).

The spectra of taro and other chloroplast ferredoxins show absorption maxima at 420 and 465 m μ . The visible absorption spectra of adrenodoxin and flavin-free mitochondrial non-heme iron proteins are similar to those of chloroplast ferredoxins (115). The extinction coefficients of chloroplast ferredoxins and the mitochondrial enzymes (freed from flavin nucleotides where present) are between 4000-5000 cm² moles⁻¹ per atom of iron (76). The EPR signals of ferredoxins and the mitochondrial non-heme iron proteins, in the reduced state, are similar. There is thus a body of evidence to suggest that the chromophoric group in chloroplast ferredoxins is related to that of a wide variety of very important enzymes in many diverse forms of life.

The capacity of taro ferredoxin to photo reduce NADP decreases with decrease in the relative visible absorbancy of the protein (Fig. 1). The protein obtained by adding excess of CMB to taro ferredoxin and dialyzing off the iron and CMB-labile sulfide complex was found to be completely inactive in the photo-reduction of NADP. Thus the iron and sulfide groups which are essential to give the maximum visible absorption to taro ferredoxin is essential also for the electron transfer activity of the protein. This has been substantiated by other workers using spinach (27) and alfalfa

ferredoxins (62).

San Pietro and associates have titrated the iron in spinach ferredoxin against chelating agents in the presence of excess CMB. The CMB blocked the sulfhydryl groups of the protein which may otherwise reduce the iron in the process of titration. Reactions were carried out with native ferredoxin and ferredoxin photo reduced with illuminated chloroplasts. One molecule of native ferredoxin, in the presence of CMB, combined with two equivalents of the ferric chelating agent, Tiron. After photo-reduction and blocking of the SH groups by CMB, one molecule of spinach ferredoxin combined with one equivalent of the ferrous chelating agent, ortho-phenanthroline (84). From these studies San Pietro and associates have concluded that native spinach ferredoxin contains two atoms of ferric iron, one of which is reversibly reduced to ferrous state on illumination with chloroplasts.

The maximum decrease in visible absorbancy of spinach ferredoxin during the photoreduction was about 50%. The visible absorbancy of spinach ferredoxin decreased about 50% immediately following the anaerobic addition of sodium dithionite. The visible absorbancy of taro ferredoxin also decreases by 50% following the addition of dithionite. Chance and San Pietro (108) have observed similarities in the absorbancy changes in spinach ferredoxin caused by photo-bleaching and by treatment with sodium dithionite. Mossbauer spectra of the oxidized and reduced spinach ferredoxin studied

by San Pietro (102) showed clearly that only one of the two irons per molecule is susceptible to chemical reduction by sodium dithionite. Whatley et al. (83) have shown that the photoreduction of NADP by spinach ferredoxin is an one electron transfer reaction which proceeds according to the equation:

All these observations suggest that the oxido-reduction of ferredoxin is associated with a ferric-ferrous valency change in one of the iron atoms present in the molecule. However, it has not been established either with ferredoxins or with other non-heme iron electron transfer proteins that the iron-sulfide group alone is functional in the electron transfer reaction. In C. pasteurianum, some of the electron transfer reactions mediated by ferredoxin can be carried out by flavodoxin (111), a protein which does not contain iron and inorganic sulfide, or by rubredoxin (112), a protein which does not contain inorganic sulfide. Thus the electron transfer reactions of non-heme iron proteins can be mediated by other proteins devoid of iron or/and inorganic sulfide.

It is difficult to determine accurately the number of cysteinyl groups in ferredoxins by spectrophotometric titration. Conflicting reports have appeared in the literature regarding the sulfhydryl content of the plant (62, 27, 32) and bacterial (22, 89, 25) ferredoxins, estimated by titration against organic mercurials. The number of mercurial equiva-

lents combining with a molecule of ferredoxin as determined by the titration is usually less than the value calculated from the cysteine (amino acid analysis) and labile sulfide content (chemical analysis) of the protein. There are many possible reasons for this discrepancy. The titre value may be affected by the uncertainty in the determination of the protein concentration. Protein concentrations determined by the phenol color reaction (41) or by the method of Warburg and Christian (79) are inaccurate unless proper corrections are made. Loss of labile sulfide occurs during storage of the protein. There is also the possibility of formation of a disulfide bond during the titration. Palmer et al. (96) have reported the formation of a disulfide bond in spinach ferredoxin when the protein is treated with strong urea solution.

The nature of the binding of iron and labile sulfide in plant ferredoxins is still not definitely established. In 1965, Phillips et al. (101) proposed a structure for the active site of clostridial ferredoxins with all the seven iron atoms in molecule arranged linearly and bound together via sulfur bridges furnished by all the eight cysteine residues and six inorganic sulfide atoms. These authors found from magnetic susceptibility and Mossbauer spectral studies that all the seven iron atoms are in strong field ferric, but they exist in two structurally nonequivalent environments of two and five atoms each. In the model proposed, the two terminal iron atoms were differently situated from the five iron atoms

in the interior of the bridge. The model also explains the following observations. 1) The iron in bacterial ferredoxins can be exchanged with ^{59}Fe only after treatment of the protein by an organic mercurial (44). The mercurial removes the inorganic sulfide and displaces the iron atoms bound, presumably, to the cysteine groups. 2) Ferredoxin cannot be carboxymethylated in the native state, or in the presence of urea, with iodoacetate indicating the absence of free cysteine groups in the protein (44). The positions of the cysteine residues were ascertained after the determination of the amino acid sequence of C. pasteurianum ferredoxin by Tanaka et al. (26).

The above model is not free from criticism. Investigations by X-ray analysis of the structure of M. aerogenes ferredoxin (113) indicate that the iron atoms are not arranged in a linear fashion in the protein. Bacterial ferredoxins, like other non-heme iron electron transfer proteins, are supposed to contain equivalent amounts of iron and inorganic sulfide, but the model proposed by Phillips et al. does not give any idea about the location of the seventh atom of sulfide.

The spectral and chemical properties of plant ferredoxins suggest that the nature of iron and sulfur binding in these proteins may be different from that of bacterial ferredoxins. The absorption spectra of taro and other plant ferredoxins show maxima at 465, 420, 330 and 277 m μ . The spectra of

bacterial ferredoxins show maxima at 390, 300 and 280 mμ. It is not yet definitely known whether the 390 mμ absorption peak of bacterial ferredoxins is due to an increase in the iron content or due to the presence of a different chromophoric group in these proteins. The EPR spectra of taro and other plant ferredoxins in the reduced state are slightly different from the corresponding spectra of bacterial ferredoxins (96). The circular dichroism and optical rotatory dispersion spectra of spinach ferredoxin was found to be qualitatively and quantitatively different from those of bacterial ferredoxins (103). Taro ferredoxin contains two atoms of iron and five cysteine groups (from amino acid analysis) compared to seven atoms of iron and eight cysteine groups per mole of bacterial ferredoxins. DTNB titrations show that a molecule of taro ferredoxin combines with about six equivalents of the reagent in the presence of guanidine hydrochloride (Table VII). The DTNB titre was not altered when EDTA was added to the reaction mixture. However, with C. acidi-urici ferredoxin, it was found that DTNB reacts with all the labile sulfide of the protein in the presence of guanidine hydrochloride, and with the labile sulfide and the cysteinyl groups in the presence of EDTA and guanidine hydrochloride (86). There is thus a difference in the reactivity of DTNB toward the two types of ferredoxins.

Any structure proposed for the iron and sulfur binding in plant ferredoxins should explain the following observations.

- 1) The production of temperature-sensitive EPR signals by the reduced protein with a g value around 1.94. The participation of iron and sulfur in the generation of the signal has been shown by isotopic substitution experiments (114).
- 2) The presence of two atoms of sulfide per mole of ferredoxin which can be liberated by acidification of the protein and which can be estimated as H_2S by Fogo and Popowski method.
- 3) The inaccessibility of the cysteinyl groups of native ferredoxin for reaction with iodoacetate, observed with alfalfa ferredoxin (62), which suggests that the cysteinyl groups are blocked in the native protein.
- 4) The results of mercurial titrations with taro ferredoxin which does not suggest the presence of disulfide groups in the protein.
- 5) The ease with which iron and sulfide are displaced from taro ferredoxin by mercurials.
- 6) Native spinach ferredoxin in neutral solution does not react easily with ferrous or ferric iron chelating agents.

Two structures have been proposed for spinach ferredoxin to explain the electron paramagnetic resonance signal generated by the reduced protein. Gibson et al. (93) consider the resonance signal to arise from high spin ferric ions in an octahedral or tetrahedral field. These authors suggest that the two iron atoms in ferredoxin strongly interact with one another through one or more sulfur ligands. The reduced complex may be of the type,

Fe^{3+} ($d^5, S = 5/2$)-sulfur ligand (s)- Fe^{2+} ($d^6, S = 2$). This structure can explain the 'g' values and the temperature dependence of the EPR signal and also the one electron transfer mechanism of ferredoxin.

Palmer and associates (116) have found from quantitative measurements of the EPR signal of spinach ferredoxin that there is only one unpaired electron associated with the iron atom giving the EPR signal. These authors assume that the signal is produced by low spin ferric ion in a tetrahedral ligand field and propose the following structure for the active site of ferredoxin (96).

According to Palmer et al. (96) mercurials can trap the mercaptide ion and hence produce Fe (III) as in structure I. The iron thus released reacts rapidly and quantitatively with Tiron, a ferric specific chelator. In strong urea solution the iron is accessible to bathophenanthroline sulfonate, a ferrous specific chelator but not to Tiron. In strong urea the Fe (III) of native ferredoxin is converted to Fe (II) as in structure II which reacts quantitatively with the ferrous chelator. During this process, a disulfide bond is formed from two cysteine sulfhydryl groups. The authors detected the presence of a disulfide bond in the product by amperometric analysis. Iodoacetate cannot react with the cysteinyl groups of the native protein since they are in combination with the iron atoms. Carboxymethylation of ferredoxin does not take place even in the presence of urea, since the two cysteinyl groups liberated are immediately oxidized to a disulfide bond. However, when the ferredoxin is incubated for a long time with urea, mercaptoethanol, and EDTA, all the iron atoms are released from the protein, and the free cysteinyl groups are maintained in the reduced state by the mercaptoethanol. Iodoacetate now reacts readily and quantitatively with the cysteinyl groups of the denatured ferredoxin.

According to Palmer and associates, Fe (III) in an environment of four sulfide ligands (structure III) and Fe (I) with one disulfide and two sulfide ligands (structure IV) are formally indistinguishable and both structures are capable

of generating the EPR signals observed with reduced plant ferredoxins. Palmer's group have observed that bis-hexamethylbenzene-Fe (I) exhibits an EPR spectrum similar to the spinach ferredoxin.

The structure proposed by Palmer et al. fails to show specifically the nature and location of the labile sulfide in ferredoxins. A molecule of spinach ferredoxin contains five cysteine residues and two moles of inorganic sulfide. It appears from the model that all the six sulfur atoms shown belong to cysteine residues. The spectra of plant ferredoxins show an absorption maximum at 330 m μ . Villarejo and Westley (117) have noticed such an absorption around 330 m μ in a mixture of cysteine and sodium sulfide which produces an organic persulfide of the type RSSH. Neither cysteine nor S⁻ was found to absorb at wavelengths greater than 300 m μ . Persulfides react with dilute acids liberating hydrogen sulfide. Thus there is a possibility for the presence of a CyS-S-H group in the structure of ferredoxins.

Blumberg and Peisach (118) have pointed out that quantum mechanics does not permit the EPR signals that have been observed in the region $g = 1.9-2.0$ to be ascribed to iron alone in any configuration whatsoever. These authors, however, suggest that the g value of a free radical can depart significantly from 2.0023, the g value of the free electron, when the free radical species has chelated to it an ion with an even number of d electrons in the low spin state. The signal

observed with reduced taro ferredoxin, therefore, may arise by the bonding between Fe^{2+} (with 6 d electrons in the low spin state) and sulfur atoms. It should be mentioned here that though the signal with $g = 4.45$ observed with native taro ferredoxin may be due to the presence of an impurity, the signal of the reduced protein is definitely from the iron sulfide chromophore as seen by comparison with the signals obtained from reduced spinach ferredoxin (96). Proteins which give the signal at $g = 4.3$ do not generate the $g = 1.94$ signal on reduction. The apparent difference in the signals observed with spinach ferredoxin in our work may be due to the temperature dependence of the signals.

Bayer and associates (109) in Germany were able to prepare spinach apoferredoxin by incubating native ferredoxin solution at pH 5.4 with α - α -bipyridyl and separating the products by column chromatography. The apoferredoxin was catalytically inactive and did not possess iron, labile sulfide, or absorbancy in the visible region of the spectrum. Ferredoxin was reconstituted from the apoprotein by treating the apoprotein with mercaptoethanol followed by sodium sulfide and Mohr's salt. The chemical composition, electron transfer activity, and optical and EPR spectra of the reconstituted ferredoxin were the same as those of native ferredoxin. Based on these findings, Bayer et al. (109) have suggested structure I for native ferredoxin and structures II to IV for the reduced protein.

The authors consider reduced spinach ferredoxin existing as a resonance hybrid of structure II to IV. The radical forms II and III can be split off from the protein by treatment with $\text{Na}_2\text{S}_2\text{O}_4$ or bipyridyl. If R_2 is a hydrogen atoms, then the persulfide linkage in the native protein can explain the 330 mu absorption maximum, and the liberation of hydrogen sulfide on acidification of the protein. These properties of the protein cannot be explained if $R_2\text{S}$ is a cysteinyl residue. If R_2 is a hydrogen, then there should be three free cysteine groups in the molecule of the native protein available for reaction with iodoacetate. But experimental evidence shows that iodoacetate does not react with native ferredoxin (62). According to the Bayer formula, iron in native ferredoxin is in the ferrous state. The structure could not explain the quantitative reaction of spinach ferredoxin with Tiron in the presence of CMB. The authors themselves have not given enough experimental evidence to support the structure.

There are many similarities between plant ferredoxins and mammalian xanthine oxidase. The visible absorption spectra, optical rotatory dispersion spectra, and circular dichroism spectra of the two proteins are similar (76). The EPR spectra of plant ferredoxins and xanthine oxidase are similar with temperature sensitive resonance signals at $g = 1.94$ (119). It has been found that the extinction coefficient of FAD and molybdenum free xanthine oxidase at 450 mμ is $5000 \text{ cm}^2 \text{ moles}^{-1}$ per iron (76) which agrees well with the extinction coefficients of plant ferredoxins at this wavelength. Both contain iron and labile sulfide in equivalent amounts. It has been observed that the optical absorption and magnetic resonance of the two proteins are due to similar chromophores situated in similar asymmetric environments (76). Massey (87) and associates have proposed the following structure for the iron sulfide linkage in xanthine oxidase.

The following changes are postulated to occur during the reaction of the protein with dilute acids or mercurials.

This structure can explain the absorption maxima at 330 mμ of taro ferredoxin spectrum, the absorbancy losses occurring on treatment of ferredoxin with urea and also the displacement of iron atoms from the protein by organic mercurials. The structure proposed does not give any idea regarding the ligands of iron other than the sulfide.

It is evident from the above discussion that though all the proposed structures can explain many of the properties of plant ferredoxins, none of them satisfactorily accounts for all the reactions observed with the proteins. More research and development of techniques are necessary for the enunciation of an unambiguous structure for this class of non-heme iron proteins. One possible structure which would explain some of the reactions of the taro protein is:

In this model all the cysteine residues of native ferredoxin are bound either to iron or labile sulfide and hence are not available for reaction with iodoacetate. Both iron atoms in the native protein are in the ferric valence state. One of the two iron atoms is linked to two cysteinyl groups. The second iron atom is linked to a cysteinyl group and possibly to another amino acid in the protein chain. It is difficult to predict which one of these iron atoms undergo reduction during illumination with chloroplasts. There are two persulfide linkages in the model which could account for the absorption of the protein at 330 mμ and the liberation of hydrogen sulfide on acidification. The primary structure of spinach ferredoxin (75) shows the cysteinyl residues located at positions 18, 39, 44, 47 and 76 in the sequence. It is theoretically possible to fold the spinach protein molecule and bring together the cysteinyl residues as shown in the above model.

Determination of amino acid sequence and X-ray structure will show whether the ferredoxin molecule can physically exist in this structure. Also, it is not known whether two iron atoms directly linked can give rise to the EPR signals observed with ferredoxins.

The complete amino acid sequence of C. pasteurianum and C. butyricum ferredoxins (26, 97) and the partial sequence of M. aerogenes ferredoxin (Dr. J. Tsunoda, personal communication) are known. The molecule of the two clostridial ferre-

doxins studied contain 55 amino acid residues each. Both of them have the same amino and carboxyterminal amino acids. Both have eight cyteiny1 residues (to which the iron atoms are believed to be linked) in identical positions of the molecule. Methionine, arginine, histidine and tryptophan are absent in both ferredoxins. Thus, ferredoxins isolated from the same genus have closely related structures. Preliminary investigations by Dr. J. Tsunoda of the amino acid composition of ferredoxin from Peptostreptococcus elsdeni, an organism of a different order, have shown methionine as one of the component amino acids, proving that structural differences can be found in ferredoxins from different orders of organisms.

The amino acid composition of taro, spinach (75) and alfalfa (62) ferredoxins are similar. If we assume that taro ferredoxin molecule contains only 98 amino acids (lower value in Table X), then the number of basic, aromatic and sulfur containing amino acids are identical in spinach and taro ferredoxins except for the amides. The electrophoretic mobilities of the two ferredoxins in acrylamide gel electrophoresis were the same indicating the identity in the net charge of the two proteins. It is likely, therefore, the total number of amides are also the same in taro and spinach ferredoxins. Both contain the same number of proline residues (helix-breaking residues) and almost the same number of α -helix forming amino acids (120); 62 in spinach and 61 in taro ferredoxin. The aminoterminal amino acid of taro ferredoxin, like all

other ferredoxins, is alanine. The carboxyterminal amino acid is also alanine as in spinach and alfalfa ferredoxins (75, 121). The bacterial ferredoxins so far examined have a glutamic acid residue at the carboxy terminal.

In spite of all these similarities between taro and spinach ferredoxins, one should expect differences in the primary structures of these two proteins to explain the differences in the finger print patterns obtained.

According to taxonomists (122, 123), the angiosperms would have originated in the Mesozoic era, of geologic time, roughly 165 million years ago. The monocotyledons and dicotyledons are believed to have evolved from Ranales order, one of the earliest fossil angiosperms, according to the scheme given below (123, 124).

Taro plant (*Colocasia*) belongs to the araceae family of the arales order of monocotyledons whereas spinach (Spinaceae) belongs to the chinopodeaceae family of chenopodiales order

of dicotyledons. The morphological differences between monocotyledons and dicotyledons are well known. From a comparative study of ferredoxins from various monocotyledons and dicotyledons, it could be possible to estimate the time lapse that had occurred between the evolution of each species in these two classes of plants.

The primary sequence of cytochrome c of at least twenty animal species is known. Margoliash and Schegter have discussed in detail (28) the similarities and differences of these cytochromes and their usefulness in the understanding of the evolution of animal species. No such study has been made with plant proteins. Ferredoxins are present in all plants, including algae, examined and seems to be the ideal choice for comparative study of primary structures in order to explore the phylogenetic relationship in plants. We have made a start toward this goal by studying the characteristics of ferredoxin from a monocotyledon. It is our aim to advance toward this goal by studying the characteristics of ferredoxins from plants of lower order like algae and tropical ferns. One of the recent instances of the use of protein sequence data in solving problems of taxonomy is illustrated in the report of the work of Dr. Charles Sibley of Yale University in the classification of birds from the structures of their blood and eggwhite proteins [Chemical and Engineering News, 48, 108 (1968)].

Three types of ferredoxins have been isolated so far from plants and bacteria. Ferredoxins from clostridia and other non-photosynthetic bacteria contain about 55 amino acid residues per molecule, the plant and algal ferredoxins contain about 100 amino acid residues per molecule, while the ferredoxin from photosynthetic bacteria, chromatium, contains 84 amino acid residues per molecule (125). The primary structure of spinach ferredoxin shows many similarities with that C. butyricum ferredoxin and it has been suggested that both types of ferredoxins would have been evolved from a common arche type (75). The primary structure of chromatium ferredoxin is eagerly awaited, as this would indicate whether the photosynthetic bacteria forms a bridge between the non-photosynthetic anerobes and plants. Determination of the amino acid sequence of low molecular weight mammalian proteins like adrenodoxin and testodoxin (126) and high molecular weight mammalian proteins like succinic dehydrogenase and xanthine oxidase would establish the structural relationship between the non-heme iron electron transfer proteins of various species.

APPENDIX

A. Tables. Pages 100 to 114.

B. Figures. Pages 116 to 146.

Table I
Purification of ferredoxin.

Fraction	Total volume, ml.	Total protein*, mg.	Total activity, units	Specific activity, units/mg.	Yield %	Fold Purification
Aqueous homogenate	3240	20740	3400	0.17	100	
DEAE-cellulose extract (batch)	98	725	2058	2.85	60.5	17.2
DEAE-cellulose column eluate, (final).	38	217	1444	6.6	42	40
Ammonium sulfate super- natant	46	105	1150	11.0	34	66
Sephadex G-75 eluate	11.2	32	704	22.0	20.7	134

* Protein determined by Sutherland et al. (41); no correction applied.

Table II
Ratio of Absorbancies of Plant Ferredoxins

Source of Ferredoxin	<u>Wavelength (mu)</u>		
	330-331	420-422	465
Parsley ^a	0.81	0.62	0.57
Brassica ^a	0.81	0.62	0.54
Spinach ^a	0.65	0.48	0.44
Alfalfa ^b	0.65	0.48	0.43
Taro	0.64	0.43	0.39

The values are expressed in terms of the absorbancy of the ferredoxins at 277 mu which is taken as unity. Absorbancies of taro ferredoxin were recorded in a Beckman DB spectrophotometer.

^aData taken from Fry and San Pietro (27).

^bData taken from Keresztes-Nagy and Margoliash (62).

Table IIIMolar Extinction Coefficients of Plant Ferredoxins

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

Source of Ferredoxin	<u>Wavelength(mu)</u>			
	277	330-331	420-422	465
Spinach ^a	20600	13400	9900	8900
Alfalfa ^b	19000	12200	9020	8240
Taro ^c	22500	14400	9700	8800

^aCalculated from the data given in reference 27.

^bCalculated from the data given for PPNR in reference 62.

^cValues calculated from absorbancy measurements and direct iron analyses of the same ferredoxin solution with a 420 to 277 mu absorbancy ratio of 0.43.

Table IV
Absorbancies of Ferredoxin in 8 M Urea

Conditions of incubation	Wavelength (mu)			
	277	330	420	465*
0.1 M phosphate buffer, pH 6.8, 5 mins	0.91	0.58	0.39	0.35
0.1 M phosphate buffer, pH 6.8, 40 hrs (aerobic)	0.90	0.55	0.365	0.33
Percentage of original absorbancy remaining	99	95	94	95
8 M urea, 5 mins, aerobic	0.89	0.61	0.38	0.34
8 M urea, 40 hrs, aerobic	0.88	0.20	0.06	0.05
Percentage of original absorbancy remaining	99	33	16	15
8 M urea, 5 mins, anaerobic	0.93	0.63	0.39	0.35
8 M urea, 40 hrs, anaerobic	0.90	0.345	0.017	0.135
Percentage of original absorbancy remaining	97	55	44	39

*The values with urea-treated ferredoxin were measured at 455 mu.

TABLE VTitration of Ferredoxin with Mercurials

No.	Mercurial Used	A $\frac{420}{277}$ mu of Ferredoxin	Sulfide mole/mole of Fd	Mercurial Reacted mole/mole of Fd
1	CMB			6.6 $\pm$ 0.2
2	CMB*			6.6
3	CMB	0.38	1.8	7.1
4	CMB	0.41		7.6
5	CMB	0.42		7.8
6	CMB	0.43	2.0	8.1
7	Mersalyl	0.33		6.1
8	Mersalyl*	0.33		6.3
9	Mersalyl	0.39	1.8	7.3
10	Mersalyl	0.42		7.7
11	CMB**	0.44	2.1	9.0
12	Mersalyl**	0.43		9.0

Protein concentration of ferredoxin was measured by the phenol color reaction 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 carried out in the presence of urea.

**Titration with spinach ferredoxin.

The values given are average of duplicate determinations.

TABLE VIBound Mercury in Apoferreredoxin

Moles of CMB/mole of Fd (before dialysis)	Moles of Hg/mole of Fd (after dialysis)
4	1.2
5	1.8
6	3.0
8	3.9
10	4.0
10	3.9
10.6	4.1
15.3	3.9

The values given are average of duplicate analyses, uncorrected for experimental losses of protein during dialysis.

Bound mercury was determined by atomic absorption spectrophotometry using mercuric chloride as reference standard. Ferreredoxin concentration determined from the absorbancy of the protein at 420 mu using an extinction coefficient of 9.7 per micromole per milliliter of protein at this wavelength. Ferreredoxin used had a 420 to 277 mu absorbancy ratio of 0.43.

Table VII
Reaction of DTNB with Taro Ferredoxin.

Reaction Conditions			Time for maximum color formation at 412 mμ	Maximum moles of DTNB reduced per mole of Fd	Moles of DTNB reduced per mole of Fd in 15 mins
4 M guanidine hydrochloride	EDTA	Air			
—	—	—	42 hrs	8.5	0.9
—	—	+	7 hrs	5.5	0.6
+	—	—	10 mins	5.6	5.6
+	—	+	5 mins	5.6	5.6
+	+	—	4 mins	5.2	5.2
+	+	+	1 min	3.5	3.5
—	+	—	4 hrs	2.0	0.8
—	+	+	4 hrs	2.0	0.4

Reaction mixtures contained 0.05-0.07 micromole of ferredoxin in 0.05 M Tris-HCl, pH 7.2, 1 micromole of DTNB, and 0.1 m mole of EDTA and 10 m mole of guanidine-HCl where present, in a final volume of 3 ml. Anerobic titrations were carried out as described under methods. In reactions involving EDTA, this reagent was added last. $A_{412 \text{ m}\mu}$ was measured in a Beckman DB Spectrophotometer.

Table VIII
Tryptophan Content of Ferredoxin

Reagent used	Nature of Fd	<u>Moles of Trp</u> <u>Mole of Fd</u>
^a 0.1N NaOH	Native	2.55
	Oxidized	0.9*
	Carboxymethylated	1.8*
	TCA precipitated	2.0*
^b N Bromosuccinimide	Native	1.9
	Oxidized	0.8
^c 6M guanidine hydrochloride	Native	3.9
	Oxidized	0.9

*Calculated from the molar ratio of Tyr to Trp assuming the protein contains 4 tyrosine residues.

^aReference 67

^bReference 68

^cReference 70

Table IX
Amino Acid Composition of Taro Ferredoxin

Amino acid	24 hr. mole/mole	Time of hydrolysis		Mean ^a value mole/mole	Nearest integer
		22 hr. mole/mole	72 hr. mole/mole		
Lysine	4.55	4.10	4.59	4.41	4-5
Histidine	1.10	1.00	1.00	1.03	1
Arginine	1.00	0.98	0.96	0.98	1
Aspartic Acid + Asparagine	9.80	9.86	10.04	9.90	10
Threonine	5.75	5.72	5.20	6.00	6
Serine	7.20	7.32	5.08	8.20	8
Glutamic Acid + Glutamine	16.65	16.00	16.61	16.42	16-17
Proline	4.00	4.48	4.22	4.23	4
Glycine	9.65	9.41	9.62	9.56	9-10
Alanine	6.85	7.28	6.81	6.98	7
^b Half cystine	5.10	5.30	5.06	5.15	5

Table IX (continued)

Amino Acid	<u>Time of hydrolysis</u>			Mean ^a value mole/mole	Nearest integer
	24 hr. mole/mole	22 hr. mole/mole	72 hr. mole/mole		
Valine	9.60	10.31	10.10	10.03	10
Methionine	0	0	0	0	0
Isoleucine	4.10	4.0	4.0	4.03	4
Leucine	6.0	5.85	5.92	5.92	6
Tyrosine	3.40	3.58	3.00	3.85	4
Phenylalanine	1.90	1.97	1.89	1.92	2

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

^aThe values of Threonine, Serine, and Tyrosine were obtained after extrapolation to zero hour hydrolysis the respective values at 22 and 72 hr. hydrolysis.

^bCalculated as carboxymethyl cysteine in 24 hr. hydrolysate and as 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 (4), valine (10) and leucine (6).

Table X
Amino Acid Composition of Taro, Spinach and
Alfalfa Ferredoxin.

Amino Acid	Taro	Spinach ^a	Alfalfa ^b
Lysine	4-5	4	5
Histidine	1	1	2
Arginine	1	1	1
Tryptophan	1	1	1
Aspartic acid + Asparagine	10	13	10
Threonine	6	8	6
Serine	8	7	8
Glutamic acid + Glutamine	16-17	13	17
Proline	4	4	3
Glycine	9-10	6	7-8
Alanine	7	9	10
Half cystine	5	5	6
Valine	10	7	9
Methionine	0	0	0

Table X (continued)

Amino Acid	Taro	Spinach ^a	Alfalfa ^b
Isoleucine	4	4	4
Leucine	6	8	6
Tyrosine	4	4	4
Phenylalanine	2	2	2
	<u>98-101</u>	<u>97</u>	<u>101-102</u>

^aValues of spinach ferredoxin were taken from Matsubara et al. (75).

^bValues of alfalfa ferredoxin were taken from Kerestzes-Nagy and Margoliash (62).

Table XI

Differences in the Amino Acid Composition of Plant Ferredoxins.

Amino Acid	Source of Ferredoxin		
	Taro	Spinach	Alfalfa
Lysine	4-5	4	5
Histidine	1	1	2
Proline	4	4	3
Half cystine	5	5	6
*Aspartic acid+Glutamic acid	26-27	26	27
Glycine+Alanine	16-17	15	17-18
Valine+Leucine	16	15	15
Threonine+Serine	14	15	14

*Asparagine and glutamine values were included in aspartic acid and glutamic acid respectively.

Table XII

Amino Acids Released by Hydrazinolysis of Ferredoxin.

Amino Acid	moles/mole of protein
Alanine	0.36
Glycine	0.25
Leucine	0.17
Serine	0.11
Valine	0.02
Aspartic Acid	0.02
Threonine	0.02

Hydrazinolysis was carried out by heating the protein with 95% hydrazine and hydrazine sulfate for 16 hours at 60°. The values are the average of duplicate determinations (uncorrected for experimental losses).

Table XIII

Amino Acids Liberated by Carboxypeptidase A Digestion of Ferredoxin.

Type of Ferredoxin	Protein to Enzyme ratio	Hydrolysis time	Amino acid.mole/mole.			
			Ala	Thr	Leu	Ser
Native	20:1	Zero hr	0.78	0.23	0.23	0.03
	20:1	One hr	0.83	0.42	0.43	0.07
	20:1	12 hrs	0.89	0.74	0.81	0.09
	20:1	24 hrs	0.97	0.88	0.96	0.19
	50:1	15 mins	0.31	0.08	0.08	
	50:1	30 mins	0.52	0.16	0.11	
Carboxy-methylated	20:1	one hr	0.77	0.70	0.68	0.14
	20:1	12 hrs	0.84	0.81	0.90	0.29
	20:1	24 hrs	0.85	0.84	0.91	0.35
Oxidized	20:1	24 hrs	0.94	0.56	0.54	0.27

DFP-carboxypeptidase and ferredoxin were incubated at 40°, for the times indicated and then the reaction terminated by adding acetic acid. Amino acid analysis performed in a Beckman Spinco Analyzer.

Fig. 1. Taro ferredoxin-mediated photoreduction
of NADP.

The reaction mixture contained, in a final volume of 3.0 ml, 0.50 micromole of NADP, 0.15 millimole of Tris-HCl buffer, chloroplast suspension containing 60 microgram of chlorophyll, and amounts of ferredoxins as indicated. The blank cuvettes contained the same components except ferredoxin. Illumination time was 5 mins.

(-●-●-●-) Pure ferredoxin, $A_{420 \text{ to } 277 \text{ mu}} = 0.43$.

(-○-○-○-) Aged ferredoxin, $A_{420 \text{ to } 277 \text{ mu}} = 0.38$.

(-■-■-■-) Ammonium sulfate supernatant obtained during the isolation of ferredoxin.

(-▲-▲-▲-) DEAE-cellulose column eluate, with 0.3 M Cl^- buffer, during the isolation of ferredoxin.

The protein concentration was determined by the method of Sutherland et al. (41) and corrected for excess of color as described under "Results".

Fig. 1

Fig. 2. Absorption spectra of pure ferredoxin.

Spectrum of a solution of ferredoxin (ca 0.1 micromole) in 3 ml of Tris-HCl buffer, pH 7.3, was recorded against a blank containing the same buffer in a Cary 14 Spectrophotometer.

Broken line ---- Spinach ferredoxin; Solid line _____ taro ferredoxin.

Fig. 3. Absorption spectra of 'cuts' obtained during the purification of ferredoxin.

The spectra of the protein solutions in Tris-HCl buffer were recorded against a blank containing the same buffer in a Cary 14 Spectrophotometer.

Curve A --- Spectrum of 0.3 M Cl^- eluate from DEAE-cellulose column.

Curve B --- Spectrum of 0.1 M Tris-HCl eluate before ammonium sulfate precipitation.

Curve C --- Spectrum of supernatant after ammonium sulfate precipitation.

For details about the 'cuts', see "Methods".

Fig. 3

Fig. 2

Fig. 4. Starch gel electrophoresis of taro ferredoxin.

Electrophoresis was carried out in pH 8.0 buffer, for 3 hrs. at 0° and 400-500 volts. Protein bands were detected by staining with nigrosine black. The anodic end of the gel is at the top of the photograph.

- a. Protein band obtained with freshly prepared ferredoxin

$$(A_{420 \text{ to } 277 \text{ m}\mu} = 0.43).$$

- b. Protein bands obtained with an aged preparation of ferredoxin

$$(A_{420 \text{ to } 277 \text{ m}\mu} = 0.38).$$

Fig. 4b

Fig. 4a

Fig. 5. Disc electrophoresis of ferredoxins
in polyacrylamide gels.

- a. Protein bands obtained by electrophoresis of taro (T) and spinach (S) ferredoxins in 7.5% acrylamide gel at room temperature. Gels were stained with amido black.
- b. Protein bands obtained by electrophoresis of spinach (S) and taro (T) ferredoxins in 30% acrylamide gel at room temperature.

For details of electrophoresis, see "Methods".

Fig. 5b

Fig. 5a

Fig. 6. Gel filtration of proteins in Sephadex G-100.

Proteins (ca 5 mg each) dissolved in 1 ml of 0.2 M Tris HCl buffer, pH 7.5, were layered on top of a sephadex G-100 column, at 4°, and were eluted with the same Tris buffer. The elution volume (V) of each protein was determined by measuring the absorbancy of the effluent fractions at 280 mu. The void volume (V_0) was determined by using Blue Dextran 2000. Molecular weight values are plotted on a logarithmic scale.

Fig. 7. Sedimentation analysis of proteins
in sucrose gradient.

Proteins (ca 2.5 mg each) were dissolved in 0.5 ml of 0.1 M phosphate buffer, pH 6.8, and layered on top of 5-20% sucrose gradients. After 64 hr. centrifugation at 41,000 r.p.m. 4°, the gradients were fractionated and protein concentration determined. The proteins used were: Ferredoxin (-●-●-●-), Cytochrome c (-x-x-x-) and Trypsin (-▲-▲-▲-).

Fig. 6

Fig. 7

Fig. 8. Absorption spectra of dithionite-ferredoxin mixtures.

Sodium dithionite in 100 M excess was added to a solution of ferredoxin (ca 1.5 mg) in Tris-HCl buffer maintained aerobically and anaerobically. The spectra were recorded immediately after mixing the solution, and after 24 hr. storage at 4°, against a Tris buffer blank.

Curve A --- Spectrum of Fd-dithionite immediately after mixing in air.

Curve B --- Spectrum of Fd-dithionite immediately after mixing anaerobically.

Curve C --- Spectrum of aerobically maintained mixture after 24 hr.

Curve D --- Spectrum of anaerobically maintained mixture after 24 hr.

Fig. 9. Absorption spectra of dithionite-treated ferredoxin.

Sodium dithionite in 500 M excess was added to a solution of taro ferredoxin (0.5 mg per ml of Tris-HCl buffer, pH 7.3) and the mixture kept at room temperature for one hr. Excess of dithionite was then removed by extensive dialysis against water.

Curve A --- Spectrum of pure ferredoxin.

Curve B --- Spectrum of dithionite treated ferredoxin.

Fig. 9

Fig. 8

Fig. 10. Absorption spectra of Urea-Fd mixtures.

Taro ferredoxin (ca 1.5 mg) was dissolved in 3 ml of 8 M urea. The spectrum of the solution was recorded 5 mins. after mixing, and after 40 hr. incubation at 4° against an 8 M urea blank.

a. Curve A -- Spectrum of ferredoxin in 8 M urea, 5 mins. after mixing in air.

Curve B -- Spectrum of the above solution after 40 hr. aerobic incubation.

b. Curve A -- Spectrum of ferredoxin in 8 M urea (anaerobic) 5 mins. after mixing.

Curve B -- Spectrum of the above solution after 40 hr. anaerobic incubation.

Fig. 10b

Fig. 10a

Fig. 10. Spectra of Urea-Fd mixtures (continued)

c. Curve A -- Spectrum of ferredoxin solution in phosphate buffer recorded against a phosphate buffer blank.

Curve B -- Spectrum of the above solution after 40 hr. aerobic incubation.

Fig. 11. Comparison of phosphoroclastic activity of taro and C. pasteurianum ferredoxins.

Each reaction mixture contains, in a total volume of 1 ml, 100 micromoles of pyruvate, 0.1 micromole of Coenzyme A, 25 micromoles of phosphate, 8 mg of lyophilized ferredoxin-free clastic extract, and amounts of ferredoxins as indicated. The acetyl phosphate formed was estimated as acetyl hydroxamate. For details of assay see "Methods".

_____ • _____	Taro ferredoxin.
----- ■ -----	<u>C. pasteurianum</u> ferredoxin.

Fig. 11

Fig. 10c

Fig. 12. Titration of ferredoxin with CMB.

CMB solution was added in aliquots to a solution of ferredoxin in 0.05 M phosphate buffer, pH 6.5, and after 20 min. incubation at 25° the absorbancy of the reaction mixture was measured against a buffer blank. Absorbancy values have been corrected for the absorption due to ferredoxin and CMB and also for dilution effects. Protein concentration were determined by the phenol color reaction.

-■-■-■-, Titration against sodium sulfide, 0.16 micromole.

-x-x-x-, Titration against spinach ferredoxin, 0.064 micromole, $A_{420 \text{ to } 277 \text{ mu}} = 0.44$.

-●-●-●-, Titration against taro ferredoxin, 0.039 micromole, $A_{420 \text{ to } 277 \text{ mu}} = 0.43$.

Fig. 13. Titration of ferredoxin against mersalyl.

Mersalyl solution was added in aliquots to anaerobically maintained ferredoxin and dye mixture and the absorbancy was measured after 20 min. incubation at 25°. Absorbancy values have been corrected for dilution effects. Protein concentrations were determined by the phenol color reaction.

-x-x-x-, Titration against reduced glutathione, 0.1 micromole.

-●-●-●-, Titration against spinach ferredoxin ($A_{420 \text{ to } 277 \text{ mu}} = 0.43$), 0.069 micromole.

-■-■-■-, Titration against taro ferredoxin ($A_{420 \text{ to } 277 \text{ mu}} = 0.42$), 0.039 micromole.

Fig. 12

Fig. 13

Fig. 14. Effect of CMB on the absorbancy of taro ferredoxin.

Aliquots of standard CMB solution were added to a solution of ferredoxin (1.2 mg) in phosphate buffer, pH 6.5. After 20 min. incubation at 25° the absorbancy of the reaction mixture was recorded at 255, 280, 330, 420 and 465 mu, against a phosphate buffer blank, in a Cary 14 Spectrophotometer. The values given have been corrected for dilution effect and for the absorbancy of CMB at the respective wavelengths. Calculations from the absorbancy increase at 255 mu (not shown in the graph) showed that 7.1 moles of CMB reacted with a mole of ferredoxin. The ferredoxin used had a 420 to 277 mu absorbancy ratio of 0.38.

Fig. 15. Titration of taro ferredoxin against DTNB.

Ferredoxin solution was incubated with excess of DTNB and other reagents, when present, and the absorbancy of the reaction mixture was measured at 412 mu at various time intervals. Appropriate corrections were made for the absorbancy due to ferredoxin and the reagents wherever necessary. The ferredoxin used had a 420 to 277 mu absorbancy ratio of 0.40. For details of titration see "Methods".

-o-o-o-, Titration with native ferredoxin.

-●-●-●-, Titration with reduced glutathione.

-■-■-■-, Titration with sodium sulfide.

-x-x-x-, Titration with ferredoxin in 4 M guanidine hydrochloride.

Fig. 14

Fig. 15

Fig. 16. Absorption spectra of ferredoxin
in alkali.

Native and oxidized taro ferredoxins were dissolved in 0.1 N NaOH and the spectra of the solutions were recorded in the U. V. and near U. V. regions.

Curve A -- Spectrum of native ferredoxin in phosphate buffer, pH 6.8, recorded against a phosphate buffer blank.

Curve B -- Spectrum of oxidized ferredoxin in phosphate buffer recorded against the phosphate buffer blank.

Curve C -- Spectrum of native ferredoxin in alkali recorded against the alkali blank.

Curve D -- Spectrum of oxidized ferredoxin in alkali recorded against an alkali blank.

Fig. 16

Fig. 17. Thin layer chromatography of DNP-amino acids.

The ether soluble DNP-ferredoxin hydrolysate was spotted on a thin layer of silica gel and developed in a chloroform-benzyl alcohol-acetic acid solvent system. The standard DNP amino acids used were aspartic acid (1), serine (2), glutamic acid (3), glycine (4), alanine (5), lysine (6), tyrosine (7), tryptophan (8), valine (9), leucine (10), isoleucine (11), and phenylalanine (12). The dashed spot with a high Rf value originating from the sample is due to dinitroalanine.

Fig. 18. Thin layer chromatogram of dansyl amino acids on silica gel G.

The dansyl derivative taro ferredoxin was hydrolysed with 6 N HCl. The hydrolysate was spotted on a thin layer of silica gel along with the dansyl derivatives of aspartic acid (2), glycine (3), alanine (4), tyrosine (5), and lysine (6). Spot no. 1 is due to dansyl hydroxide and no. 7 due to dansyl amine. The solvent system used was chloroform-ethyl acetate-methanol-acetic acid. The spot without a number in the chromatogram is due to dansyl alanine originating from the sample. The chromatogram was sprayed with Pauli reagent. Spot no. 5 alone changed to orange in color.

Fig. 17

Fig. 18

Fig. 19. Paper chromatography of carboxypeptidase
A digest of taro ferredoxin.

Native ferredoxin after 24 hr. digestion with carboxypeptidase A was spotted on a Whatman no. 1 paper together with standard amino acids. Descending chromatography carried out 12 hrs. using the upper phase of a butanol-acetic-acid-water (4:1:5 V/V) solvent system. The amino acids were detected by spraying the paper with a solution of ninhydrin in acetone.

Fig. 19

Fig. 20. Separation of peptides formed by the
action of chymotrypsin on ferredoxins.

S-carboxymethyl derivatives of spinach and taro ferredoxins were hydrolyzed with TLCK chymotrypsin for 8 hrs. High voltage paper electrophoresis of the digests in pyridine-acetic acid-water buffer, pH 6.4, was carried out in the first direction for 2 hrs. and descending chromatography in butanol-pyridine-acetic acid-water in the second direction for 12 hrs. The peptide spots were detected by spraying the paper with ninhydrin solution.

- a. Chromatogram obtained from taro ferredoxin digest. The shaded area represents tryptophan containing peptide.
- b. Chromatogram from spinach ferredoxin digest. For details see "Methods".

Fig. 20b

Fig. 20a

Fig. 21. Finger prints of ferredoxin after digestion with chymotrypsin.

S-carboxymethyl derivatives of spinach and taro ferredoxins were hydrolyzed with TLCK chymotrypsin. The peptides were separated by chromatography, on a Whatman no. 3 paper, in a butanol-acetic acid-water for 12 hrs. in the first direction and in butanol-pyridine-water for 14 hrs. in the second direction. Dashed boundaries represent faint ninhydrin positive spots. Shaded area represents tryptophan containing peptide detected by Ehrlich reagent.

- a. Peptide map of taro ferredoxin hydrolysate.
- b. Peptide map of spinach ferredoxin hydrolysate.

Fig. 21b

Fig. 21a

Fig. 22. EPR spectra of taro ferredoxin.

The spectrum was recorded in a Varian V 4500-10A EPR spectrometer with 100 kc per sec. field modulation. Microwave frequency, 9.5 k Mc; microwave power, 10 db; response, 0.3 sec; sweep rate, 1 min; sweep range, 1 kg; temperature, - 195°. Protein concentration was 15 mg/ml.

- a. Spectrum of native ferredoxin (A_{420} to 277 μ = 0.42).
- b. Spectrum of ferredoxin after reduction with dithionite.

Fig. 22a

Fig. 22b

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