ISOLATION AND PARTIAL CHARACTERIZATION OF
CORN FERREDOXIN

by
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STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTOR

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ACKNOWLEDGMENTS

I would like to express my appreciation to Drs. R. G. Jensen and M. A. Cusanovich for their assistance in this research.

This thesis is dedicated to my wife.
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ABSTRACT

A procedure has been developed for the isolation of corn ferredoxin, which is also applicable to the isolation of spinach ferredoxin. The molecular weight as determined by sedimentation equilibrium centrifugation is 11,000 \( \pm \) 500, with each molecule containing 2 non-heme iron atoms. The amino acid composition is: lysine\(_3\), histidine\(_2\), arginine\(_1\), aspartic acid\(_{13-14}\), threonine\(_6\), serine\(_{8-9}\), glutamic acid\(_{16}\), proline\(_4\), glycine\(_{8-9}\), alanine\(_{8-9}\), cysteine\(_4\), valine\(_{9-10}\), methionine\(_0\), isoleucine\(_5\), leucine\(_8\), tyrosine\(_{4-5}\), and phenylalanine\(_1\).

The spectrum of purified corn ferredoxin shows maxima at 277, 330, 423, and 463 m\(\mu\) with molar extinction coefficients respectively of 21.2 \( \times \) 10\(^3\), 13.3 \( \times \) 10\(^3\), 10.0 \( \times \) 10\(^3\), and 8.9 \( \times \) 10\(^3\). The ratio of absorbance at 330, 423, and 463 m\(\mu\) to that at 277 m\(\mu\) are .65, .48, and .43 respectively.

The protein denatures rapidly at room temperature in water and slowly in buffered solutions with a concomitant decrease in the absorbance ratios. The protein can be kept indefinitely when deaerated and stored frozen under nitrogen.
INTRODUCTION

Ferredoxin has been isolated from higher plants (1), algae (2), photosynthetic bacteria (3), and non-photosynthetic bacteria (4). In photosynthetic systems it is found to be an electron carrier which is reduced by electrons from the photosynthetic apparatus of the chloroplasts and oxidized by ferredoxin-NADP reductase (5) which in turn reduces NADP.¹

The ferredoxins have been divided into two classes, depending on their source, as bacterial or plant ferredoxin. The bacterial ferredoxins have been extensively characterized but the difficulties of purifying plant ferredoxins have inhibited their study.

Corn ferredoxin has now been isolated with sufficient purity to be able to compare its properties with those of the other plant and bacterial ferredoxins, and the isolation procedure presented may be applicable for the isolation of other plant ferredoxins.

¹ The following abbreviations will be used in this paper: Aₜ, absorbance; the subscript represents the wavelength in nm; NADP, NADPH, nicotinamide adenine dinucleotide phosphate and its reduced form; ATP, adenosine triphosphate; Fdₒₓ, Fd_red, oxidized and reduced form of ferredoxin; Tris, tris-(hydroxymethyl)aminomethane.
LITERATURE REVIEW

In 1939, Hill (6) reported the photolysis of water by chloroplasts using ferric oxalate as the electron acceptor thus showing that photosynthesis had the ability to provide reducing power. It wasn't until 1951, when Vishniac and Ochoa (7) were able to show that pyridine nucleotides could be reduced, that a physiologically important compound was found to be reduced by photosynthesis. The formation of reduced pyridine nucleotides was only indirectly measurable since it had to be coupled with a suitable dehydrogenase system and then by measuring the product of the coupled system, the presence of reduced pyridine nucleotides were inferred.

San Pietro and Lang (8) in 1956, found that by adding back a soluble extract from washed chloroplasts to a chloroplast suspension, the direct photoreduction of pyridine nucleotide was observable without the need for a coupling system. During the next few years several other compounds; triphosphopyridine nucleotide reducing factor (9), methaemoglobin reducing factor (10, 11), as well as the protein photosynthetic pyridine nucleotide reductase, or PPNR, found by San Pietro and Lang, were studied independently.
In 1962, Mortenson, Valentine, and Carnahan (12) isolated an electron carrier they called ferredoxin from the non-photosynthetic, hydrogen-evolving bacterium _Clostridium pasteurianum_.

The correlation between bacterial ferredoxin and the compounds found in plants then being studied was shown by Tagawa and Arnon (13). Their experiments showed that PPNR, triphosphopyridine nucleotide reducing factor and methaemoglobin reducing factor to be the same compound and that it acted as an electron carrier in the reduction of NADP. The bacterial ferredoxin could substitute in the photosynthetic reduction of NADP and the plant protein could serve as the electron carrier in the non-photosynthetic bacterial hydrogen evolution, so they classified the proteins as bacterial or plant ferredoxins.

The reduction of NADP was also dependent upon a flavoprotein, later isolated and termed ferredoxin-NADP reductase (14) which mediated the transfer of electrons from ferredoxin to NADP. Ferredoxin was shown to be reduced in the light, then reoxidized in a "dark" reaction by ferredoxin-NADP reductase, which in turn reduced NADP (5). The location of ferredoxin in the two photochemical system proposed by Duysens and Amesz (15) is shown in Figure 1.

Ferredoxin has been found in all photosynthetic cells studied to date, but only in certain
Figure 1. Proposed two light system for photosynthesis.
non-photosynthetic bacteria. The plant and bacterial ferredoxins have characteristics which are common to their class.

Oxidized plant ferredoxin has distinct absorption peaks at about 280, 330, 430, and 460 mµ (1, 16, 17, 18) while bacterial ferredoxins have a peak at about 280 mµ, generally a shoulder at 300 mµ, and a broad peak at 390 mµ (3, 4, 16, 19). All of the plant ferredoxins have a molecular weight of around 11,000, contain 2 non-heme iron atoms and 2 labile sulfur atoms per molecule (1, 17, 18, 20). The bacterial ferredoxins are generally lower in molecular weight with a range of from 6,000 to 10,000, and have between 5 and 7 non-heme iron atoms per molecule with the number of labile sulfur atoms equal to the number of iron atoms (3, 4, 16, 19). The redox potentials for the two types of ferredoxins are similar and near the reduction potential of the hydrogen electrode (21).

The amino acid composition of plant (17, 18, 20, 22), green algae (2), photosynthetic bacteria (3), and non-photosynthetic bacteria (4, 19) ferredoxins have similarities with methionine absent in all bacterial ferredoxins thus far studied and present only in cotton (18) of the plant ferredoxins isolated. The aliphatic and acidic residues predominate with low content of basic and aromatic amino acids.
The primary photosynthetic function of ferredoxin in plants appears to be as the acceptor of electrons from a recently isolated compound, ferredoxin reducing substance (FRS) which is now believed to be the primary acceptor of electrons from photosystem I (23). The ferredoxin reduces ferredoxin-NADP reductase which in turn reduces NADP, but several other functions for reduced ferredoxin have been indicated (24).

Arnon, Tsujimoto, and McSwain (25) have linked cyclic ATP formation with reduced ferredoxin and Buchanan, Kalberer, and Arnon (26, 27) claim that fructose diphosphatase is activated by reduced ferredoxin.

Two primary carboxylation reactions have been found which directly utilize the reducing power of ferredoxin for the biosynthesis of α-ketocarboxylic acids (28, 29). The α-ketoglutarate synthase reaction:

\[
\text{Succinyl-CoA} + \text{CO}_2 + \text{Fd}_{\text{red}} \rightarrow \alpha\text{ketoglutarate} + \text{CoA} + \text{Fd}_{\text{ox}}
\]

has only been found in the photosynthetic bacteria *Chlorobium thiosulfatium* and *Rhodospirillum rubrum* (29, 30).

The synthesis of pyruvate from acetyl CoA and CO₂;

\[
\text{Acetyl-CoA} + \text{CO}_2 + \text{Fd}_{\text{red}} \rightarrow \text{Pyruvate} + \text{CoA} + \text{Fd}_{\text{ox}}
\]

has been found in certain non-photosynthetic as well as
photosynthetic bacteria (24, 28, 30). Thus there are indications that the reducing power of ferredoxin can be utilized directly in some systems as well as being an electron carrier in the photo-reduction of pyridine nucleotide.
MATERIALS AND METHODS

Chemicals

Tris and NADP were purchased from Sigma Chemical Co., St. Louis, Missouri. DEAE 23 (fines reduced) was obtained from Reeve Angel, Clifton, New Jersey. Sephadex G-75 and G-25 were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey. All other chemicals were obtained from commercially available sources, and were of reagent grade.

Growth of Corn

Zea mays (Hybrid Sweet Corn Golden Cross Bantam T-51) was grown in a greenhouse in 50 x 40 x 12 cm containers in which holes had been drilled in the bottom. The seeds were planted in vermiculite, fed daily with about 1 liter of Hoaglands solution (31) and the leaves harvested 20-35 days after planting.

Isolation of Ferredoxin

The following procedure is a modification of the isolation techniques described by Mortenson (19), and Keresztes-Nagy and Margoliash (1). All steps were carried out in a cold room (4°) and the solutions contained 0.001 M mercaptoethanol and were buffered at pH 7.3 except where noted.
About 200 g of leaves were cut up with scissors then ground 4 min at high speed in a 1 quart Waring Blender in 400 ml 50% aqueous acetone which contained 2.1 g solid Tris per liter. This amount of Tris was needed to maintain a pH greater than 7 during the blending procedure. The acetone:Tris solution was cooled to -15° before adding to the blender. A total of about 400 g of leaves were ground and the homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged 10 min at 20,000 X g in a Sorvall Superspeed RC-2 B refrigerated centrifuge (-15°) in a Sorvall GSA rotor.

The supernatant was passed through a 4 X 4 cm bed of DEAE, equilibrated with 50% acetone:0.002 M Tris-HCl buffer, contained in a 60 ml sintered glass funnel. The column was washed with 0.01 M Tris-HCl buffer until the effluent was clear. The bound protein was eluted from the column with 0.1 M Tris-HCl buffer containing 0.8 M NaCl. The eluent was diluted 1:4 with 0.01 M Tris-HCl buffer then absorbed on a 2.5 X 45 cm DEAE column equilibrated with 0.1 M Tris-HCl buffer containing 0.2 M NaCl. This column was washed with about 1.5 liters of the equilibrating buffer until a diffuse red band separated from the dark material which was retained at the top of the column. The red band was then eluted with 0.1 M Tris-HCl buffer containing 0.3 M NaCl.
The tubes containing the red material were diluted 1:3 with 0.01 M Tris-HCl buffer and concentrated on a 1 X 3 cm DEAE column equilibrated with 0.01 M Tris-HCl buffer. The protein was slowly eluted with 0.1 M Tris-HCl buffer containing 0.8 M NaCl. About 1.5 ml of concentrated solution was collected and placed on a 1.5 X 100 cm Sephadex G-75 column. The ferredoxin was eluted with 0.01 M Tris-HCl buffer containing 0.2 M NaCl.

Ferredoxin separated from a slower moving fluorescent band and was then concentrated on a 1 X 3 cm DEAE column. This procedure yielded about 15 mg of ferredoxin with ratios of the optical densities at 330, 423, and 463 m\(\mu\) to that at 277 m\(\mu\) of 0.65, 0.48, and 0.43 respectively.

**Isolation of Spinach Ferredoxin**

Spinach ferredoxin was isolated by the same procedure with the following modifications. Chopped leaves were ground 2 min at high speed in 50% aqueous acetone containing 1 g solid Tris per liter. The ratio of liquid to leaves was lowered to 250 ml acetone solution per 200 g of leaves. The homogenate was then centrifuged 30 min at 20,000 X g without previous filtering through cheesecloth. The procedure from this point is the same as the corn ferredoxin isolation with approximately the same yield. The absorbance ratios of the optical densities at 330, 423,
and 463 to that at 277 mµ are .60, .48, and .43 respectively.

**Iron Determination**

Iron was determined by the method of Cameron (32). A protein sample was introduced into a 10 ml volumetric flask and 0.1 ml 70% perchloric acid and 0.1 ml 30% hydrogen peroxide added. The sample was digested 30 min at 100° then allowed to cool. Iron was reduced to the ferrous state with 0.1 ml 10% aqueous hydroxylamine. After 5 min 1 ml 0.5% 1-10 phenanthroline solution in 50% ethanol: pyridine was added immediately followed by 1 ml pyridine. The optical density was read at 509 mµ blanked against a sample containing all but the protein. A value of ε509

A value of ε

**Amino Acid Composition**

Ferredoxin samples were desalted on a 1.5 X 15 cm Sephadex G-25 column and lyophilized in 1 X 12 cm pyrex tubes having a constricted neck about 2 cm from the top of the tube. To the lyophilized material, 1 ml of constant boiling, glass distilled 6 N HCl was added and the samples freeze-thawed under reduced pressure to remove the oxygen. The deoxygenated frozen samples were then sealed while under vacuum and hydrolyzed for 24 and 48 hours at 110°. After hydrolysis the samples were again lyophilized and the
hydrolysate suspended in citrate buffer pH 2.2. The amino acid composition was determined on a Beckman 121 Automatic Amino Acid Analyzer. Three samples of Beckman Amino Acid Calibration Mixture (Type 1) were run as standards.

Sedimentation Equilibrium Centrifugation

Sedimentation equilibrium runs were made in a Beckman Model E Analytical Ultracentrifuge using the schlieren optical system. The centrifugation was carried out in a Yphantis cell (34) to which 0.01 ml of fluorocarbon was added to each sector. Added to the sample sectors was 0.11 ml of a solution of 0.01 M Tris-HCl buffer containing 0.2 M NaCl and \( \frac{1}{2}, 2, \) and 1 mg ferredoxin per ml and 0.12 ml of 0.01 M Tris-HCl buffer containing 0.2 M NaCl was added to the reference sectors. The runs were made at 4° and at 20,000 rpm. Pictures were taken at 24 and 28 hours using red-sensitive Eastman Kodak 1N plates with a red-sensitive (Wratten No. 23-A) filter placed before the light source (1), exposing the plates for 20 seconds. The patterns were read on a Nikon Profile Projector Model 6C. Calculations were made by the differential logarithmic method (35).

Isolation of Chloroplasts

All steps were carried out at about 0° in an ice bucket. Twenty g of corn leaves were cut into small
pieces then placed in a vacuum infiltrator, illustrated in Figure 2, and covered with a buffer containing 0.025 M Tris HCl pH 7.5, 0.5 M sorbitol, 0.005 M MgCl$_2$, and 0.005 M mercaptoethanol. This buffer will hereafter be referred to as sorbitol buffer. The infiltrator was attached to an aspirator and a nitrogen line under about 5 psi pressure. The infiltrator was alternately evacuated and pressurized forcing the buffer into the leaves until they turned dark green. The buffer was pressed from the leaves and they were chopped with a razor blade on a plastic block. The chopped leaves were placed in a semi-micro monel metal container fitted with an ice jacket and 30 ml sorbitol buffer added. The solution was ground 30 seconds at high speed then filtered through one layer on nylon gauze and one layer of nylon cloth.

This solution was filtered through nylon monofilament screen cloth of 25 micron mesh opening. The filtrate was centrifuged 5 min at 2000 X G in an International Portable Refrigerated Centrifuge Model PR-2 ($^\text{o}$). The supernatant was decanted and the pellet resuspended in 1 ml of sorbitol buffer. This technique provided a chloroplast suspension containing about 0.1 mg chlorophyll per 0.025 ml when assayed by the method of Vernon (36).

Spinach chloroplasts were isolated by the same procedure with the following modifications. Deveined chopped leaves were not vacuum infiltrated, they were
Figure 2. Vacuum infiltrator.
ground only 10 seconds in the blender, and the centrifuged pellet was resuspended in 1.5 ml sorbitol buffer. The yield of chlorophyll was about the same as the corn chloroplast isolation.

**Assay of Ferredoxin Activity**

To 6 ml of sorbitol buffer, a chloroplast suspension containing 0.2 mg chlorophyll, and 0.2 mg ferredoxin were added. Aliquots of 3 ml were placed in two quartz cuvets and the cells placed in a Hitachi Perkin Elmer 139 spectrophotometer. To the sample, 0.02 ml of 0.1 M NADP was added and the A_{440} recorded. The sample cell was placed in a water bath at 20°, 20 cm from the plexiglass side of the bath and illuminated with 4000 ft. candles from an Argus 500 projector with a 500 watt bulb. The samples were removed at three minute intervals and the A_{440} measured against the blank solution which contained all but the NADP.
RESULTS

Absorption Spectra

Corn ferredoxin has the typical plant ferredoxin spectra as shown in Figure 3, with absorption maxima at 277, 330, 423, and 463 μm. The molar extinction coefficients were calculated on the basis of amino acid composition and dry weight and found to be 21.2 X 10^3, 13.3 X 10^3, 10.0 X 10^3, and 8.9 X 10^3 at 277, 330, 423, and 463 μm respectively. The protein was found to denature rapidly in deionized water at room temperature with the intensity of the peaks at 330, 423, and 463 μm diminishing, while the 277 μm peak increases slightly as shown in Figure 4. The denaturation was found to occur even when the sample was frozen in 0.05 M Tris-HCl buffer which contained 0.15 M NaCl. The protein could be kept indefinitely when deaerated and frozen under an atmosphere of nitrogen.

Amino Acid and Chemical Composition

The values obtained for the amino acid composition are shown in Table 1, with the amino acid composition of alfalfa and spinach included for comparison. The tryptophan was assumed to be the same as in spinach and alfalfa ferredoxin since the absorbance ratios of the peaks in the corn ferredoxin spectrum were the same as the other plant
Figure 3. Absorption spectra of purified corn ferredoxin.
Figure 4. Denaturation of corn ferredoxin in deionized water at room temperature at various times — Curve A: zero time; Curve B: 5 hours; Curve C: 9 hours.
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<sup>a</sup>Values based on Isoleucine equal to 5 with 2 samples averaged for 24 hour hydrolysate and 3 samples averaged for 48 hour hydrolysate.

<sup>b</sup>Values for spinach ferredoxin were taken from Matsubara, Sasaki, and Chain (20).

<sup>c</sup>Values for alfalfa ferredoxin were taken from Keresztes-Nagy, Perini, and Margoliash (22).

<sup>d</sup>Estimated from absorbance ratios and comparison with other plant ferredoxins.
ferredoxins, and a different number of tryptophan residues could be expected to alter these ratios.

The iron determination yielded values of 0.88%, 1.12%, and 0.84%, on three separate samples, which corresponds to 2 iron atoms per 11,500 grams of ferredoxin. Since the labile sulfur is believed to be coordinated with the iron atoms (37) with a ratio of 1 labile sulfur per 1 iron atom, corn ferredoxin is also assumed to have 2 labile sulfur atoms; however, this was not independently determined.

**Electron Transfer Activity**

The ability of corn ferredoxin to facilitate the reduction of NADP in the presence of chloroplasts is shown in Figure 5. The rate of NADPH formation as determined from the initial slope of the curves shows approximately 100 μM NADP reduced per hour per milligram of chlorophyll using spinach chloroplasts, and 40 μM NADP reduced per hour per milligram of chlorophyll with the corn chloroplasts.

Corn and spinach ferredoxin were found to be interchangeable when either corn or spinach chloroplasts were used although the rate of photoreduction was higher with the spinach chloroplasts than with the corn chloroplasts. It was found that the results were not affected by either illuminating both sample and reference cuvet when NADP was
Figure 5. Reduction of NADP by chloroplasts -- See Materials and Methods for reaction mixture. 
\( \Delta \): corn chloroplasts, corn ferredoxin; \( \circ \): corn chloroplasts, spinach ferredoxin; \( \triangle \): spinach chloroplasts, corn ferredoxin; \( \bullet \): spinach chloroplasts, spinach ferredoxin.
omitted from the reference cuvet or by adding NADP to both samples and keeping the reference cuvet in the dark.

**Sedimentation Equilibrium Centrifugation**

Two sedimentation equilibrium runs were made on ferredoxin from separate isolations, with identical results. Due to the lability of ferredoxin, the centrifugation was carried out at 4° and no indication of polymerization was observed. A typical plot of In y/r vs. r^2 is shown in Figure 6. The partial specific volume of .706 was calculated from the amino acid composition and the specific volume of each amino acid (38). The values obtained for the molecular weight were 11,000 ± 500 for the two runs, which compares with the high and low values of 11,675 and 11,082 calculated from the amino acid composition.
Figure 6. Sedimentation equilibrium plot -- See Materials and Methods for conditions of centrifugation.
DISCUSSION

Due to contaminants, it was found that the isolation of corn ferredoxin could not be accomplished by the methods used for spinach (13) or alfalfa (1). If corn leaves were ground in water or buffer and then chromatographed on DEAE, the impurities were carried through the procedure and could not be removed by further chromatography or by precipitation. If, however, the leaves were ground in 50% acetone solution, in which 99% of bacterial cell material is insoluble (19), the purification could rapidly be accomplished on DEAE and sephadex G-75 columns. Since it appears that the use of 50% acetone solution with plant material also removes many impurities, this rapid method may also find use with other forms of plant ferredoxin.

The solid Tris was needed in the acetone solution to keep the homogenate above pH 7, below which the ferredoxin loses labile sulfur (39). It was also found that more Tris was needed in the solution than used in other procedures (1) because of the high amount of acidic compounds found in corn leaves.

The isolation could be accomplished in 36 hours and it was found unnecessary to take unusual precautions against denaturation during that time.
When spinach ferredoxin was isolated by this procedure, it was found that the Sephadex G-75 column was the limiting factor in the purification since the last few tubes which contained ferredoxin also contained impurities which had high absorbance in the ultraviolet region.

Corn ferredoxin has an adsorption spectrum which is nearly identical with the other plant ferredoxins and similar absorbance ratios and extinction coefficients.

The amino acid composition shows similarities to the amino acid content of the other plant ferredoxins, with a high number of acidic residues, low content of basic and aromatic residues, and a lack of methionine. Keresztes-Nagy et al. (22) have compared the amino acid sequence of the dicots, spinach and alfalfa, with that of bacterial ferredoxins, from an evolutionary standpoint. Since the amino acid composition of the monocot Zea mays is very similar to that of spinach and alfalfa, a comparison of the sequence of monocots and dicots, which are close together in their evolutionary development, may prove interesting.

Hatch, Slack, and Johnson (40) have labeled Zea mays with $^{14}\text{CO}_2$ and found that the first compounds labeled were C-4 acids, indicating a different $\text{CO}_2$ fixation pathway than the Calvin cycle found in other higher plants. It is thus of interest that the corn ferredoxin has the ability to replace spinach ferredoxin in the reduction of NADP.
using spinach chloroplasts and that spinach ferredoxin can replace corn ferredoxin when reducing NADP with corn chloroplasts. This implies that the difference in the CO$_2$ fixation pathway observed in tropical grasses is not due to a difference in the function of ferredoxin.
LIST OF REFERENCES


