PROGRESS REPORT

The P.I.'s current Department of Energy Grant (DE-FG05-90ER20017) had a start date of August 15, 1990, and the due date for this renewal proposal was July 1, 1992. However, this progress report was completed by June 1, 1992, as the P.I. had scheduled a trip to Neuchâtel and Zürich, Switzerland during the month of June to conduct DOE-funded research on ferredoxin:$NADP^+$ oxidoreductase and ferredoxin:thioredoxin reductase. During the approximately 21 1/2 months the grant has been in effect, 5 publications in refereed journals have appeared that resulted from work conducted on DOE-funded projects. In addition, one abstract has appeared and two additional manuscripts have been submitted. The published articles are:

1. Hirasawa, M., Chang, K.-T. and Knaff, D. B. (1991) The interaction of ferredoxin and glutamate synthase: Cross-linking and immunological studies. Arch. Biochem. Biophys. 286, 171-177.

2. Hilliard, N. P., Hirasawa, M., Knaff, D. B. and Shaw, R. W. (1991) A reexamination of the properties of spinach nitrite reductase: Protein and siroheme content heterogeneity in a purified preparation. Arch. Biochem. Biophys. 291, 195-199.

3. Chang, K.-T., Morrow, K. J., Hirasawa, M. and Knaff, D. B. (1991) Monoclonal antibody studies of ferredoxin:$NADP^+$ reductase. Arch. Biochem. Biophys. 290, 522-527.

4. Knaff, D. B., Hirasawa, M., Ameyibor, E., Fu, W. and Johnson, M. K. (1991) Spectroscopic evidence for a [3Fe-4S] cluster in spinach glutamate synthase. J. Biol. Chem. 266, 15080-15089.

5. Hirasawa, M., Robertson, D. E., Ameyibor, E., Johnson, M. K. and Knaff, D. B. (1992) Oxidation-reduction properties of the ferredoxin-linked glutamate synthase from spinach leaf. Biochim. Biophys. Acta 1100, 105-108.

The two articles submitted to Biochim. Biophys. Acta are:

6. Hirasawa, M., de Best, J. and Knaff, D. B. The effect of lysine and arginine modifying reagents on spinach ferredoxin:nitrite oxidoreductase.

7. Hirasawa, M., Chang, K.-T., Dose, M. M., Lou, B.-S., Ondrias, M. R. and Knaff, D. B. Characterization of two low potential cytochromes from bean sprouts.

The abstract is:

8. Jelesaron, I., De Pascalis, A., Knaff, D. B. and Bosshard, H. R. Macromolecular recognition in an electron-transfer complex: The binding site for ferredoxin on ferredoxin-$NADP^+$ reductase. 24th Annual Meeting of the Swiss Society for Experimental Biology, Basel, 1992.

Reprints of the published articles are included, along with a pre-print of the article on the effects of lysine and arginine modification on ferredoxin-binding that has recently been submitted to Biochimica et Biophysica Acta . No reprint of the second article submitted to Biochim.

MASTER

Biophys. Acta has been included because of space limitations and because DOE funding will not be requested to continue investigation of the bean sprout system. The abstract has not been included but the work presented at Basel on FNR/ferredoxin interactions is summarized below.

One of the primary goals of the original proposal was to map the ferredoxin-binding sites on three soluble enzymes that are located in spinach chloroplasts and utilize ferredoxin as an electron donor:Ferredoxin:NADP$^+$ oxidoreductase (FNR); ferredoxin:thioredoxin reductase (FTR) and glutamate synthase. As the availability of amino acid sequences for the enzymes are important in such studies, it was proposed that the amino acid sequence of glutamate synthase be determined. The amino acid sequences of FNR, FTR and ferredoxin are already known. An aim related to elucidating the binding sites on these enzymes for ferredoxin was to determine whether there is a common site on ferredoxin involved in binding to all of these ferredoxin-dependent chloroplast enzymes and, if so, to map it. One additional aim was to characterize thioredoxin binding by FTR and determine whether the same site on FTR is involved in binding both ferredoxin and thioredoxin. Considerable progress has been made on most of these original projects, although work conducted on FTR is still in its preliminary stages. Considerable progress has also been made in four additional areas, related to the goals of the original proposal: (1) The prosthetic groups of glutamate synthase have been thoroughly characterized for the first time. (2) Progress has been made in establishing the role of arginine and lysine residues in ferredoxin binding by a fourth soluble chloroplast enzyme, ferredoxin:nitrite oxidoreductase (hereafter referred to as nitrite reductase). (3) Techniques developed to label carboxyl groups on ferredoxin with taurine and to label lysine residues on ferredoxin-dependent enzymes by biotinylation appear to have general applicability to many proteins other than those currently under study in our laboratory. (4) Low potential heme proteins have been isolated from a non-photosynthetic plant tissue and characterized.

1. Ferredoxin:NADP$^+$ Oxidoreductase

    a. Monoclonal Antibody Studies

        One major goal of the original proposal was to develop a set of monoclonal antibodies raised against FNR in the hope that some of the antibodies would recognize epitopes at either the NADP$^+$ or ferredoxin-binding sites on the enzyme. Mapping of these epitopes would then provide specific information about the location of the two substrate-binding domains on the enzyme. Eleven independent monoclonal antibodies against FNR were generated and all were shown to inhibit electron flow, catalyzed by FNR, from NADPH to dichlophenol indophenol (DCPIP), a non-physiological electron acceptor. In contrast, none of the monoclonal antibodies inhibited FNR-catalyzed electron transfer from NADPH to ferredoxin. Direct binding assays showed that the antibodies did not interfere with either ferredoxin binding or NADP$^+$ binding by FNR. It was thus concluded that all of the monoclonal antibodies recognized an epitope or epitopes at the site on FNR where DCPIP is bound and reduced and that this site is separate from the sites where the two physiological substrates, NADP$^+$(H) and ferredoxin, bind. These results have been published in Ref. 3, cited on p.1 of the Progress Report. As the goal of this work was to isolate antibodies directed against epitopes located at the binding sites for either NADP$^+$ or ferredoxin and not against epitopes located at the site at which a non-physiological electron acceptor interacts with

enzyme, a different approach towards mapping the ferredoxin-binding site on FNR was taken. (See b)

b. Differential Chemical Modification

FNR was treated with biotin-N-hydroxysuccinimide ester at low ionic strength, either in the absence of ferredoxin or in the presence of equimolar ferredoxin. The rationale for this approach was that in the presence of ferredoxin, FNR lysine residues specifically involved in complex formation should be protected against biotinylation. Biotinylated FNR from the two labeling experiments was isolated and subjected to proteolytic digestion. The digests were passed over an avidin-affinity column, which retained only biotinylated peptides, and the biotinylated peptides were then eluted. An analysis of the elution pattern of the biotinylated peptides from a reverse phase HPLC column showed that one biotinylated peptide that was present in the digest of the free FNR sample was absent, and a second peptide was present in greatly reduced amount, in the digest of FNR biotinylated in the presence of ferredoxin. Amino acid sequencing of these two peptides revealed that FNR lysines 18 and 153 are specifically protected against biotinylation by complex formation with ferredoxin. In separate experiments, 4-N,N-dimethylamino-azobenzene-4'-isothiocyano-2'-sulfonic acid (S-DABITC) was used as a lysine modifying reagent, as it yields strongly colored derivatives. After proteolysis, the HPLC elution patterns of colored peptides obtained from FNR treated with S-DABITC in the presence and absence of ferredoxin were compared. A peptide containing S-DABITC-modified K33 and/or K35 was present when free FNR was labeled, but was absent when the FNR:ferredoxin complex was labeled. These results, which are summarized in the abstract cited on p. 1 of the Progress Report, have been interpreted in terms of the involvement of lysines 18, 153 and 33 and/or 35 in complex formation to ferredoxin. Final data analysis, including computer graphics modeling using FNR coordinates supplied by Dr. P. Andrew Karplus, are currently underway and the P.I. and Professor Bosshard plan to begin writing these results up for publication during the P.I.'s visit to Zürich this summer.

A complimentary set of experiments have been conducted in an attempt to map the site on ferredoxin involved in binding to FNR. Although this work was actually initiated somewhat prior to that on modification of FNR lysine groups, a large number of technical problems arising from the anomalous migration of ferredoxin during electrophoresis, the difficulty in digesting modified ferredoxin and the strongly acidic nature of the modifying reagent had to be overcome. However, methodology has now been developed for modifying aspartate and glutamate carboxyl groups on ferredoxin with taurine in the presence of the water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The ferredoxin I isoform was separated from ferredoxin II and used for all of these experiments. [^{3}H]-labeled taurine was used to trace modify ferredoxin in the presence of FNR and [^{14}C]-labeled taurine of equal specific activity was used to trace modify free ferredoxin. Ferredoxin was isolated from both samples and all carboxyl groups were then modified with non-radioactive taurine in the presence of EDC. The ferredoxins modified in the presence and absence of FNR were then mixed and subjected to proteolytic digestion. Peptides from the digest were separated using a reverse phase HPLC column and ^{3}H/^{14}C ratios for all peptides were measured. The rationale for these experiments was that peptides with ^{3}H/^{14}C ratios <1.0 contained aspartate and/or glutamate protected against carboxyl group modification by complex formation with ferredoxin and that these amino acids were thus likely

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to be specifically involved in complex formation with FNR. Amino acid sequencing of the peptides is now in progress and it appears likely that $^3H/^{14}C$ ratios can be determined for almost all of the aspartates and glutamates in ferredoxin. Even though the analysis of these peptides is not yet complete, several acidic amino acid residues on ferredoxin are clearly protected by complex formation with FNR against taurine modification. These residues are 29, 30, 34, 65 and 66. The acidic region near the C-terminus (Glu 92-94) is not protected. When the peptide analysis is complete, a model will be developed for the FNR-binding site on ferredoxin, using the tertiary structures available for the homologous cyanobacterial ferredoxins, and a manuscript submitted for publication.

2. Glutamate Synthase

    a. Gene Sequencing

        A Uni-Zap cDNA expression library from young spinach leaves has been screened using antibodies raised in rabbits against spinach glutamate synthase purified in our laboratory. Eight positive plaques were identified, one of which was positive after two additional screenings with the antibody and which also hybridized in Southern blots with a heterologous probe constructed from a full length cDNA clone for the maize glutamate synthase sent to us by Dr. T. Hase (University of Nagoya). After excision of the pBluescript phagemid from the Uni-Zap vector, colonies containing pBluescript with the cloned DNA insert were selected and the phagemid isolated and digested with EcoRI/XhoI. A 3.7 kb fragment resulted, from which 8 smaller restriction fragments were produced and subcloned into pUC19. Sequencing, using the Sanger di-deoxy chain termination method, has begun and 2.55 kb have been sequenced to date. Translation of the open reading frame produced an amino acid sequence that was highly homologous to that published in 1991 for the ferredoxin-dependent maize enzyme (731 of 849, or 86%, of the amino acids sequenced to date are identical in the spinach and maize sequences). Several tryptic peptides prepared from the purified enzyme have been sequenced and agree exactly with the amino acid sequence predicted from the DNA sequence. The N-terminal portion of the protein has also been sequenced. We are currently synthesizing oligonucleotides to be used to complete sequencing the 3' end of the 3.7 kb EcoRI/XhoI fragment and then will construct a homologous probe from a part of the 3.7 kb fragment and use it to probe the cDNA library for the 5' portion of the gene that is missing from the 3.7 kb fragment. It is anticipated that complete sequencing of the entire gene will take approximately one additional year.

    b. Prosthetic Group Content

        Previous studies had established that the ferredoxin-dependent glutamate synthases isolated from spinach chloroplasts and from the green alga Chlamydomonas reinhardtii contain 1 FMN and 1 FAD per enzyme molecule (M_r = ca. 150 kDa). Non-heme iron and acid-labile sulfide analyses revealed that glutamate synthase contained four sulfides and 3-4 irons, indicating the likely presence of one or more iron-sulfur clusters in the enzyme. However, no spectroscopic evidence for the presence of an iron-sulfur cluster was available. We have now obtained low temperature electron paramagnetic resonance (EPR), magnetic circular dichroism (MCD) and Resonance Raman spectra of the enzyme in both its oxidized and reduced states that show conclusively that there is a single $[3Fe-4S]^{0,+1}$ cluster present in the enzyme. Analysis

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of the cysteine spacings in the highly homologous maize glutamate synthase sequence, the observation that the spinach enzyme containing the [3Fe-4S] cluster is active, and our inability to obtain uptake of an additional iron by the spinach enzyme to yield a [4Fe-4S] cluster, all strongly suggest that the [3Fe-4S] configuration is the normal physiological structure of the cluster. This work, which represents the first demonstration of the presence of a functional [3Fe-4S] cluster in a ferredoxin-dependent glutamate synthase, has been published (Ref. 4). The oxidation-reduction midpoint potential (E_m) value of the cluster has been determined to be -170 mV (pH 7.7). The E_m values of the FAD and FMN groups are identical (to within 30 mV) and, at E_m = -180 mV (n=2, pH 7.7), are essentially isopotential with the [3Fe-4S] cluster. These redox characterizations, the first for a ferredoxin-dependent glutamate synthase, have also been published (Ref. 5).

    c. Ferredoxin Binding

    Previous work in our laboratory established that complex formation between glutamate synthase and ferredoxin could be demonstrated by co-migration during gel filtration and by perturbations in the absorbance and CD spectra of the proteins. We have now been able to use EDC to form an enzymatically active cross-linked complex between ferredoxin and glutamate synthase. In contrast to the successful cross-linking obtained at low ionic strength, cross-linking does not occur if EDC is added to a mixture of ferredoxin and the enzyme incubated at high ionic strength, confirming the electrostatic nature of the interaction between the two proteins. Absorbance and CD spectra of the cross-linked complex suggest that the complex stoichiometry is 2 ferredoxin: 1 glutamate synthase. Previous work in our laboratory had established that the ferredoxin-binding sites on glutamate synthase and nitrite reductase are similar to the ferredoxin-binding site on FNR. Consistent with this hypothesis is our recent observation that, while an antibody raised against spinach FNR recognizes spinach glutamate synthase, it does not recognize the cross-linked ferredoxin/glutamate synthase because the epitope(s) at the ferredoxin-binding site recognized by the antibody in the free enzyme are inaccessible to the antibody with ferredoxin cross-linked to this site. This work has been published (Ref. 1).

3. Ferredoxin:Thioredoxin Reductase (FTR)

    Although some preliminary success has been achieved in investigating the interaction between FTR and thioredoxin f, our efforts during this grant period have concentrated on elucidating the role of the iron-sulfur cluster found in the enzyme. EPR, MCD and Resonance Raman studies have been conducted and have confirmed the presence of a single [4Fe-4S]$^{2+}$ cluster in FTR and allowed us to model certain aspects of the cluster geometry. We have also confirmed an earlier report that the cluster could be oxidized by ferricyanide to the S = 1/2 [4Fe-4S]$^{3+}$ state with EPR g values of 2.11, 1.99 and 1.98. However, spin quantitation showed that <10% of the cluster becomes oxidized under these conditions. The extremely positive E_m value for this oxidation, +410 mV, compared to that for typical disulfide/dithiol E_m values (ca. -250 mV), make it seem highly unlikely that the [4Fe-4S]$^{2+ \cdot 3+}$ redox couple can function to carry electrons from the physiological electron donor, reduced ferredoxin, to the active site disulfide of FTR and reduce it to the dithiol state. Attempts to "super reduce" the cluster to see whether the [4Fe-4S]$^{1+ \cdot 2+}$ couple could be functional as a low potential conduit of electrons from reduced ferredoxin to the active site disulfide have been

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unsuccessful. No spectroscopic evidence for such cluster reduction was obtained even when E_h was lowered to -600 mV. These observations and the recent finding (P. Schürmann, personal communication) that two of the cysteine residues that serve as cluster ligands come from the α-subunit of FTR and the other two cysteines come from the ß-subunit, raise the possibility that the iron-sulfur cluster in FTR serves a structural function in connecting the two subunits rather than an electron transfer function. Small absorbance changes observed when the enzyme is reduced suggest the possibility that FTR contains an additional, previously unidentified prosthetic group. Current efforts are centered on attempts to obtain definitive evidence for the presence of such a prosthetic group and to complete the spectroscopic characterization of the iron-sulfur cluster. It is anticipated that a manuscript describing this work should be completed and submitted in less than six months.

4. Nitrite Reductase

a. Subunit Composition and Siroheme Content

Earlier work from our laboratory lead to a proposal that spinach nitrite reductase was a heterodimer, consisting of a $M_r=62$ kDa subunit that contained all of the electron-carrying prosthetic groups and a $M_r=24$ kDa subunit involved in ferredoxin-binding. We have now demonstrated that the $M_r=24$ kDa peptide is an adventitious contaminant and that spinach nitrite reductase is a monomeric protein that contains one siroheme group per $M_r=62$ kDa enzyme molecule. We have demonstrated that gel filtration chromatography can result in considerable loss of siroheme and that significant enzyme heterogeneity occurs due to proteolysis at several points near the N-terminus of nitrite reductase. The existence of multiple forms of nitrite reductase arising from this proteolysis appears to be exacerbated by freezing and thawing enzyme samples. These results have been published (Ref. 2) and are currently being utilized in attempts to prepare nitrite reductase in the presence of protease inhibitors so that samples free from proteolysis that are suitably homogeneous for crystallization can be obtained.

b. Ferredoxin Binding

Previous work in our laboratory provided evidence that ferredoxin contributed the negatively charged groups involved in electrostatic complex formation with nitrite reductase. It could thus be concluded that nitrite reductase must contribute the positive groups involved in complex formation, but no direct evidence was available to document this hypothesis. We have now obtained considerable evidence that both lysine and arginine residues on the enzyme play a role in binding ferredoxin. Treatment of nitrite reductase with either the arginine-modifying reagent phenylglyoxal or the lysine-modifying reagent N-acetyl succinimide, caused a substantial inhibition of nitrite reduction catalyzed by the enzyme when its physiological reductant, reduced ferredoxin, was supplied as the electron donor. In contrast, treatment of nitrite reductase with phenylglyoxal or N-acetyl succinimide had no effect on nitrite reduction catalyzed by the enzyme when the non-physiological electron donor, reduced methyl viologen was supplied. These results suggest that neither of the modifying reagents affect groups involved in binding nitrite or in the conversion of nitrite to ammonia catalyzed by the enzyme. Absorbance and CD spectra of modified nitrite reductase, the activity vs pH profile of the modified enzyme and spectra of the nitrite adduct of the modified enzyme all support the conclusion that neither reagent interferes with nitrite

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binding nor causes any major conformational change in the enzyme. Treatment of the enzyme with either phenylglyoxal or N-acetyl acetamide does interfere significantly with the ability of the enzyme to bind ferredoxin. Both phenylglyoxal-treated nitrite reductase and N-acetyl succinimide-treated nitrite reductase lost the ability to bind to a ferredoxin-Sepharose affinity column, no longer co-migrated with ferredoxin during gel filtration chromatography and did not exhibit the changes in CD spectrum in the presence of ferredoxin that are associated with enzyme/ferredoxin complex formation. Furthermore, treatment of nitrite reductase with N-acetyl succinimide eliminates the ability of the enzyme to form a catalytically active cross-linked complex with ferredoxin in the presence of EDC. These results, and a discussion of the role of nitrite reductase lysines and arginines at the ferredoxin-binding site of the enzyme, have been described in a manuscript that has been submitted for publication to Biochim. Biophys. Acta. A copy of this manuscript has been included as part of this Progress Report.

5. Low Potential Bean Sprout Cytochromes

Previous work in our laboratory provided evidence for the presence of ferredoxin and FNR in bean sprouts, a chlorophyll-free, non-photosynthetic plant tissue, and for a ferredoxin-dependent nitrite reductase in another non-photosynthetic plant tissue, spinach roots. A question of obvious interest is how ferredoxin, which is reduced in a light-driven reaction catalyzed by Photosystem I in photosynthetic tissues, is reduced in chlorophyll-free tissues. One approach that we have used to explore this question has been to search for low potential electron carriers in one of these non-photosynthetic plant tissues, bean sprouts. We have isolated, purified and characterized two previously undiscovered, highly basic cytochromes in bean sprouts. One of the cytochromes (M_r=38 kDa, E_m= -300 mV) contains one high spin heme $\underline{c}$ in which the heme iron is either 5-coordinate or contains a weakly-bound sixth ligand. The other cytochrome (M_r=12.5 kDa, E_m= -130 mV) contains a low spin heme that appears to be a derivative of heme $\underline{c}$ that may be covalently attached to the protein through a single thioether linkage instead of the two thioether linkages found in most $\underline{c}$-type cytochromes. The results of this investigation have been incorporated into a manuscript submitted to Biochim. Biophys. Acta. Neither cytochrome has a E_m value sufficiently negative to reduce ferredoxin (E_m= -420 mV) nor did either cytochrome serve as an electron donor that could replace ferredoxin in nitrite reductase or glutamate synthase-catalyzed reactions. Thus, further characterization of these cytochromes with DOE funds does not seem appropriate and future work on these cytochromes in our laboratory will be carried out using other funds.

Summary

Progress has clearly been made on all of the goals set forth in the original proposal. Although the monoclonal antibodies raised against FNR turned out not to be useful for mapping the FNR/ferredoxin or FNR/NADP+ interaction domains, good progress has been made on mapping the FNR/ferredoxin interaction domains by an alternative technique, i.e., differential chemical modification. Furthermore, the techniques developed for differential chemical modification of these two proteins - taurine modification of aspartate and glutamate residues and biotin modification of lysine residues - should be useful for mapping the interaction domains of many proteins that associate through electrostatic interactions. This is particularly true of the

biotinylation method, which allows for rapid identification of protected peptides by screening with avidin affinity chromatography. One published manuscript and one abstract based on this FNR work have appeared to date. Although no publications have yet resulted from the investigations carried out during this period on FTR, considerable progress has been made in characterizing the unique iron-sulfur cluster of this important regulatory protein and publication of these results is imminent. Perhaps the greatest progress has been made on the glutamate synthase aspect of the original goals, with three publications resulting that enhanced our knowledge of the interaction of this enzyme with ferredoxin and presented the first reasonably complete characterization of the prosthetic group of this key enzyme of plant nitrogen metabolism. It is anticipated that by the time this three year grant period ends, we also will have completed sequencing glutamate synthase from a cDNA library. Finally, progress has also been made with respect to another ferredoxin-dependent enzyme involved in the earliest steps of plant nitrogen metabolism, nitrite reductase. Questions concerning the subunit composition and heme content of the enzyme have been resolved and evidence demonstrating the involvement of lysine and arginine residues in binding ferredoxin has been obtained for the first time.

DISCLAIMER

PATENT CERTIFICATION

Texas Tech University
_____
Contractor

☐ Interim Certification
☒ Final Certification

DE-FG05-90ER20017
_____
DOE Prime and/or Subcontract Nos.

Contractor hereby certifies that:

1. All procedures for identifying and disclosing subject inventions as required by the patent clause of the contract have been followed throughout the reporting period.

2. There were no subcontracts or purchase orders involving research, development, and demonstration except as follows: [State none when applicable.]

3. No inventions or discoveries were made or conceived in the course of or under this contract other than the following (Certification includes ☒, does not include ☐ all subcontracts):

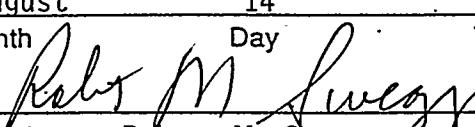   [State none when applicable.]   None

TITLE	INVENTOR	DATE REPORTED	DOE "S" NO.

4. The completion date of this contract is as follows: August 14, 1993

5. The following period is covered by this certification:

August	15	1990	to	August	14	1993
Month	Day	Year		Month	Day	Year

Texas Tech University
_____
Contractor

Signature   Robert M. Sweazy

Vice Provost for Research

Lubbock, TX   79409-1035
_____
Address

May 9, 1995
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Date of Certification

*Also include Subcontract No. if available