FINAL REPORT

Project Title: Structural Domains in NADPH: Protochlorophyllide Oxidoreductases Involved in Catalysis and Substrate Binding

Project Number: DE-FG05²-94ER20144

Project Period: 06/01/94 to 05/31/98

Principal Investigator: Michael P. Timko

We have no objection from a patent standpoint to the publication or dissemination of this material.

MP Timko

11/23/99

Office of Intellectual

Date

Property Counsel

DOE Field Office, Chicago

Until recently little direct information was available about specific structural determinants within the light-dependent NADPH: protochlorophyllide oxidoreductases (PORs) required for substrate and cofactor binding, catalytic activity, and thylakoid membrane localization. Based on our previous DOE-funded studies, during the past year we brought to fruition a number of ongoing experiments, initiated several new avenues of investigations, and overall have made considerable progress towards establishing the basic structural parameters governing POR function. Our studies to date have defined residues and domains involved in substrate and cofactor binding and catalysis, and elaborated on the mechanism for membrane localization of POR in developing plastids. Our results and their significance, as well as our work in progress, are detailed below.

A. Systematic Mutational Analysis of POR Structure

Three years ago we initiated a systematic mutational analyses of the pea POR based upon the clustered charged-to-alanine scanning mutagenesis strategy successfully applied to the study of functional determinants in a variety of other proteins. This strategy allowed us to examine the entire surface of the POR protein by exploiting the fact that clusters of charged residues are usually not buried within the folded protein structure. By altering these clusters of charges, we could subtly alter protein conformation to explore the role of this region on enzyme function. In this particular study, we were interested in identifying domains within POR required for activity (either substrate or cofactor binding, or catalysis) and thylakoid membrane assembly. This study has now been completed and a manuscript describing our results has been submitted for publication to Plant Molecular Biology (Dahlin, et al. 1998).

Our results showed that of 37 mutant PORs examined, 5 retained wild-type levels of activity, 14 were catalytically inactive, and the remaining 18 exhibited altered levels of function. Several of the mutant enzymes showed temperature-dependent enzymatic activity, being inactive at 32°C, but partially active at 24°C. Mutations in predicted α -helical regions of the protein showed the least effect on enzyme activity, whereas mutations in predicted β -sheet regions of the protein showed a consistent adverse affect on enzyme function. In the absence of added NADPH, neither wild-type POR nor any of the mutant PORs resisted proteolysis by thermolysin following assembly onto the thylakoid membranes. In contrast, when NADPH was present in the assay mixture, 12 of the 37 mutant PORs examined were found to be either fully- or partially-resistant to thermolysin post-treatment, suggesting that the mutations did not affect their ability to be properly attached to the thylakoid membrane. In general, the replacement of charged amino acids by alanine in the most N- and C-terminal regions of the mature protein did not significantly affect POR assembly, whereas all but five PORs with mutations between residues 86 and 342 were incapable of proper attachment to the thylakoid. The five mutant PORs capable of proper assembly contained mutations in regions of the protein outside of the cofactor binding pocket or active site. Failure to properly associate with the thylakoid membrane in a protease resistant manner was only weakly correlated to loss of function at 24°C. These studies were the first attempt to define structural determinants crucial to POR function and

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, make any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

intraorganellar localization. By identifying particular regions as important, it has provided us with a baseline for more critical evaluation of these regions. Over the next year we hope to fully evaluate these mutants by several different criteria in order to define how these mutations specifically affect substrate binding capacities and various kinetic properties. To do so, we will over express each mutant protein in *E. coli*, purify it and carry-out kinetic analysis as described below.

B. A System for POR Overexpression

As described in the manuscript by Martin, et al. (1997), we have established conditions for the over-expression of the pea (*Pisum sativum* L.) POR in *E. coli* as a fusion with maltose-binding protein (MBP), using the pMAL-c2 vector. Upon induction with isopropyl β -D-thiogalactoside (IPTG), the fusion protein (MBP-POR) was expressed in the bacteria and constituted 5-10 % of the total soluble cell protein. The protein was purified to greater than 90% homogeneity by a two step affinity chromatography procedure. The molecular mass of MBP-POR was determined to be approximately 80 kDa by SDS/PAGE. The fusion protein eluted from a size-exclusion chromatography on Superose 6 HR at an elution volume corresponding to a molecular mass of 150 kDa, indicating that MBP-POR behaves as a dimer in solution. MBP-POR was shown to catalyse the light-dependent reduction of protochlorophyllide to chlorophyllide with a V_{max} of 20.6 ± 0.9 μ moles/min/mg. The K_m values for NADPH and protochlorophyllide were 8.7 ± 1.9 μ M and 0.27 ± 0.04 μ M respectively.

In our initial MBP-POR fusions, removal of the MBP by Factor Xa cleavage resulted in partial proteolysis of the POR component due to internal endoprotease cleavage sites. To rectify this problem, we redesigned our expression system to include a rTEV protease site. This endoprotease is substantially more specific and we can now get complete recovery of the overexpressed protein (in the neighborhood of several milligrams/liter of culture). This has allowed us to work towards one of our stated goals of crystallization of the protein.

These studies are notable for several reasons. (1) They are the first reported kinetic measurements for the purified POR enzyme. (2) They demonstrate that the enzyme catalyzes multiple rounds of protochlorophyllide reduction and therefore is not a suicide enzyme as suggested by some competing groups in the literature. (3) They demonstrate that the native enzyme may exist as a dimer in solution, which would not be predicted based upon its structure alone. This is important since it now opens up the possibility of examining further the role of the putative dimerization domain in the protein. (4) Finally, attempts at crystallization have been initiated and we have already achieved small crystals which we are hoping to show are POR.

C. Defining a Role for the C-terminus of POR in Enzyme Activity

Numerous examples exist in the literature suggesting that C-terminal regions of proteins may play important roles in catalytic activity. Notable among these are studies involving two POR homologs, *Drosophila* ADH and bacterial 3 α , 20 β -HSD. In the former, deletion analysis showed that the C-terminal 13 amino acids are essential for activity and likely serve as part of the wall constituting the substrate binding pocket of the enzyme. In the latter case, the C-terminal 60-residues were shown to constitute part of the steroid binding pocket. Our alanine scanning mutagenesis studies have already shown that only limited regions of the C-terminus of the pea POR contributed significantly to enzyme activity. Based on these preliminary observations, Ms. Olga Karginova (a senior research assistant in my laboratory) initiated studies to look in greater detail at the role of the C-terminus in POR function and to help define the minimal catalytic unit of the enzyme. To this end, we have constructed a series of truncated POR proteins in which termination codons were introduced in the coding sequences following residues VAL-361, TYR-337, LEU-

314, and ILE-299 and the remainder of the protein was removed. Analysis of these mutants indicated that none were catalytically active, although VAL-316, TYR-337, and LEU-314 were still imported and assembled into the membrane. Thus, it is clear that the ability to import and assemble on the chloroplast membrane are not linked to catalytic function and do not require all regions of the POR protein. A manuscript describing the importation and assembly studies is currently in preparation.

We also constructed site-directed mutations and localized deletions of varying length beginning at the extreme C-terminus of the POR protein. Removal of as few as 5 residues at the extreme C-terminus impaired catalytic activity. Substitutions are being made in each of the 5 amino acids and for each of these mutant proteins, the specific activity and kinetic properties will be analyzed and the effect on protochlorophyllide binding determined. These studies should tell us more about how the C-terminus participates in enzyme function.

D. The Role of the 35-Residue Loop in POR Activity and Protein:Protein Interactions

Previous studies of tetrameric and dimeric members of the SDR family of proteins have implicated the region occupied by the 35-residue loop in the pea POR protein as a linking (dimerization/oligomerization) site for the individual chains in the oligomer. Our modeling studies (described partially in Dahlin, et al. (1998) and more completely in Holcomb, Kretsinger, and Timko, manuscript in preparation) suggest that there is ample volume available within this region of the POR protein for the loop without displacing the active site. In the mouse lung carbonyl reductase (MLCR), the individual monomers pack tightly together with the four active sites aligning to form a single cavity. We speculated that the 35-residue loop in the POR protein (residues 231-265) and its rigidity/flexibility may be important in modulating intermolecular interactions. On the one hand, the loop may prevent the POR proteins from packing too tightly in the prolamellar body which could interfere with substrate accessibility. On the other hand, the loop region might be involved in promoting interaction with other proteins, including other POR molecules, providing contact points for proper assembly on the plastid membrane. We also can not rule out the possibility that the conformation and physical properties of this surface loop may contribute directly to the structure and function of the active site. It should be emphasized that most SDR homologs do not contain this loop and so its functional role may be quite unique in POR.

To examine further the role of this region in the POR protein, Ms. Karginova has made short deletions within the enzyme which removed 1/3 of the loop progressively from either the C-terminal side or the N-terminal side. In each case the activity of the mutant POR was assayed and it was determined that all deletion mutations resulted in non-functional proteins. Since truncation of the loop may adversely affect overall protein structure, we are in the process of introducing short peptide segments back into the loop region of the protein to re-establish proper spacing. These peptide segments have similar hydrophilicity and charge properties to the various lengths of the 35-residue loop removed. The ability of these mutant proteins to carry out light-dependent protochlorophyllide reduction as well as their integration efficiency will be determined. We are also determining whether removal of this region has any effect on the ability of the POR protein to dimerize/oligomerize. Two different assays for dimerization are being used, PAGE analysis followed by Western blotting and spectrofluometric analysis. The latter is extremely sensitive and can also provide information on the orientation of the pigments in any dimer that is formed.

E. Low-Temperature Fluorescence Analysis of Intermediates in Protochlorophyllide

Photoconversion.

In addition to participating in the analysis of alanine scanning mutants, Dr. Nikolai Lebedev has developed an series of assay to specifically analyze the protein microenvironment near the substrate and cofactor binding sites in the POR protein. For his studies, Dr. Lebedev has generated MBP-POR fusions consisting of precursor (prePOR) and mature POR proteins, and N-terminal truncations. Both precursor (prePOR) and mature proteins demonstrate high levels of catalytic activity in the presence of protochlorophyllide and NADPH after purification from bacterial cell lysates by amylose affinity chromatography.

Using purified POR protein, secondary and ternary complexes were generated *in vitro* and the molecular organization of the pigment and cofactor inside *in vitro* formed complexes analyzed by pigment, NADPH, and protein fluorescence excitation and emission spectroscopy, fluorescence polarization spectroscopy, CD spectroscopy and energy transfer. The low temperature protochlorophyllide fluorescence excitation, polarization and CD spectra showed that energy transfer took place from TRP residues of the protein to the pigment. Given the location of TRP residues in the protein, this allowed us to identify a the pigment binding site at the seventh β -sheet in the C-terminal part of the protein. Fluorescence polarization studies demonstrated that binding to the enzyme fixes the NADPH configuration and localizes the cofactor in such a way that only the nicotinamide part of the molecule can interact with the pigment. According to the protein CD spectra, the enzyme folds into proper conformation in water solutions without pigment and cofactor, but these compounds induce additional alterations in its structure.

Protochlorophyllide photoconversion was also followed using low temperature fluorescence spectroscopy. The advantage of low temperature fluorescence analysis is that it allows us to trap intermediates in the photoconversion process by slowing down the efficiency of protochlorophyllide photoconversion. In doing so, we have been able to order the events that occur during catalysis. Our studies suggest that an unstable partially reduced protochlorophyllide radicle is formed initially. The source of the reductant is NADPH. This radicle is unstable at temperatures above approximately -50°C . Once the intermediate is formed, complete reduction can occur even in the absence of light energy. The source of the final reductant is likely the active site TYR. Since we have already shown that mutations in this residue abolish activity (Wilks and Timko, 1995), we can use our mutants to see whether the initial events are still capable of proceeding.

Dr. Lebedev and I have co-authored a review summarizing the current state of knowledge on the POR mechanism (Lebedev and Timko, 1998).

F. Related Studies

All of our work on chlorophyll biosynthesis and its regulation underway in the laboratory is currently being supported by our DOE funds. These studies, although peripheral to the main thrust of our structure-function analysis of POR, provide significant insight into the how and why POR function as it does. In addition to the studies described above, the work of Dr. Jeff Skinner the expression of PORA and PORB isoforms in gymnosperms was facilitated by our DOE grant. His studies established that multiple POR isoforms were present in gymnosperm species and that many aspects of the light- and developmental regulation present in angiosperms was already present in gymnosperm species. One manuscript describing this work is in press in *Plant and Cell Physiology* (Skinner and Timko, 1998), a second paper has been submitted to *Plant Molecular Biology*, and a third manuscript describing analysis of a *por* promoter in transgenic plants in preparation.

In addition, our DOE funding has helped support my writing of a review chapter describing our work on light-dependent and light-independent protochlorophyllide reduction in *Chlamydomonas* (Timko, 1998) and our ongoing studies on light-independent protochlorophyllide reduction being carried out by Bruce Cahoon in my laboratory.

A. Manuscripts Published or Submitted Acknowledging DOE Support

Wilks, H.M. and M.P. Timko. 1995. A light-dependent complementation system for analysis of NADPH: Protochlorophyllide oxidoreductase. Identification and mutagenesis of two conserved residues that are essential for enzyme activity. *Proc. Natl. Acad. Sci. (USA)* **92**: 724-728.

Yoshida, K., R.-M. Chen, A. Tanaka, H. Teramoto, M.P. Timko, and H. Tsuji. 1995. Correlated changes in the activity, amount of protein, and abundance of transcript of NADPH: protochlorophyllide oxidoreductase and chlorophyll during greening of cucumber cotyledons. *Plant Physiol.* **109**: 231-238.

Dahlin, C., C. Sundqvist, and M.P. Timko. 1995. The in vitro assembly of NADPH-protochlorophyllide oxidoreductase in pea chloroplasts. *Plant Mol. Biol.* **29**: 317-330.

Li, J. and M.P. Timko. 1996. The *pc-1* phenotype of *Chlamydomonas reinhardtii* results from a deletion mutation in the nuclear gene for NADPH: protochlorophyllide oxidoreductase. *Plant Mol. Biol.* **30**: 15-37.

Sundqvist, C., M. Seyyedi, and M.P. Timko. 1996. Chlorophyll formation in the LIP1 mutant of pea. In: **Photosynthesis: from Light to Biosphere, Vol. III**, Proceedings of the Xth International Photosynthesis Congress, (P. Mathis, ed.), Kluwer Academic Publishers, The Netherlands. pp. 1005-1008.

Martin, G.E.M., M.P. Timko, and H.M. Wilks. 1997. Purification and kinetic analysis of pea NADPH-protochlorophyllide oxidoreductase expressed as a fusion with maltose-binding protein in *Escherichia coli*. *Biochem. J.* **325**: 139-145

Lebedev, N. and M.P. Timko. 1998. Protochlorophyllide photoreduction. *Photosynthesis Res.* **58**: 5-23.

Skinner, J.S., and M.P. Timko. 1998. Loblolly pine (*Pinus taeda* L.) contains multiple expressed genes encoding light-dependent NADPH: protochlorophyllide oxidoreductase (POR). *Plant Cell Physiol.* **39**: 795-806.

Timko, M.P. 1998. Pigment biosynthesis: Chlorophylls, Heme, and Carotenoids. In: **The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas**, (J.-D. Rochaix, M. Goldschmidt-Clermont, and S. Merchant, eds.), Kluwer Academic Publishers, The Netherlands. pp. 377-414.

B. Contribution to Human Resources Training and Development.

DOE funding during this time period contributed fully or in part to the training of three postdoctoral associates, Drs. Helen M. Wilks (Ph.D., 1991, University of Bristol), Ramin Parham (Ph.D., 1996, University of Illinois) and Nikolai Lebedev (Ph.D., Moscow State University). The research efforts of three graduate students were also partially supported during this time: Jianming Li (Ph.D., 1995; Postdoctoral Associate, Salk Institute; Assistant Professor at the University of Michigan), Jeffrey S. Skinner (Ph.D., 1997, Postdoctoral Associate, Oregon State University), and A. Bruce Cahoon (Ph.D. candidate, University of Virginia).