

Proteomic Characterization of A Triton-Insoluble Fraction from Chloroplasts Defines A Novel Group of Proteins Associated with Macromolecular Structures

Brett S. Phinney[†] and Jay J. Thelen^{*,‡}

Michigan State University, Proteomics and Mass Spectrometry Facility, East Lansing, Michigan 48824 and
University of Missouri-Columbia, Department of Biochemistry, Columbia, Missouri 65211

Received November 16, 2004

Proteomic analysis of a Triton X-100 insoluble, 30 000 × *g* pellet from purified pea chloroplasts resulted in the identification of 179 nonredundant proteins. This chloroplast fraction was mostly depleted of chloroplast membranes since only 23% and 9% of the identified proteins were also observed in envelope and thylakoid membranes, respectively. One of the most abundant proteins in this fraction was sulfite reductase, a dual function protein previously shown to act as a plastid DNA condensing protein. Approximately 35 other proteins known (or predicted) to be associated with high-density protein-nucleic acid particles (nucleoids) were also identified including a family of DNA gyrases, as well as proteins involved in plastid transcription and translation. Although nucleoids appeared to be the predominant component of 30k × *g* Triton-insoluble chloroplast preparations, multi-enzyme protein complexes were also present including each subunit to the pyruvate dehydrogenase and acetyl-CoA carboxylase multi-enzyme complexes, as well as a proposed assembly of the first three enzymes of the Calvin cycle. Approximately 18% of the proteins identified were annotated as unknown or hypothetical proteins and another 20% contained “putative” or “like” in the identifier tag. This is the first proteomic characterization of a membrane-depleted, high-density fraction from plastids and demonstrates the utility of this simple procedure to isolate intact macromolecular structures from purified organelles for analysis of protein–protein and protein-nucleic acid interactions.

Keywords: chloroplast • nucleoids • Triton insoluble • organelle proteomics • multi-enzyme complexes • DNA gyrase • acetyl-CoA carboxylase

Introduction

Plastids are semi-autonomous, membrane-bound organelles found in algae and higher plants and are the site of de novo synthesis of many phytochemicals including fatty acids, isoprenoids, starch, amino acids and many secondary compounds. Many of the enzymes involved in these biosynthetic pathways are located in the soluble portion of plastids (stroma) which is divided from the cytosol by a double membrane system termed envelope membrane.¹ Plastids are believed to have an endosymbiotic evolutionary origin and one of the remnants of this ancestry is the presence of a circular genome encoding approximately 110 ORFs.² The remainder of the estimated 3600 plastid proteins are nuclear encoded.^{3,4} The estimated number of plastid proteins for a higher plant is based upon subcellular target prediction analyses of open reading frames from the *Arabidopsis thaliana* genome using organelle targeting algorithms.⁵ Nuclear-encoded chloroplast proteins are transcribed

from the nuclear genome, translated in the cytosol and imported into chloroplasts through the envelope membrane via an amino-terminal targeting peptide.^{6,7} Although over 3600 proteins are predicted to be targeted to plastids, the number of characterized plastid proteins represents only a small percentage of this predicted number.^{4,8} Despite significant efforts to characterize chloroplasts and subfractions therein, less than one-fifth of the predicted number of plastid proteins have been confirmed.

Previous proteome investigations have cataloged integral and peripheral membrane proteins associated with envelope⁹ and thylakoid¹⁰ membrane fractions. These systematic investigations have provided the first view of the principal membrane systems in plastids. A less characterized sub-plastidial compartment is the stromal space, which likely contains the majority of plastid-targeted proteins.¹ Within the plastid stroma are nonmembrane, multi-enzyme complexes and macromolecular structures, many of which have yet to be characterized. It was previously noted that a major fraction of the plastid acetyl-CoA carboxylase (ACCase) multi-enzyme complex sediments in a Triton X-100 insoluble, high-density fraction.¹¹ Compositional analysis revealed this fraction was also enriched in plastome DNA, suggestive of nucleoid co-isolation. Nucleoids

* To whom correspondence should be addressed. Jay J. Thelen, University of Missouri-Columbia, Department of Biochemistry, 109 Life Science Center, Columbia, MO 65211. Tel: (573) 884-1325. Fax: (573) 884-9676. E-mail: thelenj@missouri.edu.

[†] Michigan State University.

[‡] University of Missouri-Columbia.

are dense, heterogeneous protein-nucleic acid particles where plastome replication and transcription occur.^{12–14} The sub-plastidial location of nucleoids is a matter of speculation, although biochemical evidence indicates an association with stromal-side of the envelope membrane.¹⁵ In addition to nucleoids, membrane-depleted chloroplast subfractions likely contain high molecular weight protein complexes since the multi-enzyme ACCase was also prevalent. To verify this and begin developing mechanistic models for multi-enzyme complexes and plastid nucleoids we performed the first proteomic characterization of a membrane-depleted, high-density chloroplast fraction.

Experimental Section

Plant Material, Chloroplast Isolation and Assays. Pea seeds (*Pisum sativum* L. cv Little Marvel) were sown in a 1:1 mixture of water-saturated vermiculite and peat moss-enriched soil in a growth chamber at a constant 22° C with an 8 h photoperiod (120 W m⁻²). Intact chloroplasts were isolated from dark-adapted 7-d old pea leaves using linear Percoll gradients according to the procedure of Perry et al.¹⁶ Leaves were harvested prior to illumination so that starch accumulation was minimal. Aliquots from the various steps in the isolation procedure were saved for determination of plastid purity by immunoblotting. Chlorophyll content was quantitated as described previously.¹⁷ Protein was quantitated using a Coomassie Blue dye-binding assay¹⁸ (Bio Rad, Hercules, CA) with bovine serum albumin as standard.

SDS-PAGE and Immunoblot Analyses. Samples for SDS-PAGE were heated to 95° C for 5 min in sample buffer (2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 50 mM Tris-HCl pH 8.0, 10% (v/v) glycerol) and centrifuged at 15k × g for 5 min prior to loading onto gel. SDS-PAGE and electrophoretic protein transfer was performed under standard conditions using Hoefer SE600 electrophoresis and TE-62 transfer units, respectively. Immunoblotting was performed as described previously¹¹ using monoclonal antibodies raised against native maize mitochondrial proteins, pyruvate dehydrogenase α -subunit¹⁹ and ATP synthase β -subunit.²⁰ Polyclonal antibodies against purified, recombinant plastid proteins (pyruvate dehydrogenase α - and β -subunits), cytosolic low-molecular weight acyl-CoA binding protein and ER-bound acyl-CoA binding protein were used to determine plastid, cytosolic and ER contamination, respectively. Electronic images of blots and gels (12-bit TIFF, 300 dpi) were acquired using scanning densitometry. CBB-stained protein bands were quantified with Phoretix software (Non-linear Dynamics, U.K.) using the mode of nonspot method for background subtraction. Apparent mass calibration was performed using the cubic spline curve-fitting method.

Isolation of Triton-Insoluble Fractions from Pea Chloroplasts. Intact pea chloroplasts were thoroughly resuspended to 0.5 mg chlorophyll mL⁻¹ in ice cold lysis media (50 mM HEPES-KOH pH 8.0, 10% (v/v) glycerol, 2 mM PMSF, 2 mM benzamidine, 2 mM ϵ -amino-n-caproic acid, 2 mM EDTA, 14 mM 2-mercaptoethanol, 1% (v/v) Triton X-100), incubated on ice for 5 min, and immediately centrifuged at 30 000 × g for 30 min, 4° C. Supernatants were discarded and insoluble pellets were resuspended in the same volume of lysis media using a glass homogenizer and then centrifuged at 30k × g for 30 min, 4° C. To completely extract membranes, Triton-insoluble pellets were again resuspended in the same volume of lysis media and centrifuged (30k × g for 30 min, 4° C). The final, twice-extracted Triton-insoluble pellets were resuspended in a minimal volume

of lysis media (minus Triton) for protein quantification and proteome analyses.

Identification of Proteins by Mass Spectrometry. Gel bands were excised and subjected to in-gel tryptic digestion based on method of Shevchenko et al.,²¹ and subsequently modified by Rowley et al.,²² with the following additional modifications. The trypsin digested peptides were dried in a speedvac and resuspended in a final volume of 6 μ L of 3% (v/v) trifluoroacetic acid. Liquid chromatography/mass spectrometric analysis of each in-gel digest was accomplished using the Waters CapLC system (Waters Corporation, Milford, Massachusetts) coupled to a LCQ DECA quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) through the Picoview nano-spray source (New Objectives, Cambridge, MA). Exactly 1.0 μ L of each in-gel digest was trapped on a Michrom CapTrap cartridge (Michrom BioResources, Inc. Auburn, CA) and then backflushed on a 75 μ m id × 15 cm picofrit column packed with 5 μ m 100Å Magic C18AQ material (Michrom BioResources, Inc. Auburn, CA). The picofrit column made electrical contact through a precolumn ZDV titanium union, and terminated in a 15 μ m tip id outlet spray needle. Peptides were eluted from the column using a gradient of 5% to 50% solvent B (0.1% (v/v) formic acid, 95% (v/v) acetonitrile) in 30 min at a flow rate of 250 nl/min; mobile phase A consisted of 0.1% (v/v) formic acid. Mass spectra were acquired using the Top three double play mode of operation where the top three peptide ions detected in a MS survey scan triggered data dependent MS/MS fragmentation for obtaining product ion spectra. Uninterpreted MS/MS spectra were searched against the Viridiplantae NCBI nonredundant database (June, 2004) using Mascot (Matrix Science, Ltd, U.K.) and XTandem²³ (www.thegpm.org) database mining software. XTandem²³ searches were performed using the single amino acid substitution function to increase peptide matching. False positives were identified using the reverse sequence search function of XTandem²³ and theoretical modeling function of Probitry.²⁴

XTandem uses survival functions and expectation values to assign significance to MS/MS spectra matched to an in-silico peptide fragmentation pattern. The proteins identified in this experiment using the peptides matched by the XTandem algorithm were assessed statistically using a modified Probitry²⁴ algorithm. This algorithm assigns statistical significance to the proteins identified by taking into account such factors as the number to peptides identified, the peptide scores, the number of proteins in the database searched and the number of MS/MS spectra searched against these database.

Bioinformatic Analyses. Output identification data was manually screened based upon score and number of matched peptides. Tentative identifications with a Mascot score below 70 or fewer than 2 matched peptides were discarded. Xtandem scores below -1.3 (<95% confidence; score = log(e)) and all redundant identifications were also discarded. Primary sequences for proteins identified in this investigation were submitted to two organelle targeting algorithms, ChloroP²⁵ (www.cbs.dtu.dk/services/ChloroP/) and TargetP⁶ (www.cbs.dtu.dk/services/TargetP/). Proteins that were not predicted to be plastid localized based upon ChloroP or TargetP analyses were also analyzed using Predotar v.0.5 algorithm (www.inra.fr/predotar/). Organellar assignment and corresponding output score is noted for each protein in the supplemental table.

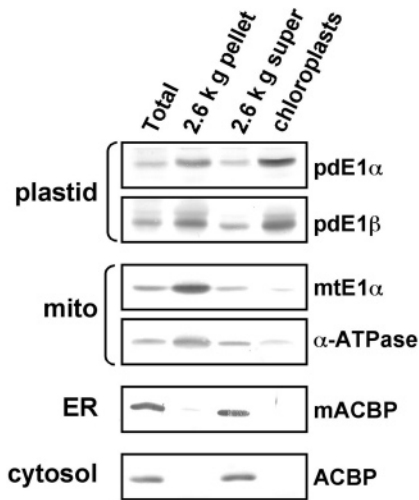


Figure 1. Validation of pea chloroplast purification by immunoblot analyses. Fractions from the chloroplast purification procedure, including the total homogenate (total), 2600 \times g pellet (2.6k \times g pellet) and supernatant (2.6k \times g super) and Percoll purified chloroplasts (chloroplasts), were quantified and equal amounts of protein (50 μ g) resolved by SDS-PAGE. Polyclonal antibodies specific to the plastid pyruvate dehydrogenase α - and β -subunits (pd E1 α and pdE1 β) were used to verify chloroplast enrichment. Monoclonal antibodies to mitochondrial pyruvate dehydrogenase α -subunit (mtE1 α) and F1-ATP synthase α -subunit (α -ATPase) were used to determine mitochondrial contamination and polyclonal antibodies to low-molecular weight (ACBP) and membrane-bound (mACBP) acyl-CoA binding proteins were used as markers for the cytosolic and ER fractions, respectively.

Results and Discussion

In Vitro Isolation Protocol Yields High Purity Chloroplasts from Pea Leaves. Integral to proteome investigations of purified organelles is experimental validation of in vitro isolation protocols and establishment of organelle purity. To determine the purity of the chloroplasts used in this investigation immunoblotting with antibodies to authentic mitochondrial, plastid, cytosol and ER proteins was employed (Figure 1). Monoclonal antibodies against maize mitochondrial pyruvate dehydrogenase α -subunit and F1 ATP synthase α -subunit detected single proteins of 43 kDa and 52 kDa, respectively, that were predominant in 2.6k \times g pellets and of low abundance in Percoll purified chloroplast fractions (Figure 1). Cytosol and ER contamination were also minimal based upon marker antibodies to low-molecular weight and membrane-bound acyl-CoA binding proteins, respectively. In contrast, plastid pyruvate dehydrogenase α - and β -subunits were highly enriched in both 2.6k \times g pellets and Percoll gradient fractions. Quantification of nonplastidial contamination, based upon these immunoblot analyses, revealed the chloroplasts used in this investigation were greater than 90% pure.

Approximately 35 Coomassie Stained SDS-PAGE Bands are Observed in Triton-Insoluble Pea Chloroplast Fractions. Previous chloroplast proteome investigations have targeted envelope and thylakoid membrane subfractions for in-depth characterization. To our knowledge, there has been no systematic analysis of a membrane-depleted, high-density chloroplast subfraction. If prepared expeditiously under stabilizing conditions, such a fraction would hypothetically contain the bulk of high molecular weight, nonmembrane macromolecular structures within chloroplasts including nucleoids, starch

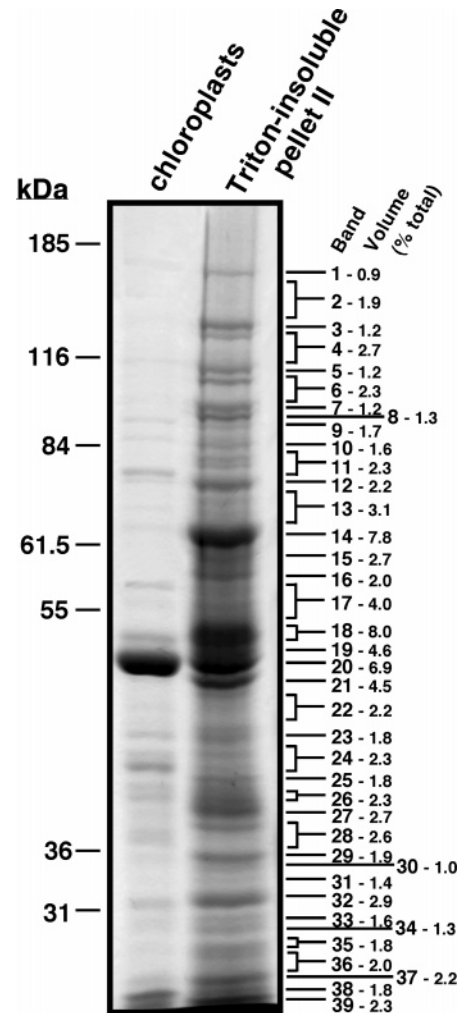


Figure 2. Polypeptide profile of Triton-insoluble 30k \times g pellet pea chloroplast preparation. Total pea chloroplasts (20 μ g) and Triton-washed, Triton-insoluble 30k \times g pellet (50 μ g) were resolved by SDS-PAGE, 9% polyacrylamide and stained with CBB. The entire Triton-insoluble lane was sectioned into 39 pieces as indicated for tryptic digestion followed by nanospray LC-MS/MS. The volume of stained protein in each section was quantified using Phoretix software (Nonlinear Dynamics) and expressed as a percentage of the total protein. Molecular weight standards are noted.

granules, and multi-enzyme complexes. Indeed, a previous investigation of the heteromeric ACCase revealed this highly labile, multi-enzyme complex could be retrieved in its intact state (based upon activity status) by de-lipidating purified chloroplasts with Triton X-100 followed by centrifugation.¹¹

Attempts to resolve proteins present in Triton-insoluble chloroplast fractions using two-dimensional gel electrophoresis resulted in substantial horizontal streaking of protein spots (data not shown), presumably due to the high nucleic acid content and hydrophobicity of proteins in this fraction. For this reason SDS-PAGE was employed instead of 2-D electrophoresis to analyze the proteins in this sample. Fractionation by SDS-PAGE and subsequent staining by Coomassie Brilliant Blue (CBB) consistently revealed 35 distinct protein bands. Five proteins of 74, 53, 52, 50, and 48 kDa corresponding to bands 14, 18, 19, 20, 21, respectively, comprised approximately 32% of the total CBB-stained protein (Figure 2). Comparison of the

SDS-PAGE band profiles with total chloroplasts revealed the protein composition of this subfraction was distinct (Figure 2).

To identify each stained protein band while also identifying those proteins below the detection of CBB, the entire lane from a one-dimensional SDS-PAGE gel was sectioned according to the predominant CBB-stained bands as well as the nonstaining 'background' regions of the gel (Figure 2). Pre-fractionation of the Triton-insoluble pea chloroplast fraction by SDS-PAGE allowed the prominent protein bands to be quantified and also reduced the complexity of each sample prior to reverse-phase capillary LC to further enhance the sensitivity of this approach. Using this approach, 341 total proteins assigned to 179 different accession numbers were identified in this investigation (Suppl. Table).

Validation of Chloroplast Purity Using Subcellular Target Prediction Algorithms. Each of the protein sequences identified in this investigation were submitted to web-based organelle targeting algorithms. Of the 179 unique proteins identified in this investigation only 15 were consensus nonplastidial, based upon ChloroP, TargetP and Predotar organellar prediction analyses (Supporting Information Table). It is likely that this number is an overestimate due to the ambiguous nature of protein annotation, particularly for unknown proteins, and the potential for dual-organelle targeting of some proteins^{26–30} which current targeting algorithms are not trained to detect. One previously characterized, dual-targeted protein is RNA polymerase which was also identified in this investigation.^{31–33} Although TargetP is considered to be a reliable organellar targeting algorithm it was observed that two known plastid proteins, oxygen-evolving enhancer protein and stearoyl-ACP desaturase, were incorrectly assigned by TargetP in the current investigation. In contrast, ChloroP and Predotar correctly localized these two proteins to plastids (Suppl. Table). Thus, it is important to verify nonplastid targeted proteins with alternative algorithms, as shown in this report, and ultimately through experimentation.

Three proteins observed in this investigation, glycolate oxidase (band 26) and glycine decarboxylase subunits aminomethyltransferase (band 24) and serine hydroxymethyltransferase (band 19), are major soluble proteins in glyoxysomes and mitochondria, respectively. The presence of these proteins in Triton-insoluble chloroplast preparations could therefore be due to protein carry-over, possibly by nonspecific binding to the outer envelope of intact plastids. As these three proteins were the few unambiguous, nonplastidial proteins identified in this investigation this explanation is more plausible than whole organellar contamination. As a whole, these data are consistent with immunoblot analyses (Figure 1) and the overall conclusion that the chloroplast preparations used in this investigation were of high purity.

Protein Composition of Triton-Insoluble Chloroplast Preparations are Distinct from Thylakoid and Envelope Membranes. Comparison of the 179 proteins identified in this investigation with those from previous proteome investigations of thylakoid¹⁰ and envelope⁹ membranes revealed only 17 (9.5%) and 42 (23%) proteins, respectively, in common with those investigations (Figure 3A). Thylakoid and envelope fractions shared 52 (23%) proteins and all three datasets had 10 proteins in common. From these data it is evident Triton-insoluble preparations are distinct from thylakoid and envelope membranes. Surprisingly, only 29% of the proteins identified here were also observed in a recent catalog of 650 chloroplast proteins identified by LC-MS/MS (Figure 3B).³⁴ The low

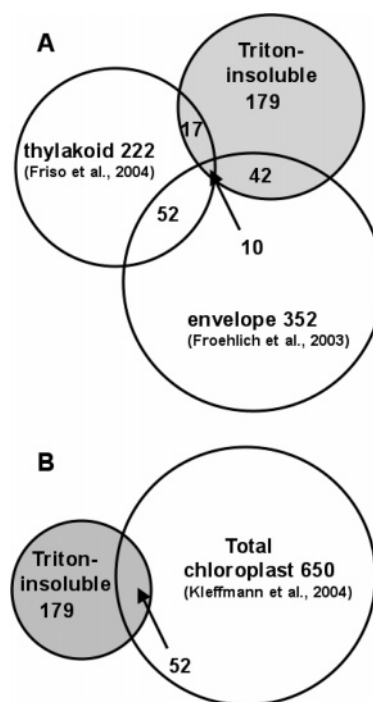


Figure 3. Venn diagram overlay of proteins identified from various chloroplast fractions. (A) Proteome analysis of purified thylakoids from *Arabidopsis* chloroplasts yielded 222 different proteins¹⁰, envelope membranes from *Arabidopsis* and pea chloroplasts resulted in 352 proteins⁹ and the current investigation produced 179 unique proteins. Total number of proteins identified and the number of coincident identifications among the proteome investigations are shown. Note that the Triton-insoluble fraction has more proteins in common with envelope than thylakoid membranes. (B) Comparison of *Arabidopsis* total chloroplast proteome³⁴ (650 proteins) with Triton-insoluble fraction. Areas of circles are proportional to number of identified proteins.

percentage of coincident proteins between these two investigations emphasizes the importance of subfractionation as a tool to extend proteome coverage.

After sorting each protein according to a fifteen class enzyme function classification scheme, adapted from the Yeast Genome Project³⁵ for the *Arabidopsis* Genome Initiative,³⁶ it was observed that 35% of the proteins identified from this investigation were involved in energy production and primary metabolism (Figure 4A). Protein synthesis and transcription classes each represented 11%, followed by cell growth/division (7%), intracellular traffic (5%) and secondary metabolism, protein destination/storage and transporters (each 4%). Unknown or hypothetical (unclassified) proteins were also a major class of proteins and they are listed in Table 1 along with the highest matching homologue from BLASTP searching of NCBI nonredundant databases. Proteins with "putative" or "like" in the Genbank identifier tag are also listed since the function of most of these proteins have yet to be confirmed.

In addition to functional classifications, proteins were also grouped according to their sub-organelle macromolecular or structural association (Figure 4B). Nucleoids contained the highest percentage of proteins (22%), although most classifications were made based upon bacterial models, as plastid nucleoids are poorly characterized structures. Multi-enzyme complexes were another abundant group of macromolecules in Triton-insoluble chloroplast preparations. Interestingly, few

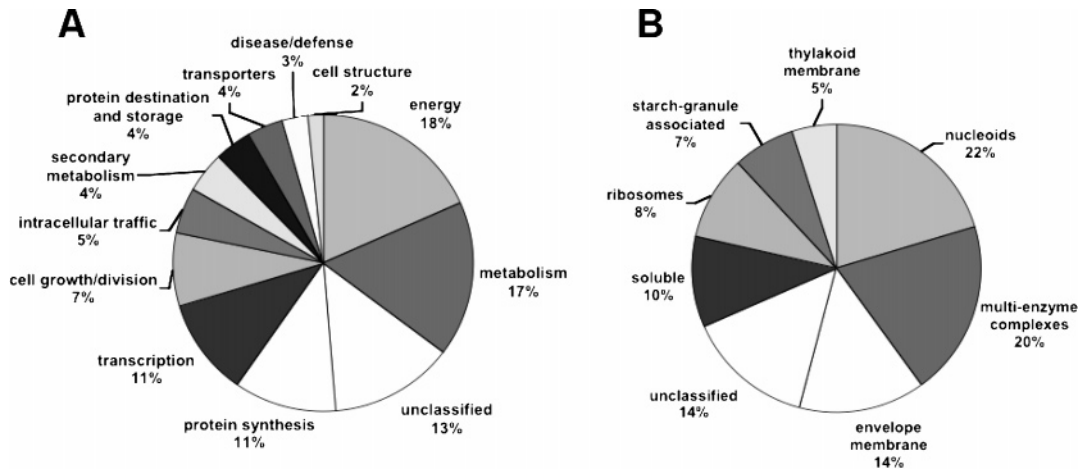


Figure 4. Enzyme function and sub-organelle structural classification of proteins identified from Triton-insoluble chloroplast fraction. Proteins annotated as unknown or hypothetical were also classified into functional and sub-organelle groups if identity was greater than 35% to a previously characterized protein. (A) Functional classification was based upon the fifteen class nomenclature scheme employed for the *Arabidopsis* genome,³⁶ which was derived from the yeast functional catalog,³⁵ with the following additional categories, secondary product metabolism and disease, defense and stress responses plus additional subcategories for plant-specific functions. (B) Classification of proteins according to sub-organelle macromolecular association according to the literature or as predicted based upon function.

of the subunits to these complexes were observed in a previous total chloroplast proteome investigation³⁴ suggesting these complexes might be present at a low copy number in chloroplasts.

Nucleoids are a Major Component of Triton-Insoluble Pea Chloroplast Preparations. An intense 68 kDa protein band (band 14) comprising approximately 8% of the total protein was identified by LC-MS/MS as one predominant protein, ferredoxin:sulfite reductase. Sulfite reductase was also a major component of bands 11, 12, 13, and 15. In addition to its primary role in sulfur assimilation for cysteine and methionine synthesis, sulfite reductase was demonstrated as one of the most abundant proteins in plastid nucleoid preparations from pea and soybean.^{37,38} In these investigations sulfite reductase was shown to be a condensing protein for plastid DNA, allowing the formation of compact protein–DNA complexes. The abundance of sulfite reductase in this fraction, in conjunction with the previous observation that nucleic acids were also prevalent¹¹ strongly supports the conclusion that nucleoids are a major component of 30k × g Triton-insoluble preparations. Interestingly, sulfite reductase was not identified from previous proteome investigations of chloroplast envelope membranes^{9,39,40} despite biochemical evidence suggesting nucleoids are tightly associated with envelope membranes.^{15,41,42}

Another class of nucleoid-associated proteins identified in this investigation were DNA gyrase-topoisomerases. Like sulfite reductase, DNA gyrases were abundant in Triton-insoluble fractions based upon intensities of CBB stained bands 7, 8, and 12. In addition to the two proteins annotated as DNA gyrase subunit A and B (accession numbers 15228353 and 15228245, respectively) two genes annotated as DNA gyrase subunit A-related (accession numbers 30681440) and DNA gyrase subunit B-like (accession number 15237634) were also identified from bands 9, 11, 13. Interrogation of the *Arabidopsis* genome with these sequences revealed only the four putative DNA gyrase genes discussed here. Interestingly, most of the sequence differences were observed at the amino-termini suggesting targeting to different organelles. The gene annotated as DNA gyrase subunit B-like (accession number 15237634) is mitochondrial-localized based upon

TargetP and Predotar prediction analyses but plastid-localized based upon ChloroP analysis, whereas accession numbers 30681440 and 15228353 are both consensus plastid localized. Although the exact role of DNA gyrases in plastid function is presently unknown, a previous investigation demonstrated these proteins are critical for nucleoid division by the use of a specific inhibitor.⁴³ The high abundance of DNA gyrases in enriched nucleoid preparations as well as the genetic redundancy also point toward an important role for these proteins in nucleoid and perhaps chloroplast division.

Other presumptive nucleoid-associated proteins identified in this investigation include putative plastid-predicted RecA DNA repair isoforms (bands 23, 24) and DNA binding proteins (bands 4, 5, 6). Another known DNA-binding protein identified in this investigation was histone. Histones are basic, low molecular weight DNA binding proteins responsible for condensing DNA.⁴⁴ However, these proteins are generally considered nuclear-localized thus it is unclear whether the polypeptide identified here is a true histone or a potentially misidentified plastid HU-protein which are similar to histones in structure and function, as demonstrated in cyanobacteria.^{45,46} The function of HU-like proteins in higher plants is not yet clear since sulfite reductase has been demonstrated to condense plastid DNA.

Transcription and Translation-Related Proteins Co-Sediment with Nucleoids. Plastid nucleoids are high-density protein–DNA particles that sediment at low centrifugal forces.^{13,47} Electron microscopy has revealed these particles are heterogeneous in structure,^{13,14,48–50} and therefore may be associated with other macromolecular structures. Previous investigations reported that purified plastid nucleoids also exhibited RNA polymerase activity although the extent of this association is not clear.^{12,13,51,52} In addition to transcription activity, it was suggested that ribosomes may also be associated with nucleoids based upon the identification of a 28 kDa ribosomal-like protein as part of nucleoid preparations from pea chloroplasts.⁴⁷

In the current investigation, numerous transcription and translation related proteins were identified in 30k × g Triton-insoluble chloroplast preparations (Figure 4). The plastid-

Table 1. Proteins Annotated as Unassigned (“unknown”, “putative”, or “hypothetical” preceding “protein” terminology in identifier tag) and Probable Proteins (“similar to”, “like”, “probable”, or “putative” in identifier) from Triton-Insoluble Purified Pea Chloroplast Fractions^a

GI no.	MW	matched peptides	ChloroP (score)	TargetP (class)	plant species	protein identity BLAST homology search (% identity)
Proteins annotated as unknown, putative or hypothetical proteins						
15242755	58,428	10	Y (0.577)	C (2)	A	metallo-beta-lactamase family protein (92%)
15226791	62,547	6	N (0.478)	C (4)	A	no similarity to known proteins
13548330	82,669	6	Y (0.536)	C (3)	A	oligoketide cyclase/lipid transport protein (17%)
15219038	37,525	5	Y (0.566)	C (2)	A	beta-tonoplast intrinsic protein-like (5%)
15236930	44,152	3	Y (0.584)	C (5)	A	putative NADPH-dependent reductase (76%)
15450597	37,525	4	Y (0.566)	C (2)	A	beta-tonoplast intrinsic protein-like (6%)
13877771	35,120	4	Y (0.543)	C (4)	A	ribosomal protein 30S subunit (52%)
15233005	34,239	2	Y (0.530)	C (2)	A	methyltransferase, putative (19%)
28416677	35,042	1	Y (0.551)	C (1)	A	hypothetical protein kinase (26%)
28393617	100,554	15	Y (0.577)	C (2)	A	metallo-beta-lactamase family protein (39%)
25278006	85,023	8	Y (0.574)	C (3)	A	UDP-N-acetylmuramoylananyl-D-glutamate-2,6-diaminopimelate ligase (50%)
26450692	60,895	3	N (0.478)	C (4)	A	no similarity to known proteins
28416497	81,000	8	Y (0.561)	C (1)	A	putative DEAD box RNA helicase (31%)
25406387	96,326	5	Y (0.542)	C (2)	A	putative chloroplast RNA processing protein (50%)
9558534	48,760	1	N (0.441)	O (3)	Os	anthranilate N-hydroxycinnamoyl/benzoyltransferase (36%)
15292887	60,145	7	Y (0.577)	C (1)	A	putative dihydrolipoamide dehydrogenase (85%)
4558676	111,490	1	Y (0.505)	C (1)	A	filament protein-related (85%)
4678934	42,275	2	Y (0.508)	M (5)	A	5'-3' exonuclease family protein (73%)
38344868	46,145	8	Y (0.566)	C (2)	Os	pyruvate dehydrogenase alpha subunit (71%)
26450902	44,831	1	Y (0.581)	C (1)	A	glyceraldehyde 3-phosphate dehydrogenase (83%)
21280837	37,378	2	Y (0.566)	C (2)	A	no similarity to known proteins
3080425	43,723	2	Y (0.584)	C (5)	A	nucleoside-diphosphate-sugar epimerase (43%)
32487387	41,183	4	Y (0.567)	C (2)	Os	protochlorophyllide reductase A, chloroplast precursor (70%)
29893583	46,354	4	Y (0.592)	C (1)	Os	50S ribosomal protein L4, chloroplast (35%)
7413642	30,567	3	Y (0.545)	C (5)	A	RNA and export factor-binding protein, putative (35%)
23296698	34,811	1	Y (0.530)	C (2)	A	methyltransferase, putative (21%)
10178029	37,206	2	Y (0.576)	C (3)	A	short-chain dehydrogenase/reductase (SDR) family protein (33%)
14597792	41,176	5	Y (0.538)	M (5)	Pp	ADP, ATP carrier protein 1 (70%)
18377498	31,823	1	Y (0.516)	C (2)	A	putative chloroplast initiation factor 3 (63%)
21592994	33,854	2	Y (0.579)	C (1)	A	ATPase involved in DNA repair (24%)
18176428	28,837	3	Y (0.570)	C (1)	A	ferritin 2, chloroplast precursor (56%)
21536493	13,259	6	Y (0.547)	C (2)	A	no similarity to known proteins
Proteins annotated as “putative” or “like” proteins						
15237634	84,615	7	Y (0.558)	M (4)	A	DNA gyrase subunit B – like protein
1522141	97,120	10	Y (0.542)	C (2)	A	unknown – similar to pentatricopeptide repeat protein
15228353	102,261	11	Y (0.589)	C (1)	A	putative DNA gyrase subunit A
6573745	172,811	4	N (0.494)	C (5)	A	unknown protein- similar to chloroplast R1 protein
30681440	104,538	2	Y (0.589)	C (1)	A	DNA gyrase subunit A -related
7488180	52,637	10	Y (0.561)	C (1)	A	probable RNA helicase
15241858	14,535	10	N (0.433)	O (1)	A	histone H2B, putative
4583548	39,939	5	Y (0.550)	C (2)	A	chloroplast FtsYhomolog
15237163	32,980	5	Y (0.506)	M (5)	A	fibrillar-like
15230982	23,834	2	Y (0.583)	C (2)	A	putative peroxiredoxin
15229443	37,780	11	Y (0.568)	C (1)	A	chloroplast ribosomal L1 – like protein
7488830	42,106	5	Y (0.589)	C (1)	P	probable malate dehydrogenase
15212142	41,494	4	Y (0.571)	C (3)	Os	putative O-acetylserine (thiol) lyase
7488841	35,878	4	Y (0.502)	C (5)	P	ribonuclease S5 homolog
15217485	42,764	11	N (0.495)	O (4)	A	putative RNA-binding protein
15230174	47,885	18	Y (0.586)	C (1)	A	putative fatty acid desaturase SBZIP, diiron protein
15228245	72,847	23	N (0.428)	O (2)	A	putative DNA gyrase subunit B
15450593	78,413	2	N (0.454)	O (2)	A	putative acyl-CoA synthetase
21536525	48,326	3	Y (0.539)	C (5)	A	dihydrolipoamide S-acetyltransferase, putative
21464567	60,757	8	Y (0.589)	C (1)	A	putative dihydrolipoamide dehydrogenase
11357296	53,781	1	Y (0.507)	C (3)	A	fructokinase-like protein
30680397	56,873	2	Y (0.561)	M (2)	A	DNA topoisomerase, ATP-hydrolyzing, putative
20453114	112,121	3	Y (0.569)	C (1)	A	putative chloroplast inner envelope protein
32129333	102,334	6	Y (0.562)	M (3)	Oj	putative DNA gyrase subunit
169128	102,711	3	Y (0.561)	C (4)	P	nuclear encoded precursor to chloroplast protein
25084218	93,726	3	Y (0.575)	C (1)	A	putative helicase
22137204	78,998	1	N (0.451)	O (4)	A	putative ABC transporter
15232029	40,551	2	N (0.432)	O (2)	A	zinc finger (C2H2 type) family protein
3738333	35,142	6	Y (0.583)	C (2)	A	putative chloroplast initiation factor 3
6634469	37,750	8	Y (0.548)	C (2)	Le	putative ascorbate peroxidase
28950693	15,082	3	N (0.431)	O (1)	A	putative histone H2B
31432862	42,766	3	Y (0.519)	C (4)	Os	putative pyruvate dehydrogenase
3850621	42,158	6	N (0.489)	O (4)	A	putative RNA binding protein
289208	47,733	1	Y (0.574)	C (2)	A	DNA repair protein (RecA homolog)

^a The genbank identification number (GI no.), predicted molecular weight (MW) and number of peptides matched from MS/MS ions searching are noted. Organelle targeting predictions are also noted. Plastid target predictions for the ChloroP algorithm were either Yes (Y) or No (N) based upon a probability score (in parentheses). Abbreviations for TargetP subcellular localization algorithm are: C, chloroplast; O, other; M, mitochondria and prediction class (1 through 5) for each protein is noted in parentheses. Plant species from which the gene originated is noted with the following abbreviations: A, *Arabidopsis thaliana*; Le, *Lycopersicon esculentum*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; P, *Pisum sativum*. BLASTP searches were performed with each unknown protein to find the highest matching homologue; percent identity is noted in parentheses.

encoded RNA polymerase β -subunit was a component of bands 4 and 5 and the plastid-encoded α -subunit was an abundant polypeptide in bands 24 and 25. Another RNA-associated protein observed in this fraction was a plastid-targeted probable RNA helicase (band 10, 11) which is involved in processes ranging from transcriptional regulation⁵³ to ribosome assembly.⁵⁴ Numerous plastid-targeted, RNA binding proteins were also identified. One particularly abundant RNA-binding protein (accession number 7446357) was the principal component of bands 33 and 34. A ribonuclease S5 homologue was observed in multiple bands and proteins identified as endoribonuclease E (band 3) and polyribonucleotide nucleotidyltransferase (band 4) were also observed in Triton-insoluble chloroplast preparations.

Plastid ribosomes were also present in Triton-insoluble chloroplast preparations based upon the number of different ribosomal proteins identified and the frequency of their identification (Figure 4, Supporting Table). In addition to ribosomal proteins three translation elongation factors and two ribonucleoproteins were also observed. These data suggest an association of the translation apparatus with nucleoids, perhaps indirectly through nascent transcripts. Alternatively, it is possible that intact ribosomes co-sediment with nucleoids due to their large mass rather than via association with nucleoids. Understanding this potential interaction will require further study, perhaps through dissociation analyses. Development of the current SDS-PAGE proteome map of should be helpful in this endeavor.

Multi-Enzyme Complexes are Enriched in 30k \times g Triton-Insoluble Chloroplast Fractions. The pyruvate dehydrogenase complex (PDC) is a labile, macromolecular assembly of at least three enzymes, pyruvate dehydrogenase (α - and β -subunits), dihydrolipoamide acetyltransferase, and lipoamide dehydrogenase. Based upon rate-zonal centrifugation, the maize mitochondrial PDC was previously estimated to be greater than 5 MDa in size,⁵⁵ due in part to a 2.7 MDa pentagonal dodecahedron central core of dihydrolipoamide acetyltransferase subunits.⁵⁶ The quaternary structure and organization of the plastid PDC is largely unknown because the plastid PDC, unlike the mitochondrial form, dissociates rapidly *in vitro*.⁵⁷ The presence of all four subunits in the membrane-depleted 30k \times g fraction characterized here (bands 17–21, 25–27) indicates this complex can be isolated under rapid extraction conditions for further structural analyses (Table 2). Since this complex sedimented at relatively low centrifugal forces association with other enzymes is possible and may signal the presence of a larger, metabolic enzyme assembly in plastids including the heteromeric acetyl-CoA carboxylase (Table 2).

The α -carboxyltransferase (α -CT) subunit to the multi-enzyme complex ACCase was detected in bands 6, 7, 8, and 12 while the β -carboxyltransferase (β -CT) subunit was observed in bands 7, 8, 9, 10, and 11 (Figure 2). Detection of CT subunits in multiple SDS-PAGE bands indicated diffuse migration during SDS-PAGE which could be the result of incomplete denaturation as these two proteins are tightly complexed by hydrophobic interactions and extensive disulfide bridging.⁵⁸ Alternatively, this could be due to incomplete chromatographic elution (also referred to as “memory effect”) from reversed-phase chromatography. Upon the basis of the number of tryptic peptides identified, the majority of α -CT was observed in bands 7 and 8 which comprised approximately 2.5% of the total protein in Triton-insoluble chloroplast preparations. The β -CT subunit was predominantly found in band 10 which repre-

sented 1.6% of total protein. In addition to the CT subunits, the biotin carboxylase (BC) and biotin carboxyl carrier protein (BCCP) subunits to ACCase were detected in bands 17, 19, 20, and 27, 28, respectively. The low number of matching peptides for BCCP could be low sequence conservation as unlike the CT subunits the pea homologues for these genes have yet to be cloned.

Bands 18, 19, and 20 were a mixed composition of ATP synthase α - and β -subunits and RuBisCO large subunit. These three proteins are present at high levels in chloroplasts and are common contaminants of *in vitro* purified envelope and thylakoid membranes.^{9,39,40,59} It is therefore plausible that these two proteins are ‘carry-over’ contaminants of Triton-insoluble fractions due to their overwhelming abundance in chloroplasts. An alternative explanation is a potential association with other proteins as part of a larger, sedimentable complex. This explanation is supported by the observation that two classes of proteins known to associate with RuBisCO, RuBisCO activase and RuBisCO binding protein subunits (groEL-related chloroplast chaperonin 60), were also prevalent (Table 2).^{59–61} Furthermore, RuBisCO was previously observed to form a complex with four other enzymes of the Calvin cycle.^{62,63} Interestingly, two of those enzymes were also observed in this investigation, phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GPDH). Surprisingly, both forms of GPDH were observed and were the major proteins in bands 24 and 26, representing approximately 4% of the total protein. GPDH was also identified as a component of bands 23, 25, and 27. Thus one possible explanation for the presence of RuBisCO, PGK, and GPDH (three soluble, stromal enzymes) in 30k \times g Triton-insoluble fractions is the isolation of an assembly of Calvin cycle enzymes plus associated chaperonins.

Conclusions

Proteomic analysis of a membrane-depleted, high-density fraction from purified pea chloroplasts resulted in the identification of 179 different proteins, almost 97% of which are known or predicted plastid proteins. This chloroplast fraction was biochemically distinct from envelope and thylakoid membranes since only 23% and 9%, respectively, of the 179 proteins were in common with previous proteome investigations of these fractions. Assignment of abundant protein bands as sulfite reductase (plastome condensing protein) and DNA gyrase confirmed nucleoids were a major component of this fraction. In addition to nucleoids, large multi-subunit complexes were also present in this fraction including ribosomes, ACCase, and PDC. The first three enzymes of the Calvin cycle, RuBisCO activase and RuBisCO binding proteins were surprisingly abundant and, if associated, could explain sedimentation in Triton-insoluble 30k \times g fractions. Indeed, these data support the previous proposal of a large, multi-enzyme assembly to facilitate metabolite channeling of reductive pentose phosphate pathway intermediates.⁶³ Despite rigorous de-lipidation with Triton X-100 detergent, sixteen envelope membrane proteins were also identified suggesting domains of this membrane system are recalcitrant to Triton extraction. Surprisingly, 32 proteins were classified as unknown or hypothetical proteins, most matching to Arabidopsis ORFs. Because this was the first proteomic analysis of a membrane-depleted, high-density chloroplast subfraction the high number of unknown and hypothetical proteins is not surprising. Since only the heteromeric ACCase was known to be localized to this chloroplast fraction, this investigation demonstrates the utility of

Table 2. Proteins Identified from Triton-Insoluble Pea Chloroplast Fraction Associated with Macromolecular Structures^a

GI no.	predicted mass	Mascot/ XTandem score	matched peptides	ChloroP (score)	TargetP (class)	Predotar	plant	protein
Multi-enzyme complexes								
<i>acetyl-CoA carboxylase complex</i>								
7619891	91,369	2141	80	Y (0.569)	C (1)		P	acetyl-CoA carboxylase alpha subunit
1703061	67,702	636	27		C (PE)		P	acetyl-CoA carboxylase carboxyl transferase subunit beta
3219361	58,808	-96.3	14	Y (0.558)	C (2)		G	biotin carboxylase precursor
3334130	27,754	81	3	Y (0.569)	C (4)	C (0.97)	G	biotin carboxyl carrier protein of ACCase
<i>pyruvate dehydrogenase complex</i>								
15223294	47,600	319	13	Y (0.573)	C (1)		A	pyruvate dehydrogenase E1 alpha subunit
15220670	44,245	130	6	Y (0.579)	C (1)		A	pyruvate dehydrogenase E1 beta subunit
15230922	50,106	184	9	Y (0.592)	C (1)		A	dihydrolipoamide S-acetyltransferase
21464567	60,757	-58.1	8	Y (0.589)	C (1)		A	putative dihydrolipoamide dehydrogenase
<i>ATP synthetase complex</i>								
114522	54,649	300	6		C (PE)		P	ATP synthase alpha chain
114560	53,147	699	25		C (PE)		P	ATP synthase beta chain
114640	41,841	293	11	Y (0.578)	C (1)		P	ATP synthase gamma chain
<i>RuBisCO and Calvin cycle complexes</i>								
2506277	63,28	649	20	Y (0.562)	C (1)		P	rubisco binding-protein beta subunit (60 kd chaperonin)
1710807	61,99	603	19	Y (0.559)	C (2)		P	rubisco binding-protein alpha subunit (60 kd chaperonin)
3893097	54,206	956	60		C (PE)		P	ribulose 1,5-bisphosphate carboxylase, large subunit
120658	43,597	835	30	Y (0.564)	C (2)		P	glyceraldehyde 3-phosphate dehydrogenase a
120663	48,580	93	29	Y (0.536)	C (2)		P	glyceraldehyde 3-phosphate dehydrogenase b
3914605	48,217	361	11	Y (0.590)	C (1)		Md	ribulose bisphosphate carboxylase/oxygenase activase
7434544	50,594	225	10	Y (0.583)	C (1)		St	phosphoglycerate kinase precursor
Nucleoids, transcription, translation								
133405	39,194	694	26		C (PE)		P	DNA-directed RNA polymerase alpha chain
11465946	121,156	522	18		C (PE)		Nt	putative RNA polymerase beta subunit
12658639	64,124	321	14	Y (0.587)	C (1)		G	ferredoxin:sulfite reductase precursor
7488820	98,786	828	21	Y (0.580)	C (1)		P	polyribonucleotide nucleotidyltransferase
7488796	69,741	918	37	N (0.463)	O (3)	C (0.50)	P	DNA-binding protein PD2
15228353	102,261	23	11	Y (0.589)	C (1)		A	putative DNA gyrase subunit A
32129333	102,334	-24.1	6	Y (0.562)	M (3)	M (0.83)	Oj	putative DNA gyrase subunit
30681440	104,538	114	2	Y (0.589)	C (1)		A	DNA gyrase subunit A -related
15228245	72,847	492	23	N (0.428)	O (2)	N	A	putative DNA gyrase subunit B
25084218	93,726	-4.7	3	Y (0.575)	C (1)		A	putative helicase
7488180	52,637	258	10	Y (0.561)	C (1)		A	probable RNA helicase
250009	48,159	241	6	Y (0.574)	C (2)		A	DNA repair protein RecA
7488841	35,878	438	14	Y (0.502)	C (5)	M (0.58)	P	ribonuclease S5 homolog
3738333	35,142	-9.9	6	Y (0.583)	C (2)		A	putative chloroplast initiation factor 3
122083	15,21	150	5	Y (0.547)	C (3)		Ea	histone H3
26453355	43,941	-14.4	5	Y (0.597)	C (1)		Le	mRNA binding protein precursor
3850621	42,158	-34.8	6	N (0.489)	O (4)	C (0.97)	A	putative RNA binding protein
7446357	32,131	396	13	Y (0.587)	C (1)		P	RNA-binding protein
1076251	31,963	-9.9	3	Y (0.586)	C (1)		M	RNA-binding protein
629557	33,937	82	4	Y (0.587)	C (4)	C (0.99)	A	RNA-binding protein RNP-D precursor
<i>ribosome-associated</i>								
15229443	37,780	114	3	Y (0.568)	C (1)		A	chloroplast ribosomal L1 - like protein
1350625	23,481	48	24	Y (0.568)	C (1)		P	50S ribosomal protein L1
3914666	30,506	80	3	Y (0.576)	C (2)		A	50S ribosomal protein L4, (R-protein L4)
400994	29,890	258	8	Y (0.531)	C (1)		P	chloroplast 50S ribosomal protein L2
400986	27,867	454	19	Y (0.554)	C (2)		P	50S ribosomal protein L15
7440606	28,352	290	12	Y (0.531)	C (1)		Nt	ribosomal protein L3 precursor, chloroplast
133913	27,167	302	13		C (PE)		P	chloroplast 30S ribosomal protein S2
13518426	23,366	100	3		C (PE)		Lj	30S ribosomal protein S4
6015084	53,131	707	28	Y (0.595)	C (1)		P	elongation factor tu (EF-TU)
133248	35,255	88	3	Y (0.552)	C (3)		Ns	31 kDa ribonucleoprotein
133247	25,330	97	4	Y (0.586)	C (1)		S	28 kDa ribonucleoprotein, chloroplast (28RNP)
Envelope membranes								
20453114	112,121	-5.9	3	Y (0.569)	C (1)		A	putative chloroplast inner envelope protein
15450593	78,413	-3.7	2	N (0.454)	O(2)	C (0.24)	A	putative acyl-CoA synthetase
729842	35,709	243	8	Y (0.575)	C(2)		P	membrane-associated 30 kDa protein
6635349	21,664	162	3	N (0.474)	C(5)	N	Ls	37 kDa chloroplast inner envelope membrane protein
7488813	110,523	1746	59	Y (0.574)	C(2)		P	import intermediate-associated 100K protein precursor
7453538	64,498	-6.9	2		C (OM)		P	Toc64 chloroplast protein translocon
21616072	56,901	-11.2	4	Y (0.540)	C (3)	C (0.26)	P	Tic62 chloroplast import protein
3915035	45,221	138	7	Y (0.558)	M (5)	C (0.94)	H	acyl-ACP desaturase (stearoyl-ACP desaturase)
1076524	96,660	690	15		C (OM)		P	chloroplast outer envelope protein OEP86 precursor
1363492	88,728	653	18		C (OM)		P	outer envelope membrane protein OEP75 precursor
1172558	29,579	554	26		C (OM)		P	outer plastidial membrane protein porin
510190	34,193	370	12		C (OM)		P	chloroplast outer envelope protein 34

^a Nonredundant proteins that could be classified as associated with multi-enzyme complexes, nucleoids (including transcription and translation), or envelope membranes are listed. Assignments are based upon the literature, where possible, and tentative otherwise. It should be noted that many of the identified proteins could be classified into multiple categories, for example, carboxyltransferase subunits to the acetyl-CoA carboxylase multi-enzyme complex are also associated with envelope membranes.¹¹ Genbank identification number, predicted mass, Mascot or XTandem (log(e)) score and number of identified tryptic peptides are shown. Subcellular target prediction analyses with deduced sequences were performed using ChloroP and TargetP prediction algorithms. If predictions from these two algorithms did not agree then a third algorithm (Predotar) was employed. Plastid targeting for the ChloroP algorithm was either Yes (Y) or No (N) based upon a probability score (in parentheses). Abbreviations for TargetP and Predotar subcellular localization algorithms are: C, chloroplast; O, other; M, mitochondria; N, neither plastid nor mitochondria. TargetP prediction class (1 through 5) for each protein is noted in parentheses. Plastid-encoded (PE) and outer membrane (OM) proteins are noted. Abbreviations for plant sources are as follows: A, *Arabidopsis thaliana*; Ea, *Encephalartos altensteinii*; G, *Glycine max*; H, *Helianthus annuus*; Ls, *Lactuca sativa*; Lj, *Lotus japonicus*; Le, *Lycopersicon esculentum*; Md, *Malus domestica*; M, *Mesembryanthemum crystallinum*; Nt, *Nicotiana tobacum*; P, *Pisum sativum*; St, *Solanum tuberosum*; S, *Spinacia oleracea*.

proteomics as a tool to rapidly dissect a protein fraction of unknown composition. This investigation will provide a foundation for future research on nucleoids and multi-enzyme complexes in plastids.

Abbreviations: ACCase, acetyl-coenzyme A carboxylase; BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; CBB, Coomassie Brilliant Blue; DTT, dithiothreitol; ESI, electrospray ionization; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RuBisCO, ribulose-1,5-bisphosphate carboxylase oxygenase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; PDH, pyruvate dehydrogenase; PDC, pyruvate dehydrogenase complex; PGK, phosphoglycerate kinase.

Acknowledgment. This investigation was supported by National Science Foundation Young Investigator Award DBI-0332418 and USDA-NRI grant 2003-00659. The authors also thank the anonymous reviewers of this manuscript for helpful comments and suggestions.

Supporting Information Available: Supporting Information Table of proteins identified from each SDS-PAGE band submitted for tandem mass spectrometry analysis. This material is available free of charge at <http://pubs.acs.org>.

References

- Douce, R.; Joyard, J. *Methods in Chloroplast Molecular Biology*; Elsevier Biomedical Press: New York, 1982.
- Sugiura, M. The chloroplast genome. *Essays Biochem.* **1995**, *30*, 49–57.
- Arabidopsis Genome Initiative. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **2000**, *408*, 796–813.
- Koo, A. J. K.; Ohlrogge, J. B. The predicted candidates of Arabidopsis plastid inner envelope membrane proteins and their expression profiles. *Plant Physiol.* **2002**, *130*, 823–836.
- Emanuelsson, O.; Nielsen, H.; Brunak, S.; von Heijne, G. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* **2000**, *300*, 1005–1016.
- Bruce, B. D. Chloroplast transit peptides: structure, function and evolution. *Trends Cell Biol.* **2000**, *10*, 440–447.
- Soll, J. Protein import into chloroplasts. *Curr. Opin. Plant Biol.* **2002**, *6*, 529–535.
- Jan van Wijk, K. Proteomics of the chloroplast: experimentation and prediction. *Trends Plant Sci.* **2000**, *10*, 420–425.
- Froehlich, J. E.; Wilkerson, C. G.; Ray, W. K.; McAndrew, R. S.; Osteryoung, K. W.; Gage, D. A.; Phinney, B. S. Proteomic study of the *Arabidopsis thaliana* chloroplast envelope membrane utilizing alternatives to traditional two-dimensional electrophoresis. *J. Proteome Res.* **2003**, *2*, 413–425.
- Friso, G.; Giacomelli, L.; Ytterberg, A. J.; Peltier, J. B.; Rudella, A.; Sun, Q.; Wijk, K. J. In-depth analysis of the thylakoid membrane proteome of *Arabidopsis thaliana* chloroplasts: new proteins, new functions, and a plastid proteome database. *Plant Cell* **2004**, *16*, 478–499.
- Thelen, J. J.; Ohlrogge, J. B. The multisubunit acetyl-CoA carboxylase is strongly associated with the chloroplast envelope through nonionic interactions to the carboxyltransferase subunits. *Arch. Biochem. Biophys.* **2002**, *400*, 245–257.
- Briat, J. F.; Gigot, C.; Lahlouche, J. P.; Mache, R. Visualization of a spinach plastid transcriptionally active DNA-protein complex in a highly condensed structure. *Plant Physiol.* **1982**, *69*, 1205–1211.
- Baumgartner, B. J.; Mullet, J. E. Plastid DNA synthesis and nucleic acid-binding proteins in developing barley chloroplasts. *J. Photochem. Photobiol.* **1991**, *11*, 203–218.
- Hansmann, P.; Falk, H.; Ronai, K.; Sitte, P. Structure, composition, and distribution of plastid nucleoids in *Narcissus pseudonarcissus*. *Planta* **1985**, *164*, 459–472.
- Sato, N.; Ohshima, K.; Watanabe, A.; Ohta, N.; Nishiyama, Y.; Joyard, J.; Douce, R. Molecular characterization of the PEND protein, a novel bZIP protein present in the envelope membrane that is the site of nucleoid replication in developing plastids. *Plant Cell* **1998**, *10*, 859–872.
- Perry, S. E.; Li, H.-S.; Keegstra, K. In vitro reconstitution of protein transport into chloroplasts. *Methods Cell Biol.* **1991**, *34*, 327–344.
- Arnon, D. I. *Plant Physiol.* **1949**, *24*, 1–15.
- Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
- Thelen, J. J.; Miernyk, J. A.; Randall, D. D. Molecular cloning and expression analysis of the mitochondrial pyruvate dehydrogenase from maize. *Plant Physiol.* **1999**, *119*, 635–643.
- Luethy, M. H.; Horak, A.; Elthon, T. E. Monoclonal antibodies to the α - and β -subunits of the plant mitochondrial F1-ATPase. *Plant Physiol.* **1993**, *101*, 931–937.
- Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **1996**, *68*, 850–858.
- Rowley, A.; Choudhary, J. S.; Marzioch, M.; Ward, M. A.; Weir, M.; Solari, R. C. E.; Blackstock, W. P. Applications of protein mass spectrometry in cell biology. *Methods Enzymol.* **2000**, *20*, 383–397.
- Craig, R.; Beavis, R. C. TANDEM: matching proteins with mass spectra. *Bioinformatics* **2004**, *20*, 1466–1467.
- Eriksson, J.; Fenyö, D. Probioty: A protein identification algorithm with accurate assignment of the statistical significance of the results. *J. Proteome Res.* **2004**, *3*, 32–36.
- Emanuelsson, O.; Nielsen, H.; von Heijne, G. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci.* **1999**, *8*, 978–984.
- Peeters, N.; Small, I. Dual targeting to mitochondria and chloroplasts. *Biochim. Biophys. Acta* **2001**, *1541*, 54–63.
- Silva-Filho, M. C. One ticket for multiple destinations: dual targeting of proteins to distinct subcellular locations. *Curr. Opin. Plant Biol.* **2003**, *6*, 589–595.
- Obara, K.; Sumi, K.; Fukuda, H. The use of multiple transcription starts causes the dual targeting of *Arabidopsis* putative monodehydroascorbate reductase to both mitochondria and chloroplasts. *Plant Cell Physiol.* **2002**, *43*, 697–705.
- Bhushan, S.; Lefebvre, B.; Stahl, A.; Wright, S. J.; Bruce, B. D.; Boutry, M.; Glaser, E. Dual targeting and function of a protease in mitochondria and chloroplasts. *EMBO Rep.* **2003**, *4*, 1073–1077.
- Chabregas, S. M.; Luche, D. D.; Farias, L. P.; Ribeiro, A. F.; van Sluys, M. A.; Menc, C. F.; Silva-Filho, M. C. Dual targeting properties of the N-terminal signal sequence of *Arabidopsis thaliana* TH11 protein to mitochondria and chloroplasts. *Plant Mol. Biol.* **2001**, *46*, 639–650.
- Kobayashi, Y.; Dokiya, Y.; Sugita, M. Dual targeting of phage-type RNA polymerase to both mitochondria and plastids is due to alternative translation initiation in single transcripts. *Biochem. Biophys. Res. Commun.* **2001**, *289*, 1106–1113.
- Richter, U.; Kiessling, J.; Hedtke, B.; Decker, E.; Reski, R.; Borner, T.; Weihe, A. Two RpoT genes of *Physcomitrella patens* encode phage-type RNA polymerases with dual targeting to mitochondria and plastids. *Gene* **2002**, *290*, 95–105.
- Hedtke, B.; Legen, J.; Weihe, A.; Herrmann, R. G.; Borner, T. Six active phage-type RNA polymerase genes in *Nicotiana tabacum*. *Plant J.* **2002**, *30*, 625–637.
- Kleffmann, T.; Russenberger, D.; von Zychlinski, A.; Christopher, W.; Sjolander, K.; Gruissem, W.; Baginsky, S. The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. *Curr. Biol.* **2004**, *14*, 354–362.
- Mewes, H.-W.; et al. Overview of the yeast genome. *Nature* **1997**, *387*, 7–84.
- The EU Arabidopsis Genome Project. Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature* **1998**, *391*, 485–488.
- Sato, N.; Nakayama, M.; Hase, T. The 70-kDa major DNA-compacting protein of the chloroplast nucleoid is sulfite reductase. *FEBS Lett.* **2001**, *487*, 347–350.
- Chi-Ham, C. L.; Keaton, M. A.; Cannon, G. C.; Heinhorst, S. The DNA-compacting protein DCP68 from soybean chloroplasts is ferredoxin: sulfite reductase and co-localizes with the organellar nucleoid. *Plant Mol. Biol.* **2002**, *49*, 621–631.
- Ferro, M.; Salvi, D.; Riviere-Rolland, H.; Vermaat, T.; Seigneurin-Berny, D.; Grunwald, D.; Garin, J.; Joyard, J.; Rolland, N. Integral membrane proteins of the chloroplast envelope: Identification and subcellular localization of new transporters. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11487–11492.

- (40) Ferro, M.; Salvi, D.; Brugiére, S.; Miras, S.; Kowalski, S.; Louwagie, M.; Garin, J.; Joyard, J.; Rolland, N. Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana*. *Mol. Cell Proteomics* **2003**, *2*, 325–345.
- (41) Sato, N.; Albrieux, C.; Joyard, J.; Douce, R.; Kuroiwa, T. Detection and characterization of a plastid envelope DNA-binding protein which may anchor plastid nucleoids. *EMBO J.* **1993**, *12*, 555–561.
- (42) Sato, N.; Rolland, N.; Block, M. A.; Joyard, J. Do plastid envelope membranes play a role in the expression of the plastid genome? *Biochimie* **1999**, *81*, 619–629.
- (43) Itoh, R.; Takahashi, H.; Toda, K.; Kuroiwa, H.; Kuroiwa, T. DNA gyrase involvement in chloroplast-nucleoid division in *Cyanidioschyzon merolae*. *Eur. J. Cell Biol.* **1997**, *73*, 252–258.
- (44) Khorasanizadeh, S. The nucleosome: from genomic organization to genomic regulation. *Cell* **2004**, *116*, 259–272.
- (45) Crevel, G.; Laine, B.; Sautiere, P.; Galleron, C. Isolation and characterization of DNA-binding proteins from the cyanobacterium *Synechococcus* sp. PCC7002 (*Agmenellum quadruplicatum*) and from spinach chloroplasts. *Biochim. Biophys. Acta* **1989**, *1007*, 36–43.
- (46) Kobayashi, T.; Takahara, M.; Miyagishima, S.; Kuroiwa, H.; Sasaki, N.; Ohta, N.; Matsuzaki, M.; Kuroiwa, T. Detection and localization of a chloroplast-encoded HU-like protein that organizes chloroplast nucleoids. *Plant Cell* **2002**, *14*, 1579–1589.
- (47) Oleskina, Y. P.; Yurina, N. P.; Odintsova, T. I.; Egorov, T. A.; Otto, A.; Wittmann-Liebold, B.; Odintsova, M. S. Nucleoid proteins of pea chloroplasts: detection of a protein homologous to ribosomal protein. *Biochem. Mol. Biol. Intl.* **1999**, *47*, 757–763.
- (48) Nakano, T.; Sato, F.; Yamada, Y. Analysis of nucleoid-proteins in tobacco chloroplasts. *Plant Cell Physiol.* **1993**, *3*, 873–880.
- (49) Yurina, N. P.; Belkina, G. G.; Karapetyan, N. V.; Odintsova, M. S. Nucleoids of pea chloroplasts: microscopic and chemical characterization. Occurrence of histone-like proteins. *Biochem. Mol. Biol. Intl.* **1995**, *36*, 145–154.
- (50) Oleskina, Y. P.; Yurina, N. P.; Mel'nik, S. M.; Belkina, G. G.; Odintsova, M. S. DNA-binding proteins and structural characteristics of chloroplast nucleoids. *Russ. J. Plant Physiol.* **2001**, *48*, 487–492.
- (51) Hallick, R. B.; Lipper, C.; Richards, O. C.; Rutter, W. J. Isolation of a transcriptionally active chromosome from chloroplasts of *Euglena gracilis*. *Biochemistry* **1976**, *15*, 3039–3045.
- (52) Briat, J. F.; Laulhere, J. P.; Mache, R. Transcription activity of a DNA-protein complex isolated from spinach plastids. *Eur. J. Biochem.* **1979**, *98*, 285–292.
- (53) Silverman, E.; Edwalds-Gilbert, G.; Lin, R. J. DEAD/H-box proteins and their partners: helping RNA helicase unwind. *Gene* **2003**, *312*, 1–16.
- (54) Charollais, J.; Pflieger, D.; Vinh, J.; Dreyfus, M.; Iost, I. The DEAD-box RNA helicase SrmB is involved in the assembly of 50S ribosomal subunits in *Escherichia coli*. *Mol. Microbiol.* **2003**, *48*, 1253–1265.
- (55) Thelen, J. J.; Miernyk, J. A.; Randall, D. D. Partial purification and characterization of the maize mitochondrial pyruvate dehydrogenase complex. *Plant Physiol.* **1998**, *116*, 1443–1450.
- (56) Thelen, J. J.; Muszynski, M. G.; David, N. R.; Luethy, M. H.; Elthon, T. E.; Miernyk, J. A.; Randall, D. D. The dihydrolipoamide S-acetyltransferase subunit of the mitochondrial pyruvate dehydrogenase complex from maize contains a single lipoyl domain. *J. Biol. Chem.* **1999**, *274*, 21769–21775.
- (57) Camp, P. J.; Randall, D. D. Purification and characterization of the pea chloroplast pyruvate dehydrogenase complex. *Plant Physiol.* **1985**, *77*, 571–577.
- (58) Kozaki, A.; Mayumi, K.; Sasaki, Y. Thiol-disulfide exchange between nuclear-encoded and chloroplast-encoded subunits of pea acetyl-CoA carboxylase. *J. Biol. Chem.* **2001**, *276*, 39919–39925.
- (59) Peltier, J.-B.; Friso, G.; Kalume, D. E.; Roepstorff, P.; Nilsson, F.; Adamska, I.; van Wijk, K. J. Proteomics of the chloroplast: systematic identification and targeting analysis of luminal and peripheral thylakoid proteins. *Plant Cell* **2000**, *12*, 319–341.
- (60) Lilley, R. M.; Portis, Jr. A. R. ATP hydrolysis activity and polymerization state of ribulose-1,5-bisphosphate carboxylase oxygenase activase (do the effects of Mg²⁺, K⁺, and activase concentrations indicate a functional similarity to actin?). *Plant Physiol.* **1997**, *114*, 605–613.
- (61) Lubben, T. H.; Donaldson, G. K.; Viitanen, P. V.; Gatenby, A. A. Several proteins imported into chloroplasts form stable complexes with the GroEL-related chloroplast molecular chaperone. *Plant Cell* **1989**, *1*, 1223–1230.
- (62) Sanchez de Jimenez, E.; Medrano, L.; Martinez-Barajas, E. RuBisCO activase: a possible new member of the molecular chaperone family. *Biochemistry* **1995**, *34*, 2826–2831.
- (63) Gontero, B.; Cardenas, M. L.; Ricard, J. A functional five-enzyme complex of chloroplasts involved in the Calvin cycle. *Eur. J. Biochem.* **1988**, *173*, 437–443.

PR049791K