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The Characterization of a Mitochondrial Acyl Carrier Protein Isoform Isolated from *Arabidopsis thaliana*[1]

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A cDNA clone was isolated from an *Arabidopsis* leaf cDNA library that shared a high degree of protein sequence identity with mitochondrial acyl carrier proteins (mtACPs) isolated from *Neurospora crassa* and bovine heart muscle. The cDNA encoded an 88-amino acid mature protein that was preceded by a putative 35-amino acid presequence. In vitro protein import studies have confirmed that the presequence specifically targets this protein into pea mitochondria but not into chloroplasts. These studies indicated that pea mitochondria were not only able to import and process the precursor protein but also possessed the ability to acylate the mature protein. The mitochondrial localization of this protein, mtACP-1, was confirmed by western blot analysis. *Arabidopsis* mitochondrial protein extracts contained two cross-reacting bands that comigrated with the mature mtACP-1 and acylated mtACP-1 proteins. The acylated form of mtACP-1 was approximately 4 times more abundant than the unacylated form and appeared to be localized predominantly in the mitochondrial membrane where the unacylated mtACP-1 was present mostly in the matrix fraction. A chloroplast fatty acid synthase system was used, and mtACP-1 was able to function as a cofactor for fatty acid synthesis. However, predominantly short- and medium-chain fatty acids were produced in fatty acid synthase reactions supplemented with mtACP-1, suggesting that mtACP-1 may be causing premature fatty acid chain termination.

ACPs are protein cofactors essential in de novo fatty acid synthesis (Ohlrogge, 1987). ACPs function to shuttle the elongating fatty acid chain through the reactions of fatty acid synthesis. These proteins are also involved in the initial desaturation and acyl transfer of 16- and 18-carbon fatty acids (Guerra et al., 1986; Ohlrogge, 1987). In animals and fungi, ACP exists as a subdomain of the multifunctional FAS polypeptide (Wakil et al., 1983), whereas in plants and most bacteria, ACPs are soluble low mol wt acidic proteins (Ohlrogge, 1987).

Although ACPs are most commonly associated with fatty acid metabolism, ACPs are also known to function in the synthesis of membrane-derived oligosaccharides (Therisod et al., 1986) and polyketides (Shen et al., 1992). ACP has also been implicated as an acyl donor involved in the activation of hemolysin, an *Escherichia coli* membrane-targeted toxin (Issartel et al., 1991).

Recently, ACPs have been reported to occur in mitochondria of *Neurospora* and bovine heart muscle (Brody and Mikolajczyk, 1988; Runswick et al., 1991; Sackmann et al., 1991). The role of mtACP is not well understood. The mtACP has been identified as a component of the NADH:ubiquinone oxidoreductase complex (complex I) of *Neurospora* (Sackmann et al., 1991) and bovine heart muscle mitochondria (Runswick et al., 1991). The acylated form of the mtACP is found to be associated with a matrix domain of the membrane-bound NADH:ubiquinone oxidoreductase complex. The function of the mtACP in complex I has not yet been determined. It has also been reported that *Neurospora* mitochondria are capable of de novo fatty acid synthesis (Mikolajczyk and Brody, 1990; Zensen et al., 1992), implying that ACP may act as a cofactor of mitochondria FAS. However, because the animal or fungal cytoplasmic FAS is thought to be sufficient to maintain fatty acid metabolism in the cell, it is unclear why this redundant pathway exists. It has also been suggested that mitochondrial FAS may be necessary for the synthesis of mitochondria-specific membrane lipids such as cardiolipin (Mikolajczyk and Brody, 1990; Zensen et al., 1992). In this regard, Brody et al. (1990) reported that in vitro labeling of *Neurospora* mitochondria with [2-^{14}C]malonic acid resulted in the radiolabel accumulating as myristoyl moieties in the cardiolipin and phospholipid fractions.

Although a plant mtACP had not been isolated, antibodies specific to the *Neurospora* mtACP are reported to cross-react with pea leaf and potato tuber mitochondrial protein extracts (Chuman and Brody, 1989). Although evidence to date supports the widely held belief that plant ACPs are predominately localized in the plastids (Ohlrogge et al., 1978), the occurrence of ACP in plant mitochondria suggests that it may be necessary to re-evaluate not only the compartmentalization of FAS but also possible alternative functions of ACP in plants.

In this report, we describe the isolation and characterization of a putative *Arabidopsis* mtACP cDNA clone. This ACP mtACP-1 shares a high degree of sequence similarity to the *Neurospora* and bovine mtACPs and contains an N-terminal extension that resembled a mitochondrial presequence. The

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Abbreviations: ACP, acyl carrier protein; cpACP, chloroplast acyl carrier protein; FAME, fatty acid methyl ester; FAS, fatty acid synthase; HAS, holo-acyl carrier protein synthetase; mtACP, mitochondrial acyl carrier protein.

subcellular localization of this ACP and its relationship to fatty acid synthesis are the focus of this research.

MATERIALS AND METHODS

Screening of the cDNA Library

The mtACP clone, pACP25, was isolated from a λ ZAP (Stratagene, La Jolla, CA) cDNA library constructed from mRNA purified from cold-acclimated *Arabidopsis* plants (Lin and Thomashow, 1992). Approximately 60,000 plaques transferred to duplicate nitrocellulose filters (Sambrook et al., 1989) were screened using a 5′ end ^{32}P-labeled (Sambrook et al., 1989) 66-nucleotide oligomer corresponding to a region containing the phosphopantetheine-binding site (Hansen, 1987). The filters were prehybridized for 2 h and hybridized overnight in 5× SSPE (1× SSPE = 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA [pH 7.4]), 1% (w/v) SDS, 0.1% (w/v) blotto at 42°C. The filters were then washed three times for 15 min in 5× SSPE, 1% (w/v) SDS at 42°C and then used to expose x-ray film overnight at −80°C with intensifying screens. pBluescript SK− (Stratagene) plasmids containing desired cDNA sequences were excised from the λ ZAP vector (Stratagene) as described by Short et al. (1988). All further manipulations were performed in pBluescript KS+ as described by Sambrook et al. (1989). The identities of positive cDNA clones were confirmed by double-stranded dideoxy sequencing with Sequenase 2.0 (United States Biochemical) (Sanger et al., 1977).

In Vitro Transcription/Translation of *Arabidopsis* Pre-mtACP-1 and Spinach Pre-cpACP-1 Precursors

The DNA sequence corresponding to the mtACP-1 precursor peptide was amplified away from the noncoding sequences of the cDNA clone pACP25 by PCR (Mullis and Faloona, 1987). The forward primer (JO107 = 5′-CGGGATCCATGGCACTGAGAAATGC-3′) and reverse primer (JO94 = 5′-CGGGATCCGCTAGACATTGGATGATT-3′) had *Bam*HI sites engineered into their respective 5′ ends to facilitate subcloning into pBluescript II KS+. The sequence was amplified with *Taq* DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA) as described by Mullis and Falloona (1987). Thirty cycles of the following thermocycle profile was used: 1 min at 94°C, 2 min at 55°C, 2 min at 72°C. The PCR product was digested with *Bam*HI, subcloned into pBluescript II KS+, and designated p0527. p0527 was linearized with *Eco*RV and used as a template to generate pre-mtACP-I transcript using T7 RNA polymerase (Boehringer Mannheim). The spinach cpACP-1 RNA template construct (Schmid and Ohlrogge, 1990) was linearized with *Bam*HI, and the pre-cpACP-1 transcript was synthesized using T3 RNA polymerase (Boehringer Mannheim). In the presence of [^{35}S]Met, 10 μg of each precursor transcript were translated using a rabbit reticulocyte lysate system (Promega).

Mitochondrial and Chloroplast Protein Import Studies

Mitochondria were purified from 400 g of 14-d-old green pea seedlings (Little Marvel; Burpee Seeds, Warminster, PA) grown at 20°C on a 12-h light/dark photoperiod as described by Fang et al. (1987). The intactness of the mitochondria was determined by the latency of Cyt *c* oxidase activity after Triton X-100 treatment (Neurburger et al., 1982).

Mitochondrial protein import reactions were performed as described by Watts et al. (1992) with the following modifications. Mitochondria corresponding to approximately 150 μg of protein were incubated for 1 h at 25°C with 20 μL of in vitro translation product in a final volume of 100 μL of 0.3 M sorbitol, 20 mM Tes, 1 mM DTT, 0.5 mM EDTA, 2 mM K_2HPO_4, 20 mM KCl, 9 mM creatine phosphate, 5.0 mM Met, 1 mM NADH, 120 μg/mL creatine kinase, 2 mM ATP (pH 7.2). The import reactions were then incubated on ice for 15 min, followed by an additional 30 min with or without 20 μg/mL proteinase K. PMSF was added to a final concentration of 10 mM, and the reactions were incubated on ice for 15 min. Intact mitochondria were reisolated by centrifugation at 5000*g* for 5 min through a 1-mL 26% (v/v) Percoll cushion containing 0.25 M Suc, 0.1% (w/v) BSA, 10 mM Tes (pH 7.2). The mitochondrial pellets were washed with 200 μL of 0.25 M Suc, 0.1% (w/v) BSA, 10 mM Tes (pH 7.2) and repelleted at 5000*g* for 5 min. The mitochondria were resuspended in 50 μL of 1× SDS-PAGE sample buffer and boiled for 5 min. Where noted, the mitochondria were resuspended in 100 mM Tris (pH 9.0), 100 mM DTT, incubated at 65°C for 30 min, and then precipitated with 5% (w/v) TCA. The resulting TCA pellet was then resuspended in 50 μL of 1× SDS-PAGE sample buffer. The mitochondrial extracts were electrophoresed on 15% SDS-polyacrylamide gels and blotted onto nitrocellulose filters (Battey and Ohlrogge, 1990). Radioactivity on the blots was detected and analyzed by phosphorimaging (Molecular Dynamics).

Spinach chloroplasts were isolated from spinach leaves as described by Roughan (1987). A 200-μL import reaction mixture containing 20 μL of in vitro translation product, 8 mM ATP, 0.33 M sorbitol, 50 mM Hepes/KOH (pH 8.0), and 100 μL of chloroplasts was incubated for 45 min at 25°C. The reactions were incubated on ice for 30 min with or without the addition of 20 μg/mL proteinase K (Boehringer Mannheim). PMSF was added to a final concentration of 10 mM, and the reactions were incubated for an additional 15 min on ice. Intact chloroplasts were reisolated by centrifugation at 1500*g* for 6 min through a 1-mL cushion of 40% (v/v) Percoll in 50 mM Hepes/KOH (pH 8.0), 0.33 M sorbitol. The chloroplast pellet was washed in 50 mM Hepes/KOH (pH 8.0), 0.33 M sorbitol and repelleted by centrifuging at 1500*g* for 6 min. The washed chloroplasts were resuspended in 50 μL of 25 mM Hepes (pH 8.0) and incubated on ice for 15 min to cause lysis. The lysed chloroplasts were then centrifuged at 14,000*g* for 10 min. The resulting supernatant was analyzed on a 15% SDS-polyacrylamide gel and blotted onto nitrocellulose as described above. The blot was then used to expose x-ray film.

Expression of pACP25 in *E. coli* and Polyclonal Antibody Production

PCR was used to facilitate the cloning of the mature mtACP-1 sequence into the *E. coli* protein expression vector pET3a (Studier et al., 1990). The forward primer (JO95 = 5′-CGGGATCCATATGTCGCACGATGATCATCTT-3′) con-

tained a *Nde*I site designed into the 5′ end. The M13 reverse-sequencing primer was used as the reverse primer. The mature peptide sequence was amplified by PCR as described above. The PCR product was cloned into the *Eco*RV site of pBluescript II KS+ and then subcloned as a *Nde*I-*Bam*HI fragment into pET3a. The resulting plasmid was designated p0914A-10.

The induced expression of mtACP-1 from *E. coli* BL21 (DE3) cells carrying the plasmid p0914A-10 by isopropyl-β-D-thiogalactopyranoside resulted in the production of an abundant protein with a molecular mass of approximately 18 kD, which was easily detected in Coomassie blue-stained SDS-PAGE gels. To produce antibodies to this protein, a 3-L culture of *E. coli* strain BL21 (DE3) containing p01914A-10 was induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside when the culture density reached an A_{600} of 0.7. The culture was incubated for an additional 6 h at 37°C, after which the cells were harvested by centrifugation at 6000*g* for 20 min. The cell pellets were washed in homogenization buffer (50 mM Mops [pH 7.5], 10 mM DTT) and resuspended in 30 mL of the same solution containing 1 mg of DNase I (Sigma). The cells were lysed in a French pressure cell at 20,000 p.s.i. The lysate was diluted to 100 mL with homogenization buffer and centrifuged at 10,000*g* for 20 min. $(NH_4)_2SO_4$ was added to the supernatant to 60% saturation and was stirred for 30 min. The insoluble material was removed by centrifugation at 10,000*g* for 30 min. TCA was added to the supernatant to a final concentration of 5% (w/v) and was then mixed slowly for 30 min. The TCA-insoluble material was pelleted at 10,000*g* for 30 min. The TCA pellet was washed with 1% (w/v) TCA and resuspended in 40 mL of 100 mM Tris (pH 9.5), 100 mM DTT. The resuspended TCA pellet was incubated at 65°C for 30 min to deacylate ACPs. The deacylated ACPs were then precipitated in 5% (w/v) TCA as described above. The washed TCA pellet was resuspended in 40 mL of 50 mM Mes (pH 6.1), 10 mM DTT. The mtACP was separated from *E. coli* ACP on a DEAE-cellulose (Whatman) column using a linear gradient of 0 to 0.5 M NaCl in 10 mM Mes (pH 6.1), 10 mM DTT. Column fractions were analyzed for ACP using an acyl-ACP synthetase assay (Kuo and Ohlrogge, 1984). Fractions containing mtACP were pooled, TCA precipitated, and resuspended in 10 mM Tris-HCl (pH 7.5). The mtACP was further purified on a Mono-Q HR5/5 column (Pharmicia) using a gradient of 0 to 0.3 M $MgCl_2$ in 10 mM Tris-HCl (pH 7.5).

Antibodies were generated by injecting the *E. coli*-expressed mtACP-1 that was emulsified with Hunter's TiterMax Research Adjuvant R1 (CytRx Corp., Norcross, GA) into New Zealand White rabbits.

Extraction of *Arabidopsis* ACPs

Arabidopsis leaf ACP extracts were prepared from 10 g of *Arabidopsis* leaves. The leaves were ground in liquid nitrogen and homogenized in 100 mL of 10 mM Hepes (pH 7.8), 10 mM DTT using a Polytron. The homogenate was centrifuged, and the supernatant was TCA precipitated and deacylated as described above. The deacylated *Arabidopsis* leaf ACP extract was resuspended in 2.5 mL of 10 mM Hepes (pH 7.8), 10 mM DTT and stored at −20°C in 500-μL aliquots.

Arabidopsis mitochondria were isolated from 18-d-old plants grown at 23°C in a 12-h photoperiod. Plants were placed in the dark for 12 h prior to mitochondria isolation. Mitochondria were extensively purified on continuous PVP-40/Percoll gradients as described by Day et al. (1985). The isolated mitochondria contained 266 μg/mL protein. The specific activity of Cyt *c* oxidase in this preparation was 77 nmol min^{-1} mg^{-1} protein. The mitochondria were 86.5% intact as determined by the latency of Cyt *c* oxidase activity before Triton X-100 treatment (Neurburger et al., 1982). The mitochondria were free of chloroplast contamination as determined by Chl content. Mitochondria extracts were deacylated as described above.

FAS Assay

Spinach chloroplast FAS was purified as described by Guerra et al. (1986). Spinach cpACP-1 and *E. coli* ACP were purified as described by Ohlrogge and Kuo (1985) and Rock and Cronan (1980), respectively. FAS assays contained 1 mM NADH, 2 mM NADPH, 1 mM acetyl-CoA, 5 mM β-mercaptoethanol, 100 μg/mL spinach leaf protein, 100 mM Tris-HCl (pH 8.0), 400,000 dpm/mL of 54 μCi/μmol [2-^{14}C]malonyl-CoA. FAS reactions were supplemented with mtACP-1 or cpACP-1 at designated concentrations. The reactions were incubated at 30°C. The reactions were then saponified with 0.1 volume of 10 M KOH and incubated at 65°C for 15 min. Then, 0.2 volume of 5 M H_2SO_4 and 1 volume of a solution of 1 mM palmitic acid in 1 M acetic acid in isopropanol were added to each sample. The saponified fatty acids were then extracted three times with 2 volumes of hexane and analyzed by scintillation counting. FAS reaction products were dried under N_2 and derivatized in 1 mL of boron trichloride at 90°C for 10 min. H_2O (1 mL) was added, and the FAME were extracted three times with 2 mL of hexane. The FAME were then separated on KC18 reverse-phase TLC plates (Whatman, Maidstone, England) developed in acetonitrile:methanol:water (65:35:0.5, v/v). The amount of ^{14}C label incorporated into each FAME species was determined by phosphorimaging analysis of the TLC plates.

RESULTS AND DISCUSSION

Isolation of a cDNA Encoding an mtACP

An oligonucleotide probe derived from a highly conserved amino acid sequence surrounding the ACP phosphopantetheine-binding site (Hansen, 1987) was used to screen an *Arabidopsis* leaf cDNA library. Forty positive cDNA clones were isolated in this screening, 12 of which were purified and sequenced. Seven of the 12 cDNAs corresponded to two previously cloned *Arabidopsis* ACP isoforms, ACP-2 (Hlousek-Radojcic et al., 1992) and ACP-3 (Lamppa and Jacks, 1991). The identity of four of the sequenced cDNAs was not conclusively determined. The remaining cDNA clone, pACP25, appeared to encode a novel *Arabidopsis* ACP isoform.

pACP25 contained a 2400-bp cDNA insert. Restriction mapping and Southern blot analysis using the 66-mer probe indicated that the ACP sequence was located in a 950-bp *Bam*HI-*Hin*dIII fragment located in the 3′ end of pACP25.

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                  m  a  l  r  n  a  i  l  r  h  l  r  v  p  v  q   16
1   GAAAGAGACGAAAATGGCACTGAGAAATGCAATTCTTCGTCACCTGAGGGTTCCGGTGCA   60
17    t  l  g  l  n  q  s  k  i  q  f  l  g  t  i  r  s  f  S  S   36
61  AACCCTAGGATTGAATCAGTCTAAAATTGGGTTCCTTGGTACGATCCGGTCATTTTCTTC  120
-37   H  D  D  H  L  S  R  E  A  V  V  D  R  V  L  D  V  V  K  S   56
121 GCACGATGATCATCTTAGCAGAGAAGCTGTCGTCGATAGAGTACTCGATGTTGTCAAGAG  180
57    F  P  K  V  D  P  S  K  V  T  P  E  V  H  F  Q  N  D  L  G   76
181 CTTCCCCAAAGTCGATCCCTCTAAGGTGACTCCTGAGGTTCATTTCCAAAACGATTTGGG  240
77    L  D  S  L  D  T  V  E  I  V  M  A  I  E  E  E  F  K  L  E   96
241 ATTAGATAGTTTGGACACAGTGGAGATAGTGATGGCTATTGAAGAGGAATTCAAGCTGGA  300
97    I  P  D  K  E  A  D  K  I  D  S  C  S  L  A  I  E  Y  V  Y  116
301 AATTCCAGACAAAGAAGCTGACAAGATCGATTCTTGCTCTCTCGCCATTGAATACGTTTA  360
117   N  H  P  M  S  S
361 CAATCATCCAATGTCTAGCTAATCGCTGTGTGCTCTCTCATGGTTTCTCCTTCCTTTTTC  420
421 TTGTATGAGGTTCTTCTCTCTTTGATAATGCCATAATGGGATTTGGCAAAAAAATCACTT  480
481 TTATTTGGAGAGCATTGTTATTGACTCTTTTAACGGTTCCAGTTTCTTAAAAAGAAAACC  540
541 TTTTTGGAATTCC
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Figure 1. Nucleotide and deduced amino acid sequence of pACP25. The putative mitochondrial presequence sequence is denoted by the lowercase, single-letter amino acid code. The underlined nucleotide sequence represents the translational initiation consensus sequence (Heidecker and Messing, 1986). The bold, uppercase, single-amino acid sequence represents the conserved sequence surrounding the phosphopantetheine attachment site.

This cDNA fragment was subsequently sequenced, and two tandem open reading frames were detected. One open reading frame encoded a protein that showed a high degree of identity to the *Pseudomonas* enzyme *N*-carbamyl-L-amino acid amidohydrolase (Watanabe et al., 1992). The second open reading frame encoded a protein showing a high degree of identity with ACP. The two reading frames were separated by an *Eco*RI site, suggesting that two independent cDNA fragments could have been inadvertently subcloned into the phage vector during the library construction. Because the two open reading frames encode two proteins that have very different functions, it is unlikely that the two open reading frames originated from the same mRNA species.

The protein encoded by pACP25, designated mtACP-1, contained the phosphopantetheine-binding domain that is highly conserved among all ACPs (Ohlrogge, 1987). A putative translation initiation sequence (Heidecker and Messing, 1986) (underlined in Fig. 1) surrounded the AUG initiation codon. No polyadenylation site consensus sequence or poly(A) tail was found in the 3′ untranslated sequence.

The mature mtACP-1 protein sequence was compared to the *Arabidopsis* cpACP sequences and the known mtACP sequences (Fig 2). Although the amino acid sequence corresponding to the region surrounding the prosthetic group attachment site (underlined in Fig. 2) was highly conserved among mtACP-1 and all other ACPs, two amino acid changes common to each of the mtACPs were detected in this region. First, at position 44 (denoted by * in Fig. 2), a highly conserved Ala seen in all cpACPs was replaced by a Leu in the mtACPs. A second alteration in the conserved sequence of the cpACPs was seen at position 54 (denoted by ^ in Fig. 2), where a highly conserved Gly was replaced by an Ala in the mtACP sequences. The region where these changes occur is totally conserved among all known cpACPs sequences but is different in mtACP-1 and the bovine and *Neurospora* mtACP sequences. In addition to these specific changes in the highly conserved phosphopantetheine-binding domain, when compared to known ACP protein sequences, mtACP-1 shared the highest degree of identity with the mtACPs. The *Neurospora* and bovine heart muscle mtACP protein sequences are 68 and 67% identical with mtACP-1, respectively, whereas the *Arabidopsis* cpACPs share less then 60% identity with mtACP-1. The *Arabidopsis* cpACP sequences share at least 69% identity among themselves (Hlousek-Radojcic et al., 1992); thus, the sequence of the mtACP-1 protein appeared to be more conserved with widely divergent organisms of three eukaryotic phylogenetic kingdoms than with cpACPs isolated from the same species, *Arabidopsis*. The alignment of mtACP-1 amino acid sequence with the *Neurospora* and bovine mtACPs sequences suggested that mtACP-1 encoded an 88-amino acid mature protein preceded by a 35-amino acid N-terminal extension. Both the calculated isoelectric point of 4.26 and molecular mass of 9914 D were consistent with other ACPs of both chloroplast and mitochondrial origin.

The 35-amino acid N-terminal extension (lowercase letters in Fig. 1) shared many of the structural features common to mitochondrial presequences as defined by von Heijne et al. (1989). These features included a high degree of α-helical nature at the N terminus, hydrophobic amino acids at the C terminus of the presequence between amino acids 24 and 34, and a highly conserved Arg at position 32. Using these criteria we hypothesized that the first 35 amino acids of the protein encoded by pACP25 represented a mitochondrial presequence.

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                        10        20        30        40        50        60        70        80
                        |         |         |         |  *      |  ^      |         |         |
At mtACP-1  SSHDDHLSREAVVDRVLDVVKSFPKVDPSKVTPEVHFQNDLGLDSLDTVEIVMAIEEEFKLEIPDKEADKIDSCSLAIEYVYNHPMSS
Nc mtACP    :AGGHLKKDQVFSRIAQVLSGFDKVN::KNI:ETA::A:::::::::::V:::::::SI:::::D::Q:H:VDK:V::ILSQ:DAN
Bt mtACP    :DAPPP:TL:GIK::::Y:L:LYD:I::E:LSVNS::MK::::::::Q:::I::M:D::GF::::I::E:LMCPQEIVD:IADKKDVYE

                        10        20        30        40        50        60        70        80
                        |         |         |         |  *      |  ^      |         |         |
At mtACP-1  SSHDDHLSREAVVDRVLDVVKSFPKVDPSKVTPEVHFQNDLGLDSLDTVEIVMAIEEEFKLEIPDKEADKIDSCSLAIEYVYNHPMSS
At cpACP-1        AKQETIEK:SAI::KQLSLT:D:KVVAETKFA:I:A:::::::::GL::::NIGMAEEK:Q::ATVEQ:A:LIEELINEKK
At cpACP-2        AKPET::K:CA::RKQLSLKEADEITAATKFAA::A:::::::::GL::::GI:MAEEK:QS:ATVEQ:AALIEELIFEKAK
At cpACP-3        AKPET::K:CA::RKQLSLKEADEITAATKFAA::A:::::::::GL::::GI:MAEEK:QS:ATVEQ:AALIEELLGGKAK
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Figure 2. Alignment of mtACP-1 protein sequence with known mtACPs (top) and with the *Arabidopsis* cpACPs (bottom). :, Conserved amino acids; * and ^, highly conserved Ala and Gly residues observed in all cpACPs but that have been replaced by Leu and Ala residues (respectively) in the three mtACPs. The underlined sequence represents the conserved sequence surrounding the phosphopantetheine attachment site. At mtACP-1, *A. thaliana* mtACP; Nc mtACP, *N. crassa* mtACP (Sackmann et al., 1991); Bt mtACP, bovine mtACP (Runswick et al., 1991); At cpACP-1, *A. thaliana* cpACP-1 (Post-Beittenmiller et al., 1989); At cpACP-2, *A. thaliana* cpACP-2 (Lamppa and Jacks, 1991); At cpACP-3, *A. thaliana* cpACP-3 (Lamppa and Jacks, 1991).

E. coli Expression of pACP25

The cDNA sequence encoding the mature mtACP-1 protein was subcloned into the *E. coli* protein expression vector pET3a. mtACP-1 could be clearly separated from the *E. coli* ACP by DEAE-cellulose anion-exchange chromatography and was purified to near homogeneity as determined by silver staining of SDS-PAGE gels (data not shown). The *E. coli* mtACP-1 migrated as an 18.0-kD doublet when analyzed by SDS-PAGE. Pure ACPs have been reported to sometimes migrate as doublets during PAGE (Jackowski and Rock, 1987). The purified mtACP-1 protein was used to generate polyclonal antibodies in rabbits. The resulting antibodies were highly specific for mtACP-1. Although the antibodies did cross-react with other ACP isoforms because of the high degree of similarity shared among all ACP isoforms, these antibodies showed a marked preference for mtACP-1. At a 1:500 dilution, the crude antiserum was sufficient to detect as little as 10 ng of mtACP-1 on a western blot (data not shown).

In Vitro Protein Import Studies

To confirm the targeting specificity of the mtACP-1 presequence, in vitro mitochondrial and chloroplastic protein import studies were conducted. Pea mitochondria and spinach chloroplasts were incubated in the presence of radiolabeled apo-mtACP-1 or spinach apo-cpACP-1 precursor proteins with and without the addition of proteinase K. Proteins imported into the organelles were protected from digestion by exogenously added protease and were detected in mitochondrial or chloroplast extracts by autoradiography. The identities of processed proteins were determined by mobilities on SDS-PAGE gels relative to mobilities of ACP standards.

The mtACP-1 presequence proved to be sufficient for targeting protein import into pea mitochondria. Although several radiolabeled proteins appeared to be associated with the reisolated mitochondria after incubation with in vitro translated apo-mtACP precursor (Fig. 3, lane 1), only two proteins, with apparent molecular masses of 18 and 12 kD, were protected from protease treatment (Fig. 3, lane 2).

The 18-kD radiolabeled protein comigrated with the mature holo-mtACP-1 standard (Fig. 3, lanes 2 and 5), and the 12-kD band appeared to migrate slightly higher than the mature palmitoyl-mtACP-1 standard (Fig. 3, lanes 2 and 4). Because the 12-kD band did not migrate exactly with the palmitoyl-mtACP-1 standard, it is possible that the mtACP acylated during the import reaction was composed of a fatty acid of a different chain length, since acyl-ACPs are known to migrate on native PAGE differently, depending on the fatty acid chain length (Post-Beittenmiller et al., 1990). To confirm that the 12-kD protein did correspond to an acylated form of mtACP-1, postimport mitochondrial extracts were deacylated by treatment with 100 mM DTT at pH 9.0 (Lakin-Thomas and Brody, 1985). Upon deacylation, the 12-kD radiolabeled band disappeared, whereas the presence of the 18-kD band was not affected (Fig. 3, lane 3). Thus, it appears that the pea mitochondria were not only able to import and process the mtACP-1 precursor but also able to acylate the mature mtACP-1 protein. These results contrast with chloroplast protein import experiments in which acylated forms of ACP were not detected (Fernandez and Lamppa, 1990a, 1990b). Although *Neurospora* mitochondria have been reported to be capable of de novo fatty acid synthesis (Mikolajczyk and Brody, 1990; Zensen et al., 1992), it is not known whether the acyl chain esterified to the mature mtACP-1 is derived from mitochondrial de novo fatty acid synthesis or from an acyl transfer reaction from a preformed acyl group.

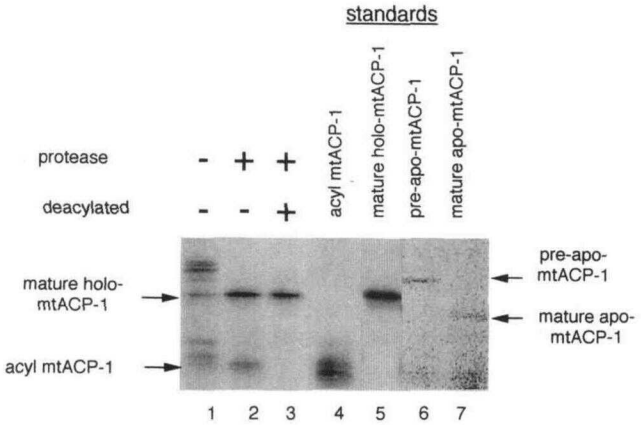

Figure 3. In vitro protein import of mtACP-1 precursor into pea leaf mitochondria. Mitochondrial protein import studies were performed as described in "Materials and Methods." The radiolabeled proteins were detected on protein blots using a Molecular Dynamics phosphorimaging system. Lanes 1 to 4 were derived from images obtained when the background to signal range was set at 10 to 1000 counts. Lanes 6 and 7 were derived from images obtained when the background to signal range was set at 10 to 50 counts. Lane 1, Import mixture containing pea mitochondria and radiolabeled mtACP-1 precursor. Lane 2, Same as lane 1, but the mitochondria were incubated with proteinase K after import. Lane 3, Same as lane 1, but the mitochondria were treated with proteinase K and then deacylated (Lakin-Thomas and Brody, 1985). Lane 4, Acyl-mtACP-1 standard that was synthesized in vitro from mtACP-1 and [^{14}C]palmitic acid using *E. coli* acyl-ACP synthetase (Kuo and Ohlrogge, 1984). Lane 5, mtACP-1 protein standard derived from *E. coli*-expressed pACP25 and detected by western analysis. Lane 6, Radiolabeled mtACP-1 precursor standard synthesized from rabbit reticulocyte lysates and transcripts derived from p0527. Lane 7, Radiolabeled apo-mtACP-1 synthesized from rabbit reticulocyte lysate and transcripts derived from p0914A-10.

It is interesting that no radiolabeled protein was detected in the postimport mitochondrial extract that comigrated with the mature apo-mtACP-1 standard (Fig. 3, lane 7). This suggests that the pea mitochondria are able to very efficiently add the phosphopantetheine prosthetic group to imported mtACP-1 protein. These results contrast with chloroplast protein import studies in which the complete conversion of the apo-cpACP I to the holo form was not seen (Fernandez and Lamppa, 1990a, 1990b). However, because the mitochondrial protein uptake reactions were incubated for a longer time than the typical ACP chloroplast import reaction, we cannot rule out the possibility that the complete conversion of the apo-mtACP-1 to the holo form was not time dependent. Our results indicate that the mitochondria may contain HAS, the enzyme responsible for the addition of the

prosthetic group. Elhussein et al. (1988) reported that HAS activity was associated with both cytoplasmic and plastid cell fractions with relatively little HAS activity being detected in the mitochondria. Although the results shown in Figure 3 strongly suggest that the pea mitochondria possess HAS, it is possible that the mtACP precursor was modified prior to import by contaminating cytoplasmic HAS present in the mitochondria preparation. However, it is not likely that the prosthetic group was added during the in vitro translation reaction, since it is known that the rabbit reticulocyte lysates contain no HAS activity (L. Savage and D. Post-Beittenmiller, unpublished data). In additional control experiments it was shown that the spinach cpACP-1 precursor was not able to direct protein import into the mitochondria (data not shown).

When the mtACP-1 precursor was incubated with spinach chloroplasts, it was able to bind to the outer chloroplast envelope (Fig. 4, lane 1); however, after protease treatment, no chloroplast import had occurred (Fig. 4, lane 2). However, as expected, the spinach cpACP-1 precursor was imported and correctly processed by spinach chloroplasts (data not shown).

Subcellular Localization

To confirm the mitochondrial localization of mtACP-1, western analysis of *Arabidopsis* leaf extracts was performed. Blots of *Arabidopsis* protein extracts run on 15% SDS-PAGE gels were probed with antibodies specific for mtACP-1. To simplify the number of potential forms of mtACP-1, protein extracts were first deacylated (Lakin-Thomas and Brody, 1985). An 18-kD doublet that cross-reacted with the mtACP-1 antibodies was detected in deacylated whole leaf and deacylated purified mitochondria extracts (Fig. 5, lanes 1 and 2). These proteins comigrated with the *E. coli*-expressed mtACP-1. The anti-mtACP-1 antibodies weakly cross-reacted with proteins that migrated to the known positions of the *Arabidopsis* cpACP isoforms (Battey and Ohlrogge, 1990) in whole leaf extracts (Fig. 5, lane 1). Based on the total ACP content of *Arabidopsis* leaf, as estimated from acyl-ACP synthetase, and the level of mACP-1, as approximated from western blots, we determined that the mtACP-1 may constitute as much as 10% of the total *Arabidopsis* leaf ACP.

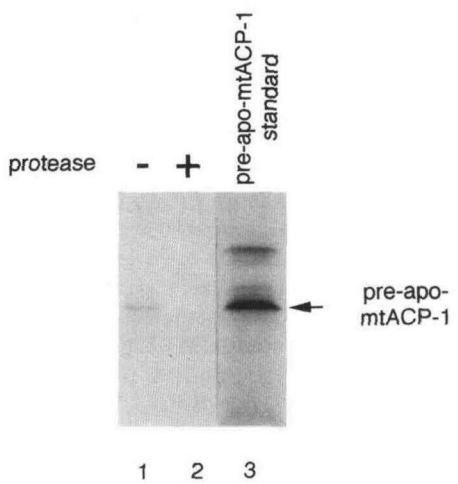

Figure 4. In vitro protein import studies of the mtACP-1 precursor into spinach chloroplasts. Chloroplast protein import studies were performed as described in "Materials and Methods." The radiolabeled proteins were detected by autoradiography. Lane 1, Import mixture containing spinach chloroplasts and radiolabeled mtACP-1 precursor. Lane 2, Same as lane 1, but the chloroplasts were incubated with proteinase K after import. Lane 3, Radiolabeled mtACP-1 precursor standard synthesized from rabbit reticulocyte lysates and transcripts derived from p0527.

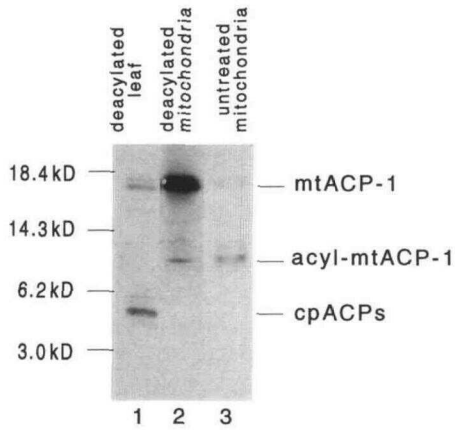

Figure 5. Western blot analysis of *Arabidopsis* leaf subcellular fractions. *Arabidopsis* whole leaf and mitochondrial protein extracts were purified as described in "Materials and Methods." Where noted, the extracts were deacylated by treatment with 100 mM DTT at pH 9.0 for 30 min at 65°C. Lane 1, Deacylated leaf protein extract; lane 2, deacylated mitochondrial protein extract; lane 3, untreated mitochondrial protein extract.

In addition to the unacylated form of mtACP-1, intact *Arabidopsis* mitochondria contained a cross-reactive protein that comigrated with the acyl-mtACP-1 synthesized in vitro (Fig. 5, lane 3). As estimated from the staining intensity of western blots, the acylated form of mtACP-1 appeared to be present at approximately 4 times the level of free mtACP-1 in purified mitochondria. This result differs from spinach cpACPs in which the free ACP accounted for approximately 60% of the cpACP pool (Post-Beittenmiller et al., 1990). Further analysis showed that the acylated form of mtACP-1 was associated primarily with the mitochondrial membrane fraction. The 100,000*g* pellet of the lysed mitochondria appeared to contain the majority of the acylated mtACP-1 along with a small amount of free mtACP-1 (Fig. 6, lane 2). The free mtACP-1 was present in relatively higher amounts than the acylated form in the 100,000*g* supernatant fraction (Fig. 6, lane 3). The membrane localization of the acylated mtACP-1 was consistent with the report that the *Neurospora* and bovine mtACPs were present only in the acylated form when associated with the NADH:ubiquinone oxidoreductase membrane complex (complex I) (Runswick et al., 1991; Sackmann et al., 1991); further evidence is necessary to determine whether mtACP-1 is also associated with complex I in *Arabidopsis* mitochondria.

FAS Assays

The role of mtACPs, if any, in mitochondrial de novo fatty acid synthesis is unclear. In animals and fungi fatty acid

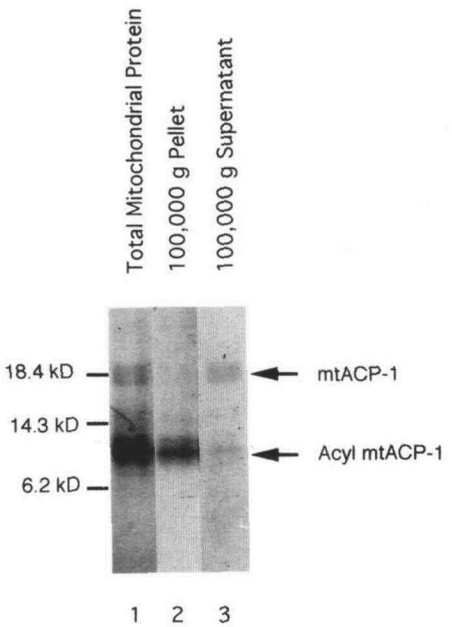

Figure 6. Western blot analysis of mitochondrial membrane and matrix fractions. *Arabidopsis* mitochondria were purified as described in "Materials and Methods." The mitochondria were lysed by sonication and fractionated by centrifugation at 100,000g. Lane 1, Unfractionated mitochondria; lane 2, 100,000*g* pellet from sonicated mitochondria; lane 3, 100,000*g* supernatant from sonicated mitochondria.

synthesis has long been thought to occur solely in the cytoplasm, whereas the plastids have been considered to be the specific locale for fatty acid synthesis in plants. However, the recent isolation of mitochondrial ACPs and acyl-ACPs has opened up the possibility of an additional subcellular compartment for fatty acid synthesis.

Although *Neurospora* mitochondria have been reported to be capable of de novo fatty acid synthesis (Mikolajczyk and Brody, 1990; Zensen et al., 1992), no direct evidence exists to show that mtACPs function as cofactors for mitochondrial fatty acid synthesis. Brody and Mikolajczyk (1988), however, reported that the *Neurospora* mtACP was able to function in an *E. coli* FAS system. To determine whether or not *Arabidopsis* mtACP-1 was able to act as a cofactor in FAS, in vitro chloroplast FAS activities were compared using mtACP-1 and spinach cpACP-1 concentrations ranging from 0.5 to 25 μM. The assays were performed using concentrations of chloroplast FAS extracts that would allow linear rates of FAS activity during a 15-min period. Although the rates of fatty acid synthesis varied depending on the FAS preparation, mtACP-1 was in all cases able to act as a cofactor for spinach chloroplast FAS when compared to spinach cpACP-1 (Fig. 7). These results suggest that, despite its structural divergence, mtACP-1 could function as a cofactor for chloroplast FAS.

The profiles of the fatty acids synthesized by the chloroplast FAS in the presence of mtACP-1 and spinach cpACP-1 were compared. As expected, FAS reactions incubated with spinach cpACP-1 yielded predominately long-chain fatty acids (Fig. 8A). However, when FAS reactions were supplemented with mtACP-1, short-, medium-, and long-chain fatty acids were synthesized (Fig. 8B). Caproic acid, lauric acid, myristic acid, and palmitic acid each made up approximately 20% of the total fatty acid, whereas octanoic and stearic acids each represented approximately 10%. A similar fatty acid profile was observed at mtACP-1 concentrations ranging from 0.5 to 5.0 μM (data not shown). To determine whether the short- and medium-chain fatty acids derived from the FAS reactions supplemented with mtACP-1 were present as acyl-ACPs, the reactions were extracted prior to saponification to analyze only free fatty acids (Fig. 8). These analyses showed that the short- and medium-chain fatty acids produced in reactions containing mtACP-1 were not esterified to mtACP-1. The results suggest that the fatty acid products produced on mtACP-1 were prematurely cleaved from the ACP. Although the accumulation of fatty acids of intermediate-chain lengths were also observed when plant cpACPs were used to supplement an *E. coli* FAS system (Simoni et al., 1967), the fatty acids were apparently present as acyl-ACP intermediates and not as free fatty acids.

The observation of hydrolyzed short- and medium-chain fatty acids in the FAS assays supplemented with mtACP-1 was particularly surprising because the acyl-ACP thioesterases are known to be specific for long-chain acyl-ACPs (Ohlrogge et al., 1978). It may be possible that mtACP-1 facilitates release of shorter chain fatty acids from ACP through its presentation of the acyl-ACPs to acyl-ACP thioesterases. Brody and Mikolajczyk (1988) reported that 3-hydroxytetradecanoyl-ACPs have been isolated from *Neurospora* mitochondria, suggesting that medium-chain acyl-ACP intermediates do occur on mtACPs. Furthermore, in vivo and in vitro labeling of *Neurospora* mitochondria with [2-^{14}C]-malonic acid has resulted in the synthesis of radiolabeled short- and medium-chain acyl-ACPs and free myristic acid (Mikolajczyk and Brody, 1990). However, additional in vivo labeling experiments in *Neurospora* (Zensen et al., 1992) indicated that long-chain acyl intermediates were synthesized

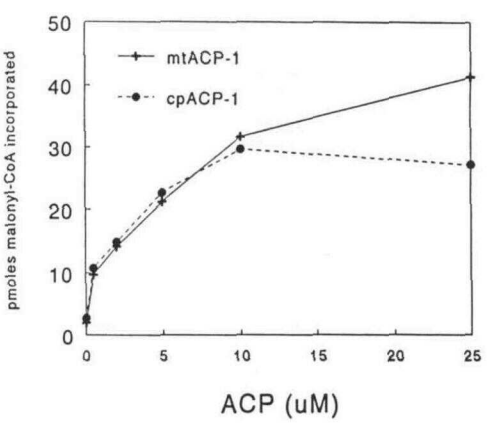

Figure 7. Utilization of mtACP-1 and spinach cpACP-1 by chloroplast FAS. Reactions (75 μL) were incubated for 15 min at 30°C. Activity was measured as incorporation of [^{14}C]malonyl-CoA into the lipid fraction. Lipid extracted into hexane following saponification and neutralization of the reaction mixture was counted by liquid scintillation.

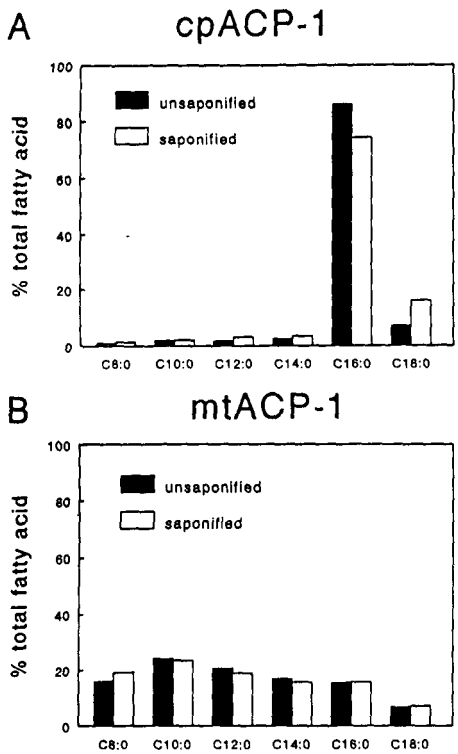

Figure 8. Product analysis of FAS reactions supplemented with mtACP-1 or spinach cpACP-1. ^{14}C-labeled fatty acids derived from unsaponifed and saponified FAS reactions supplemented with either 22.5 μM mtACP-1 or 22.5 μM spinach cpACP-1 were analyzed by reverse-phase TLC and quantitated by phosphorimaging. A, The FAME profiles derived from unsaponified (solid bars) and saponified (open bars) FAS reactions supplemented with spinach cpACP-1. B, The FAME profiles derived from unsaponified (solid bars) and saponified (open bars) FAS reactions supplemented with mtACP-1. In this experiment, the total ^{14}C incorporation into the FAMEs derived from the unsaponified and saponified FAS reactions supplemented with mtACP-1 was 24,624 and 28,543 dpm, respectively. Unsaponified and saponified FAS reactions supplemented with cpACP-1 had total ^{14}C incorporations of 48,906 and 43,746 dpm. FAS (1 mL) reactions were incubated for 1 h. C8:0, Caproic acid; C10:0, deconoic acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:0, stearic acid.

on mtACP. Acyl-ACPs isolated from bovine mitochondria were reported to have an acyl moiety with a molecular mass of 302 D, again suggesting that a long-chain acyl intermediate could be synthesized on mtACPs (Runswick et al., 1991). Because of these divergent results, it is difficult to ascertain the significance of the accumulation of short- and medium-chain fatty acids in the chloroplast FAS assays supplemented with mtACP-1.

From the in vitro chloroplast FAS experiments it was apparent that mtACP-1 was structurally close enough to cpACP to at least initiate de novo fatty acid synthesis but sufficiently dissimilar enough to participate differently in the fatty acid termination reactions. Although the results of these experiments could not predict what role if any mtACP-1 plays in mitochondrial de novo FAS, they do suggest that mtACP-1 is capable of acting as a cofactor in a FAS system. Further experiments are necessary to determine whether mtACP-1 does participate in mitochondrial de novo FAS.

CONCLUSION

The cumulative results presented in this paper strongly support the conclusion that mtACP-1 represents a mitochondrial localized ACP isoform. First, mtACP-1 has been shown to be more closely related to the *Neurospora* and bovine mtACPs than to the *Arabidopsis* cpACPs. Second, the protein import studies confirmed the specificity of the mtACP-1 presequence for import into mitochondria. And finally, the detection of mtACP-1 by western blot analysis in mitochondrial extracts and the apparent membrane association of the acylated form of mtACP-1 are both consistent with characteristics reported for the *Neurospora* and bovine mtACPs (Runswick et al., 1991; Sackmann et al., 1991).

Although the function of mtACP-1 has not been determined, mtACP-1 is clearly effective as an FAS cofactor in in vitro chloroplast FAS studies. However, further studies using isolated mitochondria will be required to determine whether mtACP-1 plays a similar role in the mitochondria. Also, because of the high degree of structural similarity that mtACP-1 shares with other mtACPs, it is possible that the plant mitochondria ACPs may also be associated with NADH:ubiquinone oxidoreductase. The apparent membrane localization of acyl mtACP-1 is consistent with the association of the *Neurospora* and bovine mtACPs with the NADH: ubiquinone oxidoreductase membrane complex (Runswick et al., 1991; Sackmann et al., 1991). Regardless of the outcome, further characterization of the function of mtACP-1 will expand present ideas of the roles ACPs play in plant metabolism.

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