cDNA cloning, primary structure and gene expression for H-protein, a component of the glycine-cleavage system (glycine decarboxylase) of pea (*Pisum sativum*) leaf mitochondria

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We have isolated and characterized cDNA clones encoding the H-protein of the glycine-cleavage system of pea (*Pisum sativum*) leaf mitochondria. The deduced primary structure revealed that the 131-amino-acid polypeptide is cytoplasmically synthesized with a 34-amino-acid mitochondrial targeting peptide. The lipoate-binding site was assigned to be lysine-63, as deduced from a sequence comparison with several lipoate-bearing proteins. The expression of the gene encoding H-protein was shown to occur specifically in the leaf tissue, with light exerting an additional effect by increasing the mRNA levels severalfold. Two polyadenylation sites were found in the mRNA, and a single-copy gene encoding the H-protein was detected in pea genome.

INTRODUCTION

Mitochondria from plant leaves are distinct from all other mitochondria in that these organelles are capable of oxidizing glycine at extremely rapid rates (Douce *et al.*, 1977). The oxidation of glycine by mitochondria represents an important step in the metabolic pathway of photorespiration (Lorimer & Andrews, 1980; Husic *et al.*, 1987). This amino acid is oxidized in the matrix space by the glycine-cleavage system to produce CO_2, NH_3, NADH and 5,10-methylenetetrahydropteroyl-L-glutamic acid (Bourguignon *et al.*, 1988). The latter compound reacts with a second molecule of glycine to form serine and tetrahydropteroyl-L-glutamic acid in a reaction catalysed by serine hydroxymethyltransferase. The glycine-cleavage system has been purified from plants (Sarojini & Oliver, 1983; Walker & Oliver, 1986; Bourguignon *et al.*, 1988) and animals (Kikuchi & Hiraga, 1982) and is composed of four different component proteins referred to as the P- (a homodimer of 97 kDa peptides containing pyridoxal phosphate), H- (a 15.5 kDa lipoamide-containing protein), T- (a monomer of 45 kDa) and L- (a homodimer of 60 kDa peptides containing FAD) proteins. The H-protein provides the attachment site for the lipoic acid cofactor that interacts with the different active sites in the complex. The glycine decarboxylase is present in low amounts in etiolated pea (*Pisum sativum*) leaves, but increases dramatically upon exposure to light. Glycine decarboxylase represents a large proportion of the matrix protein in green leaf mitochondria (approx. 30–50 % of the total amount of matrix proteins) and with matrix concentrations approaching 0.2 g/ml it actually alters the density of the organelles (Day *et al.*, 1985; Bourguignon *et al.*, 1988). On the basis of the effect of cycloheximide on the biosynthesis of glycine decarboxylase, Walker & Oliver (1986) concluded that P-, H-, T- and L-proteins were nuclear-encoded.

To characterize further this complex and to isolate and identify genes that specify enzymes involved in glycine decarboxylation, we have isolated, as a first step, cDNA clones encoding the entire H-protein and deduced its primary structure. We found that the H-protein is specifically synthesized in leaf tissue. In addition, we detected a single-copy gene coding for H-protein in pea.

MATERIALS AND METHODS

Plant material

Pea (*Pisum sativum* L. var. Douce Provence) plants were grown in vermiculite at 28 °C (light period; Philips lamps model TLD 58W83) and at 22 °C (dark period) in a growth cabinet, with either a 12 h light period or constant darkness.

Isolation of mitochondria

Mitochondria were isolated and purified from pea leaves as described by Douce *et al.* (1987), using self-generating Percoll gradients and a linear gradient of 0–10 % (w/v) polyvinylpyrrolidone 25 (top to bottom). The mitochondria were found in a tight white band near the bottom of the tube, whereas the thylakoids remained near the top of the tube. The mitochondria were subsequently concentrated by differential centrifugation. The purified mitochondria were suspended in a medium containing 0.3 M-mannitol, 10 mM-phosphate buffer, pH 7.2, 1 mM-EDTA and 1 mM-β-mercaptoethanol at approx. 100 mg of protein/ml. O_2 uptake was measured at 25 °C with a Clark-type oxygen electrode (Hansatech, King's Lynn, Norfolk, U.K.). The reaction medium contained 0.3 M-mannitol, 5 mM-MgCl₂, 10 mM-KCl, 10 mM-phosphate buffer, pH 7.2, 0.1 % (w/v) defatted BSA and known quantities of mitochondrial proteins, in a total volume of 1 ml.

Purification and chemical cleavage of H-protein

High-molecular-mass proteins from pea mitochondrial matrix retained on an XM-300 Diaflo membrane ('matrix extract') were prepared as described by Neuburger *et al.* (1986). The H-protein of the glycine-cleavage system has been purified to apparent homogeneity from the matrix extract by using gel filtration, ion-exchange and phenyl–Superose f.p.l.c. (Bourguignon *et al.*, 1988). The identity of H-protein was determined by measuring the amounts of [¹⁴C]bicarbonate fixed to the carboxy-group carbon atom of glycine during the exchange reaction (in the presence of saturating amounts of P-protein) (Bourguignon *et al.*, 1988). For cleavage at methionine residues, freeze-dried H-protein

was dissolved in 70% (v/v) formic acid to give a 1% (w/v) protein solution, and a 100-fold molar excess of CNBr over methionine residues was added (Gross, 1967). After 18 h at 4 °C in the dark the sample was partly dried under N_2, diluted 10-fold with water and freeze-dried. After freeze-drying, the walls of the tube were wetted with aminoethanol, left for 10 min at room temperature and then dried under vacuum. The reaction mixture was dissolved in 70% (v/v) formic acid, diluted 1:3 with running buffer [70% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid] and applied to a reverse-phase column (RP 300 aquapore octyl; Browlee Laboratories, Foster City, CA, U.S.A.). Peptides isolated by h.p.l.c. were freeze-dried and stored at −20 °C before analysis. The sequence of amino acids at the N-terminus of H-protein and of peptides produced by chemical cleavage was performed on a model 477A gas-liquid-phase protein sequenator (Applied Biosystems) equipped with a model-120A on-line phenylthiohydantoin amino acid analyser. The amino acid composition was determined using a model-6300 Beckman analyser on six aliquots (1 μg each) of the H-protein after hydrolysis in the presence of constant-boiling HCl at 110 °C.

Immunochemical studies of H-protein

Purified H-protein was checked for purity in SDS/7.5–15%-(w/v)-acrylamide-gradient gels. Bands corresponding to H-protein were excised and pooled. The polypeptide was eluted by electrodialysis and injected as an emulsion with Freund's adjuvant into rabbits for raising antibodies. Booster injections (1 mg of native enzyme) were given at 20-day intervals. Blood was collected 10 days after the third injection. IgG fractions were purified from rabbit antisera by ion-exchange chromatography as described by Saint-Blancard et al. (1981). Pooled fractions containing IgG were dialysed and concentrated by ultrafiltration (XM-50 membrane; Amicon). Purified IgG fractions (0.5 ml; 200 mg·ml⁻¹; in 10 mM-Tris/HCl, pH 7.2) were stored at −80 °C until used.

Protein and nucleic acid extraction

Protein extracts were prepared by the method of Hurkman & Tanaka (1986). Tissues were ground to a fine powder in liquid N_2 with a mortar and a pestle. Powder (2.5 g) was adjusted to 5 ml with extraction buffer [0.7 M-sucrose/0.5 M-Tris/HCl (pH 7.6)/50 mM-EDTA/0.1 M-KCl/2% (v/v) 2-mercaptoethanol/2 mM-phenylmethanesulphonyl fluoride]. Phenol (1 vol.) was added and the mixture was gently shaken on a rotary wheel at room temperature for 20 min. The phases were separated by centrifugation and the phenol phase re-extracted twice with extraction buffer. Finally the proteins were precipitated from the phenol phase by adding 5 vol. of 0.1 M-ammonium acetate in methanol at −20 °C. The precipitate was washed three times with methanolic 0.1 M ammonium acetate and once with acetone. The proteins were solubilized in SDS sample buffer and stored at −80 °C.

For RNA preparation, 10 g of tissue were frozen in liquid N_2 and homogenized in a mixture of 20 ml of 50 mM-Tris/HCl (pH 9.0)/1% (w/v) SDS and 20 ml of phenol with a Polytron homogenizer, followed by phenol, phenol/chloroform and chloroform extraction. RNA was precipitated by adding LiCl to 2 M and centrifuging. After solubilization in water, RNA was recovered by ethanol precipitation. The mRNA was purified by oligo(dT)–cellulose chromatography (Maniatis et al., 1982).

Nuclear DNA was prepared from purified leaf nuclei (Watson & Thompson, 1986). The DNA was purified by two CsCl centrifugations, followed by dialysis with 10 mM-Tris/HCl (pH 8.0)/1 mM-EDTA.

cDNA cloning

The lambda gt11 cDNA library was purchased from Clontech Laboratories (FL 1100b). The cDNA lambda gt10 library was constructed from light-grown leaf mRNA by using an Amersham cDNA synthesis kit (RPN 1256) and lambda gt10 cloning kit (RPN 1257). Expression screening of the lambda gt11 library was done as described by Huynh et al. (1985) using rabbit antisera raised against the purified H-protein. Two positive clones were purfied (Lg11, Lg21) and sequenced. An internal fragment of 519 bp was subcloned in a pUC18 plasmid (Ph 519) and used to screen the lambda gt10 library. Two positive clones were subcloned in pUC18 and sequenced (Pgh12 and Pgh13).

DNA sequencing

The plasmid sequencing reaction was performed as described by Zhang et al. (1988) using the phage-T₇ DNA polymerase (Tabor & Richardson, 1987). Occasionally an ambiguous band pattern has been resolved with 7-deaza-dGTP according to the manufacturer's (Pharmacia) instructions.

Western-, Northern- and Southern-blot gel analysis

Protein extracts were separated by SDS/PAGE in the buffer system described by Laemmli (1970). The proteins were electrophoretically transferred to nitrocellulose (Hybond C Extra; Amersham). The membrane was blocked with 5% (w/v) defatted dry milk in TBST [20 mM-Tris/HCl (pH 7.5)/0.5 M-NaCl/0.05% (v/v) Tween 20] and incubated with rabbit antisera against H-protein (1:1000 in TBST). After washing with TBST, the bound antibody was revealed by incubation with anti-rabbit IgG–alkaline phosphatase conjugate (Promega) (1:7500 in TBST), followed by washing in TBST and incubation with alkaline phosphatase colour-development solution (Promega).

Northern transfer of RNA denatured with glyoxal to Hybond N membranes (Amersham) was carried out as described by Maniatis et al. (1982). The nucleic acid hybridization solution was 50% (v/v) formamide/0.2% (w/v) polyvinylpyrrolidone (M_r 40000)/0.2% (w/v) BSA/0.2% (w/v) Ficoll (M_r 400000)/10% (w/v) dextran sulphate (M_r 500000)/0.1% (w/v) sodium pyrophosphate/1 M-NaCl/50 mM-Tris/HCl (pH 7.5)/1% SDS/denatured salmon sperm DNA (100 μg/ml). The DNA probe (Ph 519) was ³²P-labelled using a multiprime DNA labelling system (Amersham), and hybridization was allowed to proceed overnight at 42 °C. Membranes were washed twice in 2×SSC/0.1% SDS at room temperature and once in 0.5×SSC/0.1% SDS at 65 °C and autoradiographed at −80 °C (1×SSC is 0.15 M-NaCl/0.015 M-sodium citrate). Methylene Blue staining of RNA on nylon membranes was done as described by Maniatis et al. (1982).

High-molecular-mass nuclear DNA was digested overnight with restriction endonucleases and concentrated by ethanol precipitation. Separation by agarose (0.8%) electrophoresis and Southern transfer were done as described by Maniatis et al. (1982). Hybridization with the labelled cDNA probe was conducted overnight at 37 °C in the nucleic acid hybridization solution described above. The final washes were at 60 °C in 2×SSC/0.1% SDS and autoradiography was for 1–2 weeks at −80 °C.

RESULTS

cDNA cloning and the primary structure of H-protein

A polyclonal antibody raised against the purified H-protein was used to screen a cDNA lambda gt11 library from pea seedlings (Clontech Laboratories). Two immunopositive clones (Lg11 and Lg21) were plaque-purified. After sequencing, an internal 519 bp fragment was subcloned into pUC18 (Ph 519)

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1    caggaaaaca cacactcaca tacatgaaca atatctcatc atttcatttc

                                                        M  A
51   attcttctac acactcctca caaacacaac ccttaacaat aacaATGGCA

     L  R  M  W  A  S  S  T  A  N  A  L  K  L  S  S  S  S
101  CTTAGGATGTGGGCTTCTTCAACTGCCAATGCACTCAAACTCTCCTCTTCCTCT

                                               1
     R  L  H  L  S  P  T  F  S  I  S  R  C  F  S  N  V  L
155  AGATTACATCTCTCCCCTACCTTTTCCATCTCCAGATGTTTCTCTAATGTTTTG

              10                        20
     D  G  L  K  Y  A  P  S  H  E  W  V  K  H  E  G  S  V
209  GATGGACTCAAGTACGCACCTTCACATGAATGGGTCAAGCATGAAGGATCAGTA

              30                              40
     A  T  I  G  I  T  D  H  A  Q  D  H  L  G  E  V  V  F
263  GCCACTATTGGTATCACTGACCATGCCCAGGACCATCTTGGAGAGGTTGTGTTT

                    50
     V  E  L  P  E  P  G  V  S  V  T  K  G  K  G  F  G  A
317  GTGGAGCTTCCAGAACCTGGTGTCTCAGTTACAAAGGGTAAAGGTTTCGGAGCT

        60        ★                70
     V  E  S  V  K  A  T  S  D  V  N  S  P  I  S  G  E  V
371  GTTGAAAGTGTGAAGGCAACAAGTGATGTAAACTCTCCTATTTCCGGTGAGGTT

        80                        90
     I  E  V  N  T  G  L  T  G  K  P  G  L  I  N  S  S  P
425  ATCGAGGTTAATACTGGGCTCACCGGAAAGCCTGGCCTCATCAATTCAAGCCCC

              100                       110
     Y  E  D  G  W  M  I  K  I  K  P  T  S  P  D  E  L  E
479  TATGAAGATGGATGGATGATAAAAATAAAGCCAACTAGTCCAGATGAATTAGAA

              120                       130
     S  L  L  G  A  K  E  Y  T  K  F  C  E  E  E  D  A  A
533  TCCTTGTTGGGTGCAAAAGAGTACACAAAGTTTTGTGAGGAAGAAGATGCTGCA

     H_stop
587  CATTGA tgttcatttt tcaatatatt gaactgactg tttttaatat tgt

636  actacta tctatcatct tgttgtctat tttttttcaa atgcaataga
                            ▼
683  attgcttcaa gttattgttg tttccatgcg acacttcagt gtgtcagtct

733  atctagcttg tcatgtacta attaagaagc aaatttgttt tttctcatcc

783  agttattatt ttcacttcaa tgttttcaca atgatttttg ataacaaagt

833  tcagatcggt c a_n
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of cDNA encoding H-protein of the glycine-cleavage system

Nucleotides are numbered on the left of the sequence. The nucleotide sequence is a combination of the sequences of clones Lg11 (nucleotides 69–843), Lg21 (nucleotides 52–696), Pgh12 (nucleotides 1–811) and Pgh13 (nucleotides 41–696). The inverted black triangle (▼) indicates the second polyadenylation site found in clones Lg21 and Pgh13. The deduced amino acid sequence of the protein is depicted as single-letter codes positioned above the nucleotide sequence. The first amino acid of the mature protein (serine) is indicated by the number '1'. The amino acid sequences which were determined by protein sequencing are underlined. The star (★) indicates the deduced lipoyl-lysine residue.

and further used to screen a pea leaf cDNA library in lambda gt10. In all, 20 positive phages were purified and subcloned into pUC 18 plasmids. Two of them (pgh12, pgh13) were subjected to nucleotide-sequence analysis. The nucleotide sequence of the four cDNA species analysed (Lg11, Lg12, pgh12 and pgh13) were identical in their overlapping regions. The sequence shown in Fig. 1 is a combination of these four sequences.

From the sequence analysis of several cDNAs it appears that two polyadenylation sites occur on the mRNA, at 104 and 252 nucleotides downstream from the stop codon. The hexamer AATAAA presumed to be the recognition sequence directing post-transcriptional cleavage–polyadenylation of the 3′ end of pre-mRNA (Proudfoot & Brownlee, 1976) were not detected.

The ATG triplet (nucleotides 95–97) was tentatively assigned as the initiator codon, because the surrounding sequence agrees

well with the favoured sequence flanking the functional plant initiaton codon AACA*A*U*G*GC (Joshi, 1987; Lütcke *et al.*, 1987). The synthesis of pea leaf H-protein will begin, therefore, with the dipeptide methionylalanine, in common with most plant proteins. In addition, even though the in-frame putative amino acid sequence upstream of the proposed initiator codon ATG is not interrupted by a stop codon, the amino acid composition of this region does not agree with the structural requirements of a mitochondrial targeting peptide according to the recent extensive studies by Hartl *et al.* (1989) and von Heijne *et al.* (1989).

From the putative ATG triplet (nucleotides 95–97) the sequence codes for a polypeptide of 165 amino acids. It was found that 70 amino acids determined by N-terminal sequencing of the peptide fragments generated by CNBr cleavage of the purified H-protein showed complete sequence similarity with the amino acid sequence deduced from the cDNA (Fig. 1). This confirmed our identification of the cDNA as encoding H-protein. The mature protein is a polypeptide of 131 amino acids. In addition, the amino acid composition deduced from the nucleotide sequence agreed well with the overall amino acid composition of the H-protein determined after acid hydrolysis (results not shown).

The M_r of the precursor polypeptide is 17688. When pea leaf mRNA was translated *in vitro* in a wheat-germ lysate and the labelled products immunoprecipitated, a polypeptide of 19000–20000 M_r was detected by SDS/PAGE (results not shown). Assuming the initiating ATG codon to be correctly assigned, the H-protein is predicted to contain a presequence peptide of 34 amino acids (Fig. 1). The presequence peptide exhibits a typical enrichment for alanine (12%), leucine (15%), arginine (9%) and serine (26%), and contains few isoleucine (3%), lysine (3%) and no negatively charged (aspartic acid or glutamic acid) residues. In addition, the presequence peptide presents an alternance of positively charged and hydroxylated regions constantly found in mitochondrial targeting peptides and which are essential for both the import and proteolytic-cleavage mechanisms (for a review, see Hartl *et al.*, 1989). Hence this presequence can be designated as a mitochondrial targeting peptide (von Heijne *et al.*, 1989).

Gene expression for H protein

When the polypeptides of the matrix of mitochondria from dark- or light-grown leaves are separated by SDS/PAGE (Fig. 2), the accumulation of H-, P-, T- and L-proteins of the glycine-cleavage system, as well as the serine hydroxymethyltransferase in the light-grown tissue is striking. For example, the concentration of the H-protein in light-grown leaves is estimated to

Table 1. State-3 rate of O_2 uptake by mitochondria from dark- and light-grown pea leaves

Additions were as follows: (1) 15 mM-glycine; (2) 5 mM-oxoglutarate, 0.3 mM-thiamin pyrophosphate and 0.2 mM-NAD$^+$; (3) 5 mM-pyruvate, 0.3 mM-malate, 0.3 mM-thiamin pyrophosphate and 0.2 mM-NAD$^+$; (4) 1 mM-NADH and 0.6 mequiv. of Ca^{2+}

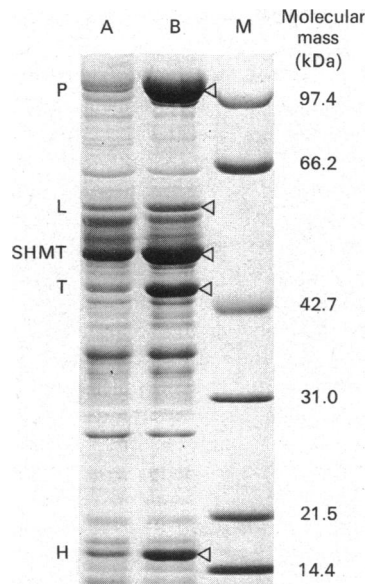| | Rate (nmol of O_2 consumed/min per mg of protein) | |
Substrate	Dark	Light
(1) Glycine	42	223
(2) Oxoglutarate	176	173
(3) Pyruvate	108	133
(4) NADH	428	390

Fig. 2. Comparison between matrix polypeptides from dark- and light-grown pea leaf mitochondria

An 80 μg portion of proteins from dark- (A) or light- (B) grown pea leaf mitochondria matrix were separated by SDS/7.5–15%-PAGE, and the gel was stained with Coomassie Blue. 'M' are molecular-mass markers from Bio-rad. Note that the matrix of green leaf mitochondria is distinguished by the presence of several pronounced bands at M_r 97000 (P-protein), 61000 (L-protein), 53000 (serine hydroxymethyltransferase, SHMT). 45000 (T-protein) and 15500 (H-protein).

Fig. 4. Organ-specific expression of H-protein mRNA

Total RNA was size-fractionated and hybridized with the ^{32}P-labelled cDNA probe. (a) Autoradiogram (13 days at −80 °C). (b) Methylene Blue staining of the nylon membrane after autoradiography. The lanes with leaf RNA (dark or light) contained 1.5 μg of RNA, whereas the other lanes contained 15 μg of RNA. The triangles (◀) indicate the faint signals observed with the root or stem tissues. Upper bands are due to cross-hybridization with ribosomal RNA.

Fig. 3. Effect of light on the abundance of H-protein mRNA

Carefully calibrated amounts (2 μg) of mRNA were size-fractionated and hybridized with the ^{32}P-labelled cDNA probe. A, mRNA from dark-grown leaves, 8 days old; B, mRNA from light-grown leaves, 8 days old; C, mRNA from dark-grown leaves, 8 days old, after 20 h of light. Autoradiography was for 2 h at −80 °C.

Fig. 5. Western-blot analysis of H-protein in different tissues

Protein extracts (50 μg) were subjected to SDS/12%-PAGE and transferred to nitrocellulose. H-protein was immunodetected with a specific antiserum. The extreme right-hand lane (H) contained 0.5 μg of pure H-protein.

be roughly 10-fold higher than in dark-grown leaves. To confirm this observation we have also measured the oxidation of different substrates by mitochondria isolated from the dark- or light-grown leaves (Table 1). On a protein basis, oxoglutarate, pyruvate and NADH were oxidized at the same rate in both types of mitochondria. In contrast, glycine oxidation in green-leaf mitochondria was much higher than that observed in mitochondria from etiolated leaves. However, the rate of glycine oxidation measured in the mitochondria from dark-grown leaves (42 nmol/min per mg of protein) reveals that these organelles contain a substantial amount of glycine-cleavage system, indicating that this complex is also synthesized in etiolated leaves maintained in total darkness.

To determine whether the difference in the accumulation of glycine-cleavage complex and in enzymic activity corresponds to a change in the amount of mRNA, a Northern analysis of mRNA for H-protein was carried out using a ^{32}P-labelled 519 bp fragment (nucleotides 151–670) of the cDNA as probe. Total
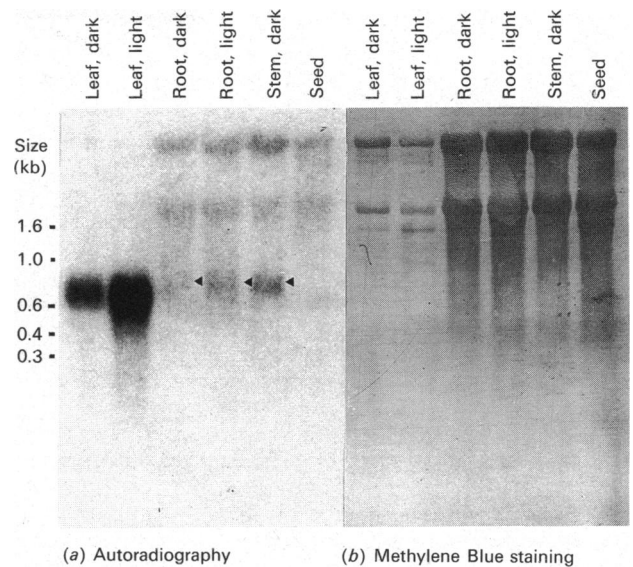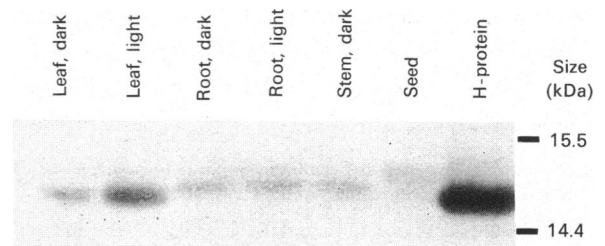
RNAs were prepared from the leaves of 8-day-old pea seedlings which were grown in total darkness, on a light/dark program or which were illuminated for 20 h after 8 days of darkness. The result of Northern analysis performed under stringent conditions are shown in Fig. 3. An mRNA of 700 nucleotides was easily detected in the three samples after only 2 h autoradiography at −80 °C. Clearly the light-grown leaves contain more mRNA for H-protein (roughly 3–5 fold) than the dark-grown leaves, and after a 20 h light period an increase in the mRNA level was observed. These results may indicate that light could play a role in the induction of H-protein mRNA. However, the steady-state level of H-protein mRNA in etiolated pea leaves was far from negligible, suggesting that some other factor could also modulate the expression of the H-protein.

To detect a possible tissue-specific expression, we performed a Northern analysis of total RNA from different tisues: leaf, root, stem and seed (Fig. 4). Large differences were observed between the different tissues. The H-protein mRNA level was found to be
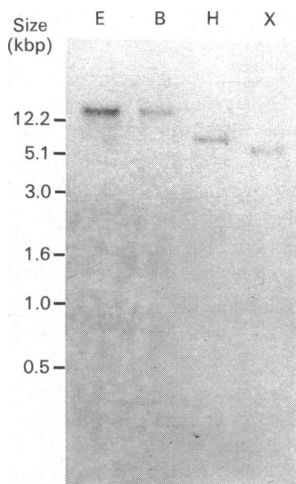
Fig. 6. Southern-blot analysis of pea genomic DNA

A genomic DNA gel blot of pea DNA (10 μg) digested with EcoRI (E), BamHI (B), HindIII (H) and XbaI (X) was hybridized with the ³²P-labelled cDNA probe as described in the Materials and methods section. The final wash was carried out in 2 × SCC/0.1 % SDS at 60 °C. Autoradiography was for 2 weeks at −80 °C.

negligible in the root, stem and seed tissues (a faint signal after 13 days autoradiography). The marked accumulation of H-protein mRNA in leaf tissue was not due to unequal sample loading or RNA degradation in the other samples, as evidenced by the Methylene Blue staining of the nylon membrane after autoradiography (Fig. 4).

We were interested to determine whether these differences could be observed at the level of protein synthesized. Samples of each tissue were subjected to SDS/PAGE, transferred to nitrocellulose, and probed with H-protein-specific antibodies. Fig. 5 shows a protein-gel-blot analysis of H-protein present in proteins isolated from various tissues of pea. The protein was detected in the leaves of dark- and light-grown plants with the same mobility as the purified protein. The ratio between the two signals is in good agreement with the Northern analysis of mRNA. Surprisingly, a signal was detected in the other tissues, but with a lower mobility, and we cannot exclude a cross-reaction with another protein, because glycine-oxidation rates observed in mitochondria from root and stem tissues are almost negligible (Henricson, 1989).

Southern-blot analysis of nuclear DNA

The DNA probe derived from the cDNA (nucleotides 151–670) was used in a Southern-hybridization experiment with pea nuclear DNA digested with several restriction endonucleases (Fig. 6). In all lanes a single band was detected, even after a low stringency wash, a result suggesting that a single-copy gene codes for H-protein in pea.

DISCUSSION

We report the characterization of cDNAs for H-protein, a constituent of the glycine-cleavage system involved in the photorespiratory pathway of 'C₃' plants (Lorimer & Andrews, 1980; Husic et al., 1987). This research follows previous work of this laboratory dealing with the isolation and biochemical characterization of the glycine-cleavage system from pea leaf mitochondria (Neuburger et al., 1986; Bourguignon et al., 1988).

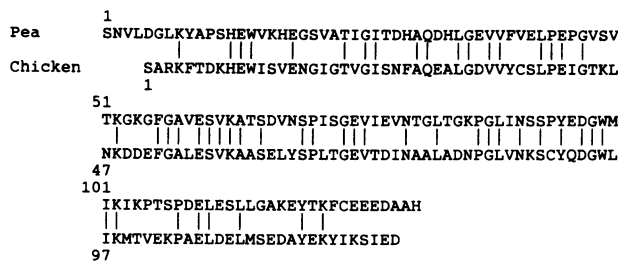Sequencing of four different clones revealed the presence of two polyadenylation sites in the mRNA, which could be a

Fig. 7. Sequence similarities between pea and chicken H-proteins

The sequence of chicken H-protein was determined by Fujiwara et al. (1986). Sequence identities are depicted as vertical bars. Residue numbers for pea and chicken H-protein are given on the left.
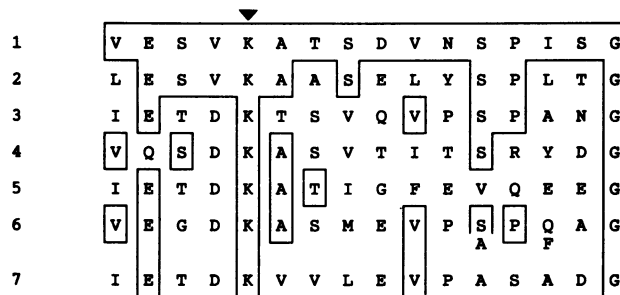

Fig. 8. Primary structures surrounding lipoyl-lysine residues in several proteins

1, Pea H-protein (the present work); 2, chicken H-protein (Fujiwara et al., 1986); 3, bovine 2-oxoglutarate dehydrogenase complex, E2 component (Bradford et al., 1987); 4, human, bovine branched-chain-α-oxoacid dehydrogenase complex, E2 component (Hummel et al., 1988; Lau et al., 1988); 5, rat pyruvate dehydrogenase complex, E2 component (Gershwin et al., 1987; Yeaman et al., 1988); 6, Escherichia coli pyruvate dehydrogenase complex, three domains of E2 component (Stephens et al., 1983); 7, E. coli 2-oxoglutarate dehydrogenase complex, E2 component (Spencer et al., 1984). Sequence identities with pea H-protein are indicated by boxes. The inverted black triangle (▼) indicates the lipoyl-lysine residue.

common feature for plant genes, as suggested by Dean et al. (1986). The absence of clear polyadenylation signals in both cases certainly reflects the high level of flexibility in the processing and polyadenylation of plant genes. We assigned the ATG (nucleotides 95–97) to the initiation codon on the basis of the following evidence: it agrees perfectly with the proposed plant initiation consensus sequence (Joshi, 1987; Lütcke et al., 1987) and the upstream in-frame sequence does not meet the criteria of a mitochondrial targeting peptide (von Heijne et al., 1989). However, further experiments and gene-sequence data are required in order to characterize the initiation codon. The primary structure of the protein deduced from the nucleotide sequence indicates that the H-protein is synthesized as a precursor with a 34-amino-acid presequence whose structure agrees with the requirements of a mitochondrial targeting peptide (Hartl et al., 1989; von Heijne et al., 1989). The overall sequence similarity at the amino acid level between the H-protein from pea (131 amino acids) and chicken (125 amino acids) (Fujiwara et al., 1986) is moderate (42 %; Fig. 7). However, in the region between amino acid residues 56 and 76 of the pea sequence and residues 52 and 72 of the chicken sequence, this percentage reaches 67 %. Bacterial H-protein and chicken H-protein have been shown to

contain lipoic acid as a prosthetic group (Robinson *et al.*, 1973; Kochi & Kikuchi, 1974; Fujiwara *et al.*, 1979). Later the lipoic acid was shown to be covalently linked to a lysine residue identified in the amino acid sequence of chicken H-protein (Fujiwara *et al.*, 1986). To assess possible sequence conservation, we compared the amino acid sequences of the H-protein from pea with the chicken H-protein and with several enzymes bearing lipoyl-lysine domains (Fig. 8). It is noteworthy that lysine-63 of the pea sequence is conserved in all lipoate-bearing protein sequences. Furthermore, the same lysine residue has been shown to be covalently linked to the lipoic acid prosthetic group in the chicken H-protein (Fujiwara *et al.*, 1986). Despite the lack of supporting evidence, it is tempting to assign a similar role for lysine-63 on the pea H-protein. Also, not only is glycine-74 conserved among all species studied, but it is always located 11 amino acids residues downstream from lysine-63. It should be stressed that, according to a recent review by Yeaman (1989), little is known about the location (cytosolic compartment? matrix compartment?) or the nature of the ligase(s) responsible for the attachment of the lipoate moiety. The availability of a cDNA for the H-protein of the higher-plant glycine decarboxylase complex will allow more refined investigations into mitochondrial import and lipoic acid attachment at the molecular level.

Glycine oxidation was shown to occur preferentially in green tissues (Arron & Edwards, 1980; Gardestrom *et al.*, 1980; Morohashi, 1987), and a dramatic accumulation of the glycine-cleavage system in mitochondria from green leaves and not in etiolated leaves was demonstrated (Day *et al.*, 1985; Walker & Oliver, 1986). Clearly, in accordance with previously published data, these results indicate that the glycine-cleavage system accumulates in mitochondria from green leaves and not in etiolated leaves, as shown by the measurement of substrate oxidation (Table 1) and SDS/PAGE analysis of matrix proteins (Fig. 2). Day *et al.* (1985) and Bourguignon *et al.* (1988) have shown that this accumulation led to an increase in the mitochondria density on Percoll gradients. The question that arises is whether light regulates gene expression of glycine-cleavage-system proteins as it does for numerous phototrophically engaged proteins (for a review, see Kuhlmeier *et al.*, 1987). The availability of a cDNA probe urged us to investigate steady-state RNA levels of the H-protein, which is a mitochondrial enzyme engaged in a light-driven metabolic pathway. The Northern analysis of mRNA from dark- and light-grown leaves shows a 3–5-fold light-induced increase in the transcripts levels (Fig. 3), a result supported by Western-blot analysis of leaf proteins (Fig. 5). However, this difference is small in comparison with the prominent light induction of genes for the small subunit of ribulose-1,5-biphosphate carboxylase oxygenase, the chlorophyll-*a*/*b*-binding protein or other chloroplast proteins (Kuhlmeier *et al.*, 1987). Interestingly, a very low level of transcripts was found in root, stem and seed (Fig. 4), suggesting preferential expression in the leaf. This result correlates well with the glycine-oxidation rates measured in different organs of spinach (*Spinacia olenacea*) (Gardestrom *et al.*, 1980) or pea (Henricson, 1989). It would appear, then, that the mRNA for H-protein accumulates preferentially in the leaf tissue. The existence of two types of cDNAs differing by the length of their 3′ untranslated regions was deduced from the nucleotide-sequence analysis and raises the question as to whether the two mRNA species are differentially regulated in an organ-specific manner, as was shown recently for rice (*Oryza sativa*) lectin mRNA (Wilkins & Raikhel, 1989). So far we have not been able to discriminate between the two mRNA species in Northern blots, perhaps because of a large difference in the amounts of the transcripts. However, the slightly higher molecular mass of the mRNA detected in the root and stem tissues is noteworthy (Fig. 4).

The general conclusions that can be drawn concerning gene expression for H-protein in pea are: (*a*) the expression occurs predominantly in the leaf; (*b*) light exerts an additional effect by increasing the level of transcripts. The latter effect could result more from the cellular differences between etiolated and green tissues rather than from a specific light induction at the molecular level. Further experiments will be required to define the components of the signal-transduction chains that lead to light responsiveness and to organ specificity. Cloning of the gene is needed, and the characterization of *cis*-acting elements should allow one to distinguish between the light- and organ-specific factors responsible for H-protein-gene expression in pea.

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