Purification and Primary Amino Acid Sequence of the L Subunit of Glycine Decarboxylase

EVIDENCE FOR A SINGLE LIPOAMIDE DEHYDROGENASE IN PLANT MITOCHONDRIA*

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In order to purify the lipoamide dehydrogenase associated with the glycine decarboxylase complex of pea leaf mitochondria, the activity of free lipoamide dehydrogenase has been separated from those of the pyruvate and 2-oxoglutarate dehydrogenase complexes under conditions in which the glycine decarboxylase dissociates into its component subunits. This free lipoamide dehydrogenase which is normally associated with the glycine decarboxylase complex has been further purified and the N-terminal amino acid sequence determined. Positive cDNA clones isolated from both a pea leaf and embryo Agt11 expression library using an antibody raised against the purified lipoamide dehydrogenase proved to be the product of a single gene. The amino acid sequence deduced from the open reading frame included a sequence matching that determined directly from the N terminus of the mature protein. The deduced amino acid sequence shows good homology to the sequence of lipoamide dehydrogenase associated with the pyruvate dehydrogenase complex from Escherichia coli, yeast, and humans. The corresponding mRNA is strongly light-induced both in etiolated pea seedlings and in the leaves of mature plants following a period of darkness. The evidence suggests that the mitochondrial enzyme complexes: pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and glycine decarboxylase all use the same lipoamide dehydrogenase subunit.

Lipoamide dehydrogenase (LDH)† is a FAD-containing polypeptide, which catalyzes the reduction of the lipoamide group in a number of multisubunit enzyme complexes. It has been extensively studied in both bacterial and eukaryotic species as the E3 component of α-ketoad dehydrogenase complexes (pyruvate dehydrogenase (PDC), 2-oxoglutarate dehydrogenase (2-ODC), and branched-chain 2-oxo acid dehydrogenase) and as the L subunit of glycine decarboxylase (for review, see Carothers et al., 1989). Considerable interest has been focused on the relationship between LDH and the different multisubunit enzymes, specifically as to whether all mitochondrial multisubunit complexes which contain LDH share a common LDH subunit or whether each complex uses a different LDH subunit encoded by a separate gene.

In the most well characterized bacterial system, Escherichia coli, LDH is a component of two multisubunit complexes: PDC and 2-ODC and is encoded by a single gene (lpd). The lpd gene together with the aceE and aceF genes, which encode two other subunits of PDC, form an operon (see Guest et al., 1984, for review). In other bacterial species the situation appears more complicated. In Pseudomonas putida up to three different LDH genes have been identified (Burns et al., 1989). The LPD-Glc gene encodes the lipoamide component of 2-ODC and probably PDC and GDC. LPD-Val is specific to the branched chain oxo acid dehydrogenase complex (Sokatch et al., 1983; Sokatch and Burns, 1984) and the function of LPD-3 is unknown (Burns et al., 1989). Freundenberg et al. (1989) have recently identified an atypical lipoamide dehydrogenase subunit associated with GDC from the anaerobic bacteria Eubacterium acidaminophilum. This appears, however, to be the only example characterized to date in which an LDH subunit has been identified as being specific to GDC.

The situation in eukaryotes is similar to E. coli. A mutant in the yeast lipd1 gene abolishes all LDH activity together with PDC and 2-ODC activity (Dickinson et al., 1986). In mammals, immunological data (Matuda and Saheki, 1985), reconstitution studies (Sakurai et al., 1970), and observation on human genetic disorders (for review, see Stansbie et al., 1986) all indicate that PDC, 2-ODC, and branched chain oxo acid dehydrogenase all use the same lipoamide subunit. Much less work has been done on the L subunit of eukaryotic GDC.

Although some immunological data have suggested that in the rat liver a separate LDH polypeptide exists specifically for GDC (Carothers et al., 1987). Walker and Oliver (1986) have shown that yeast LDH restores glycine decarboxylase activity when added to the P, H, and T subunits from pea and that a monoclonal antibody shown to act on the L subunit of GDC, selected on the basis of its ability to inhibit glycine decarboxylase activity, inhibited PDC with equal efficiency. Such evidence suggests that in pea leaf mitochondria that the L subunits of PDC and GDC are very similar. No direct measurements of cross-reactivity or sequence comparisons can be made, however, since no lipoamide dehydrogenase subunit preparation has been demonstrated to be that exclusively from GDC.

(Received for publication, July 23, 1991)
The pea leaf represents an excellent system in which to study GDC since, due to its key position in the photosynthetic pathway, GDC may represent 30-50% of the total mitochondrial matrix protein (Oliver et al., 1990). Like bacterial and mammalian GDC, pea GDC is a multisubunit complex composed of different numbers of four subunits: P, T, H, and L (LDH) (Oliver et al., 1990), which may be purified as a loosely bound complex (Neuberger et al., 1986). Information on the interaction of the GDC subunits with one another and on the control of GDC expression have been hampered by the lack of clones for each subunit. The sequence of cDNAs, encoding the H (Kim and Oliver, 1990; Macherel et al., 1990) and P (Turner et al., 1991) subunits from pea are, however, now available, as well as the sequence of a cDNA encoding the T subunit from bovine liver (Okamura-Ikeda et al., 1991). The aim of this work was to identify a clone encoding the L subunit of GDC, to examine the relationship of this subunit to the lipoprotein dehydrogenases of other mitochondrial multisubunit complexes (PDC and 2-ODC), and to look at its expression compared to other subunits of GDC.

**EXPERIMENTAL PROCEDURES**

Growing of plant material, manipulation of light and dark regimes, nucleic acid extractions, cDNA cloning, and Southern, Northern, and Western blotting were all as described previously (Turner et al., 1991) except that RNA for Northern blots was size fractionated on agarose gels following denaturation with glyoxal (Covey et al., 1981).

**Protein Extractions**—Fully expanded leaves and roots from pea seedlings and developing embryos (~100 mg fresh weight per embryo) were extracted in 5 mM Tris, pH 7.5, with 0.15 g of polyvinylpyrrolidone g^-1 fresh weight of tissue. Extracts were spun at 10,000 × g for 10 min at 4°C and the supernatants retained. Protein concentrations were determined using the dye-binding assay (Bradford, 1976).

**Purification of Lipoprotein Dehydrogenase**—Mitochondria were purified from pea leaves and the matrix proteins isolated according to Bourguignon et al. (1988). Mitochondrial matrix extract was applied to a Mono Q HR5/5 column equilibrated with 10 mM potassium phosphate, pH 6.5, 1 mM EGTA, and 1 mM β-mercaptoethanol (buffer A) and the column washed through with 5 column volumes of buffer A. Proteins were eluted at room temperature with a linear gradient (30 ml) of increasing potassium phosphate (10-400 mM in buffer A) at a flow rate of 1 ml min^-1. The loading and wash volumes were pooled and 1-ml fractions were collected through the gradient. All fractions were assayed for LDH, PDC, and 2-ODC. The LDH activity was eluted as a single peak at 210 mM phosphate. The fractions containing LDH activity were pooled, diluted with 10 volumes of distilled water, and loaded onto a second Mono Q HR5/5 column equilibrated with 20 ml Tris, pH 8.0. The column was eluted with a linear gradient of KCl (0-500 mM in buffer A) and the LDH activity eluted as a single peak at 280 mM KCl. The N-terminal amino acid sequence of the protein was determined as described previously (Turner et al., 1991).

**Enzyme Assays**—Glycine decarboxylase was assayed by the decarboxylation of [14C]glycine as described by Walker and Oliver (1986). Lipoamide dehydrogenase was assayed according to Motokawa and Kikuchi (1974). Activity of PDC was determined by the spectrophotometric method described by Randall and Miernyk (1990) and 2-ODC was assayed in the same way except 2 μM o-oxoglutarate replaced pyruvate.

**Immunological Techniques**—Antisera to the LDH was raised in a rabbit by injecting 600 μg of the purified protein. Injections in Freund's adjuvants, isolation of the IgG fraction, and immunoprecipitation experiments were as described previously (Turner et al., 1991).

**DNA Sequencing and Sequence Comparison**—The cDNA clone pGDLL1 was sequenced completely on both strands as previously described (Turner et al., 1991). The clone pGDLE1 was sequenced from each end and using an internal oligonucleotide primer as indicated (Fig. 1). Sequence alignments were performed using a modification of the program CLUSTAL (Higgins and Sharp, 1988), and sequence relationships were calculated as described by Hein (1990) using sequences available from the EMBL data base.

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**RESULTS AND DISCUSSION**

**Isolation of LDH Associated with GDC**—When the matrix extract of pea leaf mitochondria was fractionated on a Mono Q column using a linear gradient of phosphate, over 93% of the LDH activity loaded was recovered and the vast majority (97%) of this recovered activity was found in a single peak at 210 mM phosphate (Table I). The protein was at least 80% pure at this stage as deduced from Coomassie-stained SDS-polyacrylamide gels. The remainder of the recovered activity (2%) was found in the wash fractions. The PDC and 2-ODC activities were only detectable in the wash fraction although only slightly more than half of the loaded activity of the latter two enzymes was recoverable (Table I).

The large peak of LDH activity eluted at 210 mM phosphate on the Mono Q column is considered to be that of the enzyme associated with the glycine decarboxylase complex for the following reasons. Bourguignon et al. (1988) have shown that under conditions of low ionic strength 97% of the LDH activity from pea leaf mitochondrial matrix extract can be retained behind a Diaflo XM-300 membrane along with all the other subunits of the glycine decarboxylase complex and PDC. If purified exogenous LDH is added to the same matrix extract it will pass through with quantitative recovery of the added activity (Oliver et al., 1990). The implications of these experiments are that LDH does not exist as a free protein in the matrix of pea leaf mitochondria but is bound into large multisubunit enzyme complexes. Three such complexes exist in pea mitochondria, GDC, PDC, and 2-ODC. Of these complexes, PDC is known to be stable and requires the presence of 1 M salt to dissociate completely the LDH (E2) subunit from the complex (Randall and Miernyk, 1990), whereas GDC can be fractionated into subunit components on a Sephacyr S-300 column in the presence of 50 mM KCl (Bourguignon et al., 1988).
al., 1988). Although the recoveries of PDC and 2-OIC were only slightly more than 50%, only a small proportion (3%) of LDH activity co-fractionated with these enzyme complexes (Table 1) and so even if the recoveries observed were due to dissociation of these enzyme complexes, any possible contribution of LDH from them to the LDH eluting at 210 mm phosphate must be small. In addition, during the purification of individual GDC subunits, Bourguignon et al. (1988) showed that a very large proportion of LDH activity could be purified independently of PDC activity when matrix extract from pea leaf mitochondria was fractionated on a size basis (Sephacryl S-300 column under conditions in which the glycine decarboxylase complex dissociates) rather than on a charge basis as in our experiment.

The LDH activity was further purified on a second Mono Q column to yield a single band on a 12% SDS-polyacrylamide gel of M, 60,000 which is similar to those reported previously for this enzyme from pea leaf mitochondria (59,000, Walker and Oliver, 1986; 61,000, Bourguignon et al., 1988). Seventeen amino acids were unambiguously determined at the N terminus. There was no indication of heterogeneity in the sequence due to the presence of other minor proteins, consistent with the amino acid sequence being only from the LDH subunit of GDC.

In order to examine the ability of the anti-L IgG to inhibit LDH and GDC activities, the antibody was used to immuno-precipitate the L subunit from the mitochondrial extract and the supernatant assayed for the enzyme activity. At the appropriate concentration of the antibody it is possible to remove all detectable lipoamide dehydrogenase activity in the extract (Fig. 1b) at which point greater than 95% of GDC activity remaining presumably reflects the ability of the antibody to immunoprecipitate the L subunit of GDC. The low level of GDC activity remaining presumably reflects the ability of GDC to function with only the P, H, and T subunits as described previously (Walker and Oliver, 1986). It is interesting to note, however, that where the antibody to matrix extract ratio is such that only partial inhibition of LDH activity occurs (Fig. 2a), there is consistently greater inhibition of LDH activity than GDC activity. The above observation may result from the fact that under the conditions used for these experiments, the action of LDH is not rate-limiting for GDC activity. Consistent with this, the extracts used have a much higher activity for LDH than GDC (see legend to Fig. 1) and Walker and Oliver (1986) have shown that the addition of exogenous LDH to mitochondrial extract does not increase the rate of GDC activity.

Isolation of a cDNA Clone for LDH—The LDH antibody recognizes a single band of M, 60,000 on Western blots of total protein extract from pea leaves, embryos, and roots separated on an SDS-polyacrylamide gel (Fig. 7a). In order to isolate a cDNA clone for LDH the antibody was used to screen λgt11 pea cDNA libraries constructed from both pea leaf and embryo mRNAs. Positive clones were subcloned into Bluescript for further analysis and a leaf cDNA (pGDLL1) of around 1.8 kb selected for further analysis. The sequence (Fig. 3) of this clone contained a large open reading frame, and the amino acid sequence derived from it included a sequence matching exactly that derived directly from the N terminus of the mature protein. Although the deduced amino acid sequence includes the entire sequence of the mature polypeptide, there are only 11 residues upstream of this. No methionine residue is present at or near the N terminus of the open reading frame, consistent with the absence of part of the N-terminal leader sequence from the clone. In order to determine the sequence of the entire open reading frame, a second clone (pGDL1) from the embryo-derived library was examined. Although this clone was of similar length (1.8 kb), sequence analysis of this clone contained a large open reading frame, and the amino acid sequence derived from it included a sequence matching exactly that derived directly from the N terminus of the mature protein. Although the deduced amino acid sequence includes the entire sequence of the mature polypeptide, there are only 11 residues upstream of this. No methionine residue is present at or near the N terminus of the open reading frame, consistent with the absence of part of the N-terminal leader sequence from the clone. In order to determine the sequence of the entire open reading frame, a second clone (pGDL1) from the embryo-derived library was examined. Although this clone was of similar length (1.8 kb), sequence analysis of this clone contained a large open reading frame, and the amino acid sequence derived from it included a sequence matching exactly that derived directly from the N terminus of the mature protein.
Cloning of the L. Subunit of Glyceraldehyde 3-Phosphate

clones has been determined (Fig. 1) they are identical. Copy number experiments (see below) show that the 3' end of both cDNAs hybridize to only one gene and this gene contains an internal 1.3-kb HindIII fragment consistent with the restriction map of the cDNAs (Fig. 1). The sequence of pGDLE1 contains an extra 90 nucleotides at the 5' terminus not present in pGDLL1. This region extends the open reading frame derived from pGDLL1 and includes 2 in-frame methionine residues separated by an alanine residue. The most upstream methionine of the two shows a good match to the plant consensus translation initiation site (Joshi, 1987). If this methionine is the initiation site there is a 31-amino acid leader sequence, rich in serine (20%) with the ability to form an amphipathic helix, consistent with it being a mitochondrial targeting sequence (von Heijne, 1986).

Although the deduced amino acid sequence shown here is the first published LDH sequence from plants and the first sequence of a LDH subunit known to be associated with GDC, it shows good sequence homology with other published LDH subunit sequences (Fig. 4). Rice et al. (1984) predicted the presence of four domains in the structure of LDH, and their presence has been confirmed more recently by the elucidation of the three-dimensional structure of LDH from bakers' yeast (Takenaka et al., 1988). These four domains, which correspond to the active site, the FAD- and NAD-binding sites, and the interface region are all well conserved in the pea LDH sequence (Fig. 4).

The evolutionary relationship of various LDH subunits is well documented (see Carothers et al. (1989) for review) as is the possibility that a wide number of FAD dehydrogenases, including glutathione reductase and mercuric reductase, evolved from a common progenitor (Brown et al., 1983; Fox and Walsh, 1983; Stephens et al., 1983). A relationship based upon the comparison of the amino acid sequences of the pea LDH with the amino acid sequence of LDH genes from other organisms is shown (Fig. 5). Of particular interest is the relationship between the sequence from pea with that of yeast, human, and pig, since these are all mitochondrial LDHs and the latter three are known to be components of PDC. Such a relationship, which mirrors the evolutionary relationship of the organisms themselves, is consistent with these genes all serving the same function and with a common subunit being used by GDC and PDC. It is not possible, however, to exclude the possibility that an event, such as gene duplication, has occurred resulting in different enzyme complexes using the product of different LDH genes. If this is the case, however, it must have occurred relatively recently, subsequent to the divergence of plants from the other species.

Expression and Organization of the LDH Genes—The copy number of the LDH genes was determined by probing Southern blots of pea genomic DNA cut with HindIII or BamHI together with pGDL1 digested with KpnI loaded to give a signal corresponding to 0.5-4 copies/haploid genome. Two fragments of the LDH cDNA clone pGDL1 were used as probes (see Fig. 1): LH3, a fragment of pGDLL1 which covers 60% of the coding region (top) and LN4, a fragment from the 3' noncoding region of pGDLL1 (bottom). Numbers on the left-hand side indicate the position and size (base pairs × 103) of standard markers.

![Alignment of the pea LDH sequence with that of the yeast, human, and E. coli PDC E subunits](image)

**Fig. 4. Alignment of the pea LDH sequence with that of the yeast, human, and E. coli PDC E subunits.** The region around the active site cysteines as well as part of the interdomain contact region and the binding sites of FAD and NAD are all indicated. Dots represent residues identical to that of the pea sequence.

**Fig. 5.** Relationship of other LDH subunits to that of the pea sequence based upon sequence divergence. Other than the *P. putida* (a component of branched chain oxa acid dehydrogenase complex) and pea (described here) LDHs, all LDH molecules shown are known to be a component of PDC. Length of horizontal lines are proportional to the amount of sequence divergence, see "Experimental Procedures."
LH3 corresponds to two genes, the 3' fragment LNd is specific for a single gene and may therefore be used to examine the expression of only a single gene. The hybridization patterns of a large number of pea genotypes have been examined, and no change in the hybridization pattern is seen between high (0.1 \times SC at 65 °C; 1 \times SC is 0.15 M NaCl, 0.015 M sodium citrate) and low stringency (2 \times SC at 50 °C), indicating that there are no other closely related genes.

To examine the tissue specific expression of the LDH genes, both the LH3 and LNd fragments were used to probe a Northern blot containing mRNA from various tissues. Both probes show an identical pattern of expression (Fig. 7b), indicating that if the second LDH gene is expressed it is either expressed at a relatively low level or both LDH genes are expressed in a tightly coordinated manner. The level of hybridization is similar in both embryos and leaves, with the highest level of expression in roots (Fig. 7b). Such an observation argues against the product of this LDH gene being exclusive to GDC in pea for the following reasons. First, the mRNAs for the P and H subunits are barely detectable in embryo and roots (Macherel et al., 1990; Turner et al., 1991).

Second, the rate of glycine oxidation by mitochondria from pea root apices is very low compared to that of mitochondria from leaves (Walton and Woolhouse, 1986), while the amount of LDH detected on Western blots of proteins from these tissues is comparable (Fig. 7a).

In order to determine the affect of light on the level of LDH mRNA, a Northern blot containing mRNA from etiolated seedlings exposed to light for various periods of time was probed with pGDLL1. The LDH mRNA accumulates rapidly and appears to precede that of the GDC H subunit mRNA (Fig. 7c, i). Since the exposure of etiolated seedlings to light also causes an increase in leaf development, the same blot also included mRNA from the leaves of mature plants which had been placed in the dark for 60 h, then re-exposed to light. Although the level of LDH declines in the dark, the fall is not as dramatic as that observed for the H subunit (Fig. 7c, ii). Once the plants are returned to the light, however, the induction of LDH mRNA is dramatic and far exceeds the induction seen for the H subunit mRNA (Fig. 7c, ii). It is tempting to speculate that the induction of L subunit mRNA must precede that of the H subunit to ensure that there is sufficient LDH available for the essential function of PDC and 2-ODC even in the presence of a comparatively large amount of GDC.

Conclusion—We have cloned a cDNA which corresponds to the LDH subunit of GDC. Sequence comparison, together with the organization and expression of the corresponding gene all indicate that the same LDH subunit may be shared in GDC, PDC, and 2-ODC. The corresponding mRNA is strongly light-induced in leaves and yet is found in comparable levels in other tissues. Since it is required to maintain an adequate supply of LDH to all of the complexes which require it and these complexes may all be expressed at different levels under different circumstances, the LDH gene must have a complex mechanism of regulation.

Acknowledgments—We thank Dr. Pat Barker (Institute of Animal Production and Grassland Research, Babraham, Cambridge) for performing the N-terminal amino acid sequencing; Dr. G. Murphy for technical advice on DNA sequencing and sequence alignments; and Dr. A. Smith and Dr. D. Murphy for useful comments on the manuscript.

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