Glycine decarboxylase and pyruvate dehydrogenase complexes share the same dihydrolipoamide dehydrogenase in pea leaf mitochondria: evidence from mass spectrometry and primary-structure analysis

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In order to compare the dihydrolipoamide dehydrogenase associated with the pyruvate dehydrogenase complex (E3) with that associated with the glycine decarboxylase complex (L-protein), we report for the first time the purification and characterization of the E3 component from pea leaf mitochondria. The first 30 amino acids of the N-terminal sequence of the mature E3 protein are identical with those of the mature L-protein of the glycine decarboxylase complex. Electrospray ionization-mass spectrometric analysis of E3 and the L-protein gave exactly the same molecular mass of $49\,753 \pm 5$ Da. We have also confirmed the primary structure of the L-protein, in par-

ticular the C-terminal sequence, deduced from the cDNA published by Bourguignon, Macherel, Neuburger and Douce [(1992) *Eur. J. Biochem.* **204**, 865–873]. Western-blot analysis shows that specific polyclonal antibodies raised against the L-protein recognize specifically both E3 and L-protein but not the porcine dihydrolipoamide dehydrogenase. We conclude that, in pea leaf mitochondria, the pyruvate dehydrogenase and glycine decarboxylase complexes share the same dihydrolipoamide dehydrogenase. We have also confirmed by MS analysis that the FAD is not covalently bound to the enzyme.

INTRODUCTION

Two multienzyme complexes play a fundamental role in plant leaf respiration: the pyruvate dehydrogenase complex (PDC; EC 1.2.4.1), which regulates entry of carbon into the tricarboxylic acid cycle and related metabolism [1], and the glycine decarboxylase complex (GDC; EC 2.1.2.10), which catalyses with serine hydroxymethyltransferase (EC 2.1.2.1) the mitochondrial step of photorespiration [2–5]. These two complexes contain a dihydrolipoamide dehydrogenase (dihydrolipoamide-NAD⁺ oxidoreductase; EC 1.8.1.4; LPD) called E3 in the case of the PDC and L-protein in the case of GDC. E3 is also the flavoprotein component of the multienzyme 2-oxoglutarate acid and branched-chain 2-oxo acid dehydrogenase complexes [6,7]. LPD has been extensively studied in several organisms and several situations may exist. For example, *Escherichia coli* contains a single LPD for pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase encoded by the *lpd* gene [8]. Indeed mutants of *E. coli* deficient in LPD lack both pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activities [9]. A similar situation is observed in *Saccharomyces cerevisiae* [10]. In contrast, *Pseudomonas putida* contains up to three different LPDs called LPD-Val, LPD-Glc and LPD-3 [11–15]. LPD-Val is the specific E3 component of branched-chain oxo acid dehydrogenase, LPD-Glc is the E3 component of 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase and the L-factor of the glycine oxidation system. However, LPD-3 differs in mol-

ecular mass, amino acid composition and N-terminal amino acid sequence from the two other LPDs and seems to be associated with an unknown multienzyme system. On the other hand, Freudenberg et al. [16] have isolated from *Eubacterium acidaminophilum* an atypically small LPD which seems to be specifically involved in glycine metabolism. This enzyme seems to be a component of the glycine decarboxylase and glycine reductase complexes [17]. Surprisingly, in mammals the situation is not yet clarified. Otulakowski and Robinson [18] have published the sequence of the human and porcine LPD as being the L-protein of GDC and E3 of the α -oxo acid dehydrogenase complexes, but there is immunological evidence for two LPDs in rat liver mitochondria, one of which may be the L-protein component of GDC [19]. In plants, in particular pea leaf mitochondria, Turner et al. [20] and Bourguignon et al. [21] have strongly suggested that there is a single LPD, encoded by a single gene, involved in the 2-oxo acid multienzyme complexes and GDC. Turner et al. [20] have shown that the L-protein of GDC is composed of 498 amino acids with a calculated molecular mass of 53 125 Da. In contrast, Bourguignon et al. [21] give, for the same protein from a different pea cultivar, a calculated molecular mass of 49 721 Da and a composition of 470 amino acids. The peptide sequence differs at the C-terminal region of the protein. In fact, a close examination of the cDNAs corresponding to the L-proteins isolated by the two groups shows that they differ by only one nucleotide. Studies by Kopriva et al. [22] and ourselves (unpublished work) have revealed allelic variation between the

Abbreviations used: ESI-MS, electrospray ionization-mass spectrometry; FAB, fast-atom bombardment; GDC, glycine decarboxylase complex; LPD, E3, dihydrolipoamide dehydrogenase; PDC, pyruvate dehydrogenase complex; DTT, dithiothreitol; TFA, trifluoroacetic acid.

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The nucleotide sequence published by Bourguignon et al. [21] has the accession number X63464 in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases. The nucleotide sequence published by Turner et al. [20] has been corrected and has the accession number X62995.

nucleotide and amino acid sequences of the T-protein gene in the two pea cultivars used by Turner et al. [20] and Bourguignon et al. [21]. In this case there are changes to the amino acids at three positions but not to the length of the predicted open reading frame.

To clarify the situation we set out to discover which L-protein sequence is correct or whether there are two different genes encoding the mitochondrial L-protein differing by one nucleotide. To achieve this we used electrospray ionization-mass spectrometry (ESI-MS) to determine the mass of the native L-protein along with HPLC/ESI-MS analysis of peptides generated by its chemical (CNBr) cleavage. In order to confirm the C-terminal sequence of the L-protein, we purified the C-terminal peptide and submitted it to Edman degradation. In contrast with the data of Turner et al. [20] and Bourguignon et al. [21], Taylor et al. [23] have shown by immunological detection that E3 from pea mitochondrial PDC has a molecular mass of 67 kDa. We have therefore investigated further whether E3 isolated from PDC is the same protein as the L-protein associated with GDC in pea leaf mitochondria.

MATERIALS AND METHODS

Purification of mitochondria and preparation of a matrix extract

Pea (*Pisum sativum*) leaf mitochondria were isolated from 12-day-old plants by differential centrifugation and purified on Percoll/polyvinylpyrrolidone gradients [24]. The soluble (matrix) enzymes were released from the mitochondria by three freeze/thaw cycles, and, after removal of membranes by ultracentrifugation, the resulting crude extract was concentrated on an Amicon membrane as described by Bourguignon et al. [3].

Assay of LPD

Assay mixtures for measuring LPD activity contained 30 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.5, 2 mM dithiothreitol (DTT), 2 mM NAD^+ , 5 mM dihydrolipoate and enzyme in a final volume of 0.5 ml. The reaction, carried out under strict anaerobic conditions, was initiated by the addition of dihydrolipoate and the progress of the reaction was monitored, at 30 °C, by the increase in absorbance of NADH at 340 nm (Uvikon 810; Kontron). Dihydrolipoic acid was prepared from lipoic acid (DL-6,8-thioctic acid; Sigma) as described by Kochi and Kikuchi [25]. Protein concentration was determined by the method of Lowry et al. [26] with BSA as standard.

Assay of PDC

Assay mixtures for measuring PDC activity contained 30 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.5, 2 mM DTT, 5 mM MgCl_2 , 0.2 mM CoASH, 0.5 mM thiamine pyrophosphate, 2 mM NAD^+ , 15 mM pyruvate and enzyme in a final volume of 0.5 ml. The reaction was initiated by the addition of pyruvate and the progress of the reaction was monitored, at 30 °C, by the increase in absorbance of NADH at 340 nm (Uvikon 810; Kontron).

Purification of the LPD associated with PDC (E3)

The matrix extract (280 mg of protein) was first fractionated on a Sephacryl S-300 gel-filtration column (2.5 cm × 90 cm; Pharmacia) equilibrated with buffer containing 10 mM Mops, 10 mM Tris/HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 50 mM KCl and 1 mM PMSF. The column was connected to a Pharmacia FPLC system and the proteins were eluted with the same buffer at 4 °C (flow rate 0.5 ml/min; fraction size 3 ml). PDC activity was collected among the first proteins eluted from

the column (total volume of fractions 11 ml). The pooled fractions (called Sephacryl fraction in Table 1) were applied to an HR 5/5 Mono Q column (Pharmacia) previously equilibrated with buffer A (20 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.5, 1 mM EDTA, 2 mM DTT). The proteins were eluted with a linear potassium phosphate gradient (0.02–1 M in buffer A over 120 min; 0.3 ml/min; fraction size 0.6 ml). During this chromatography step the PDC protein components seem to be dissociated and PDC activity is lost. At this stage, the activity of the LPD associated with PDC (E3) was followed. The fractions containing E3 activity (3 ml), which were eluted as a single peak at 0.3 M potassium phosphate, were added to 3 ml of buffer containing 20 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.5, 1 mM EDTA, 2 mM DTT and 2 M $(\text{NH}_4)_2\text{SO}_4$. This fraction (called Mono Q fraction in Table 1), containing E3 in the presence of 1 M $(\text{NH}_4)_2\text{SO}_4$, was applied to an HR 5/5 phenyl-Superose column (Pharmacia) previously equilibrated with 20 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.5, containing 1 mM EDTA, 2 mM DTT and 1 M $(\text{NH}_4)_2\text{SO}_4$. The proteins were eluted with a decreasing linear gradient of $(\text{NH}_4)_2\text{SO}_4$ (1 M to 0; 0.3 ml/min; fraction size 0.6 ml). Under these conditions E3 was not retained by the column but was eluted just after the void volume. At this stage of purification, E3 was pure as judged by Coomassie staining of SDS/polyacrylamide gels.

Purification of the L-protein of GDC from green leaf mitochondria

A two-step protocol (gel-filtration and ion-exchange chromatography on a Mono Q column) is generally used to purify the L-protein to homogeneity [3]. However, when the total amount of protein in the matrix extract is too high (above 200 mg of matrix protein), a third chromatographic step must be performed to eliminate contaminating proteins. The fractions containing the L-protein, eluted from the Mono Q column (the second step of purification), were therefore loaded on a phenyl-Superose column under the same conditions as for the last step of purification of E3 associated with PDC. This chromatographic step allows the purification of L-protein to homogeneity.

Purified LPD from porcine heart was purchased from Sigma and the purity of the protein was checked by SDS/PAGE analysis.

SDS/PAGE and immunoblot analysis

Preparation of the polyclonal antibodies raised against the L-protein of GDC and their purification by affinity chromatography, and details of the Western-blot experiments, were as previously described [21]. SDS/PAGE analysis was performed as described by Laemmli [27].

CNBr cleavage and fragment separation

For cleavage at methionine residues, freeze-dried purified L-protein from GDC was dissolved in 70 % (v/v) trifluoroacetic acid (TFA) resulting in a 1 % (w/v) protein solution which was incubated in the dark at 4 °C in the presence of a 1000-fold excess of CNBr over methionine residues. After 24 h, the sample was partly dried under N_2 , diluted 10-fold with water and freeze-dried. The resulting peptides were separated using an Applied Biosystems (140B) high-performance liquid chromatograph fitted with a C_8 Brownlee column (2.1 mm × 100 mm; 5 μm). The flow rate was 200 $\mu\text{l}/\text{min}$ with a linear gradient of 3–50 % acetonitrile (0.1 % TFA) over 50 min. After detection at 215 nm, fractions were collected and solvent was removed by evaporation under vacuum.

MS analysis

HPLC/ESI-MS analysis

Combined reverse-phase HPLC/MS analysis of the native L-protein from GDC and E3 from PDC were performed using a Perkin–Elmer Sciex API III triple-quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray (ionspray) source. HPLC/MS analyses were achieved using a Phoenix 20 (Carlo Erba; Fisons Instruments) fitted with an in-house-fabricated column (Nucleosil C₈; 0.25 mm × 150 mm; fused silica). The flow rate was 5 µl/min with a linear gradient of 9–90% acetonitrile (0.1% TFA) over 45 min. The effluent from the UV detector was directly introduced into the electrospray source via a fused-silica capillary (50 µm × 80 cm). Approx. 4.5 µg of the native E3 or L-protein was injected on to the column. The mass spectra of the proteins were recorded in the mass-to-charge ratio (*m/z*) 700–1700 range.

Fast-atom-bombardment (FAB) MS

Some fractions containing the small peptides resulting from the CNBr cleavage of the L-protein, eluted from the C₈ HPLC column, were analysed using a VG ZAB SEQ (Fisons Instruments) mass spectrometer equipped with a FAB ion source and a caesium gun (30 keV) (see ref. [28] for more details).

Amino acid sequencing

The L-protein purified from GDC and the E3 purified from PDC (100 pmol) were used for direct sequence analysis by automated serial Edman degradation with an Applied Biosystems 477 gas/liquid-phase protein sequencer. A CNBr fragment of the L-protein with a mass of 1057 Da measured by FAB-MS was also microsequenced.

RESULTS AND DISCUSSION

Purification and MS analysis of the L-protein associated with GDC

A three-step protocol (gel-filtration chromatography on a Sephacryl S-300 column, ion-exchange chromatography on a Mono Q column and chromatography on a phenyl-Superose column) was used to purify the L-protein to homogeneity. The final specific activity of the L-protein, in the presence of dihydrolipoate as substrate, was 15.6 µmol of NADH formed/min per mg of protein. The native L-protein of GDC from pea leaf mitochondria was analysed by ESI-MS. The extracted mass spectrum is shown in Figure 1. A coherent series of multiply charged ions, related to molecules bearing from 30 to 49 charges, is clearly observed for this protein. The family of ion peaks is converted into a single peak corresponding to the neutral molecule by using a deconvolution algorithm (inset of Figure 1A). The measured mass of the L-protein of pea GDC is 49753 ± 5 Da (470 amino acids) which is very close to the value (49721 Da) deduced from the cDNA sequence obtained by Bourguignon et al. [21]. The result from this experiment indicates that the two subunits that compose the L-protein are identical and are not covalently linked to each other. Indeed the apparent molecular mass of the native enzyme was estimated by gel-filtration chromatography and non-denaturing PAGE to be 120 kDa for the pea L-protein. It is worth noting the great difference (around 10 kDa) between the real mass of the protein measured with the mass spectrometer and that estimated by SDS/PAGE analysis (59 kDa).

On the other hand, alignment of the cDNA sequence encoding the L-protein reported by Turner et al. [20] (clones pGDLL1 and

pGDLE1) with the sequence published by Bourguignon et al. [21] shows perfect identity except for one nucleotide (G, nucleotide 1535) introduced into the sequence reported by Turner et al. [20], which allows, on translation, the formation of a mature protein of 498 amino acids instead of 470. Resequencing of clones pGDLL1 and pGDLE1 in both directions using specific primers confirms that the extra G in the sequence of Turner et al. [20] is an error, and the sequence should be ¹⁵³²GCCCAT instead of GCCGCATT (using the numbering of Turner et al. [20]). The two cDNA sequences obtained by the two groups are therefore identical and are derived from the same gene.

In order to confirm the primary structure, in particular the C-terminal sequence of the L-protein, a preliminary study of the purified L-protein cleaved chemically with CNBr has allowed the purification by HPLC of a peptide with a mass of 1057 Da as measured by FAB/MS. This peptide was entirely sequenced by Edman degradation and the sequence obtained (Ala-Thr-Tyr-Asp-Lys-Pro-Ile-His-Ile) matched perfectly the C-terminal sequence of the L-protein deduced from the nucleotide sequence published by Bourguignon et al. [21]. In order to confirm the suggestion of Bourguignon et al. [21] and Turner et al. [20] that pea leaf mitochondria contain a single E3 for mitochondrial PDC, GDC and 2-oxoglutarate dehydrogenase complex, we purified the LPD from PDC (E3).

Purification of the E3 component of PDC

A three-step protocol was devised to purify the E3 component of PDC. The first step was fractionation, on a gel-filtration column (Sephacryl S-300), of the soluble mitochondrial proteins containing PDC activity. During this chromatographic step, PDC, which is very large (5000–6000 kDa; see ref. [29]), remained intact and was eluted just after the void volume. The specific activity of the PDC Sephacryl fraction was 160 nmol of NADH formed/min per mg of protein. LPD activity associated with PDC was also measured (Table 1) and represented a small amount of the total LPD activity present in the matrix extract. On the other hand, the major peak of LPD activity (around 90% of the matrix activity) corresponding to the L-protein (see above) was eluted later during this chromatographic step [3,21]. Indeed, L-protein emerged from the column between the P-protein of GDC (approx. 200 kDa) and the T-protein (approx. 40 kDa). The Sephacryl fraction containing the PDC activity was directly chromatographed on an ion-exchange Mono Q column. Finally, the Mono Q fraction containing LPD activity associated with PDC (see Table 1) was loaded on a phenyl-Superose column and E3, not retained by the column, was eluted in the first fractions. The specific activity of E3 at this stage (15.8 µmol of NADH formed/min per mg of protein in the presence of dihydrolipoate and NAD⁺) was almost identical with the values obtained for the L-protein of GDC (15.6 µmol of NADH formed/min per mg of protein) and for the purified porcine heart LPD (15.2 µmol of NADH formed/min per mg of protein).

SDS/PAGE and Western-blot analysis

SDS/PAGE analysis (Figure 2A) revealed that E3 from pea leaf PDC, eluted from the phenyl-Superose column, was purified to homogeneity and has a molecular mass around 59 kDa. The purified L-protein of pea GDC and the LPD from porcine heart analysed under the same conditions had identical apparent molecular masses (Figure 2A). Polyclonal antibodies, raised against L-protein of pea GDC and purified by affinity chromatography, were tested against the three proteins. Figure 2(B) shows that, on a protein basis, the antibodies recognized E3 and L-protein from pea leaves with the same intensity, but only a very

Figure 1 ESI-MS of purified L-protein isolated from GDC (A) and purified E3 isolated from PDC (B)

The net charge of some of the ions is indicated above the peaks which correspond to multiprotonated forms (from 30 to 49 charges) of protein. The insets show the reconstructed molecular mass profiles obtained by using a deconvolution algorithm.

low immunological reaction was detected with porcine LPD (not visible on the Figure).

Amino acid sequencing and MS analysis of E3 from PDC

The N-terminal sequence of the purified L-protein from pea leaf mitochondria was determined using a gas-liquid sequencer and gave the following single sequence of 30 amino acid residues: Ala-Ser-Gly-Ser-Asp-Glu-Asn-Asp-Val-Val-Ile-Ile-Gly-Gly-Gly-Pro-Gly-Gly-Tyr-Val-Ala-Ala-Ile-Lys-Ala-Ala-Gln-Leu-

Gly-Phe. This sequence confirmed that published by Turner et al. [20] and Bourguignon et al. [21]. The first 30 amino acid residues of the N-terminal sequence of the purified E3 protein from pea leaf mitochondria were also determined and were identical with those of the L-protein. The purified E3 enzyme was analysed by ESI-MS in the same way as the L-protein of GDC. The extracted mass spectra are shown in Figure 1(B). The mass of 49754 ± 5 Da measured for the E3 component of pea PDC is exactly the same, within experimental error, as that obtained for the purified L-protein (49753 Da). Interestingly, in all experiments

Table 1 Purification of the E3 component of PDC from pea leaf mitochondria

LPD activity was determined by measuring spectrometrically NADH formation in the presence of dihydrolipoate as substrate (see the Materials and methods section).

Purification step	Total protein (mg)	Total activity (μ mol of NADH formed/min)	Specific activity (μ mol of NADH formed/min per mg)
Matrix extract (L + E3)	280	53.2	0.19
Sephacryl fraction (E3)	14.6	5.0*	0.34
Mono Q fraction (E3)	0.3	3.3	11.0
Phenyl-Superose fraction (E3)	0.071	1.1	15.8

* The low activity recovered in the Sephacryl fraction is attributable to the elimination of the L-protein during this chromatographic step.

Figure 2 SDS/PAGE and immunoblot analysis of E3 protein of pea PDC, the L-protein component of pea GDC and porcine heart LPD

(A) Proteins were resolved by SDS/PAGE and visualized by staining with Coomassie Brilliant Blue R250. Lane M, Bio-Rad markers; lane 1, 1.0 μ g of purified E3 from PDC; lane 2, 1.0 μ g of purified L-protein from GDC; lane 3, 1.0 μ g of purified porcine heart LPD. (B) The same proteins were resolved by SDS/PAGE and transferred to nitrocellulose membrane. Western-blot analysis was performed using immunopurified polyclonal antibodies raised against the L-protein.

performed with different batches of purified E3 or L-protein, the mass measured by MS was always different by 32 Da from that calculated from the L-protein cDNA. A similar difference was also observed for porcine LPD. Indeed, the mass of native porcine LPD measured by MS is higher than the mass of 50198 Da deduced from porcine LPD cDNA published by Otulakowski and Robinson [18] (result not shown). This difference is probably attributable to a post-translational modification of an amino acid (such as oxidation, hydroxylation or formylation). We intend to supplement the mass determination of the native L-protein with an exhaustive MS analysis of the peptides generated by cleavage of the protein with CNBr or protease followed by sequencing (by tandem MS or Edman degradation) of the peptide(s) with deviating mass. Interestingly, the MS data

indicate that FAD, which has a mass of 720 Da, is not covalently bound to the pea LPD and becomes detached during the course of MS analysis. The predicted molecular mass calculated from the cDNA sequence is 49721 Da, and when the molecular mass of FAD is included the total molecular mass is 50441 Da.

Conclusion

We have purified to homogeneity both the mature L-protein of GDC and the E3 component of PDC from pea leaf mitochondria. The results presented here allow us to conclude, from several lines of evidence, that the PDC and GDC from these organelles share the same LPD. First, E3 and L-protein have the same specific activity and behave identically on ion-exchange and phenyl-Superose chromatography and during SDS/PAGE. Secondly, E3 and L-protein share antigenic determinants that are not present on the porcine enzyme. Thirdly, and most importantly, the two proteins have identical N-terminal peptide sequences and their molecular masses are indistinguishable by MS.

The fact that a unique LPD is involved in several multienzyme complexes (GDC, PDC and probably 2-oxoglutarate dehydrogenase complex) in higher-plant mitochondria raises some interesting questions about the regulation of synthesis and control of the distribution of this unique enzyme associated with different complexes. Recent work carried out in our laboratories indicates that the expression of LPD in pea leaves is regulated by a mechanism that appears to be different from that involved in the synthesis of specific components (P-, T- and H-proteins) of GDC. Indeed, Northern-blot analysis of RNA from different tissues such as leaf, stem, root and seed using L-protein cDNA as a probe indicates that LPD mRNA accumulates in all tissues whereas expression of the genes encoding H-, T- and P-proteins occurs specifically in leaf tissue [20,21,28,30]. Similarly in developing wheat leaves, accumulation of L-protein is different from that of the other GDC polypeptides [31].

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