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The organisation and expression of the genes encoding the mitochondrial glycine decarboxylase complex and serine hydroxymethyltransferase in pea *(Pisum sativum)*

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Summary. Restriction fragment length polymorphisms have been used to determine the chromosomal location of the genes encoding the glycine decarboxylase complex (GDC) and serine hydroxymethyltransferase (SHMT) of pea leaf mitochondria. The genes encoding the H subunit of GDC and the genes encoding SHMT both show linkage to the classical group I marker *i*. In addition, the genes for the P protein of GDC show linkage to the classic group I marker *a*. The genes for the L and T proteins of GDC are linked to one another and are probably situated on the satellite of chromosome 7. The mRNAs encoding the five polypeptides that make up GDC and SHMT are strongly induced when dark-grown etiolated pea seedlings are placed in the light. Similarly, when mature plants are placed in the dark for 48 h, the levels of both GDC protein and SHMT mRNAs decline dramatically and then are induced strongly when these plants are returned to the light. During both treatments a similar pattern of mRNA induction is observed, with the mRNA encoding the P protein of GDC being the most rapidly induced and the mRNA for the H protein the slowest. Whereas during the greening of etiolated seedlings the polypeptides of GDC and SHMT show patterns of accumulation similar to those of the corresponding mRNAs, very little change in the level of the polypeptides is seen when mature plants are placed in the dark and then re-exposed to the light.

Key words: Gene expression (glycine decarboxylase serine hydroxymethyltransferase) – *Pisum sativum* – RFLP mapping

Introduction

The mitochondrial glycine decarboxylase complex (GDC) catalyses the cleavage of glycine to yield CO_2, NADH and 5,10-methylene-tetrahydrofolate (methylene-THF). Studies on both mammalian liver (Kikushi 1973) and the leaves of green plants (Walker and Oliver 1986a) have shown that four different polypeptides are involved in the reaction: P (a pyrodoxal-containing protein of $M_r \sim 100\,000$), H (a lipoamide-containing protein of $M_r \sim 15\,000$), T (a protein of $M_r \sim 45\,000$ required for the transfer of the methylene group from P to tetrahydrofolate) and L (an FAD-containing lipoamide dehydrogenase of $M_r \sim 60\,000$) (Bourguignon et al. 1988; Kikuchi 1973; Hiraga and Kikuchi 1980; Okamura-Ikeda et al. 1982; Walker and Oliver 1986a). In addition, in the mitochondria from green leaves the enzyme serine hydroxymethyl transferase (SHMT) is coupled to GDC, completing the reaction by transferring the methylene from THF to glycine to form serine. Serine hydroxymethyltransferase exists as a tetramer of four identical subunits of M_r 53 000 (Schirch and Peterson 1980; Bourguignon et al. 1988).

In the leaves of green plants the mitochondrial glycine cleavage reaction is part of the photorespiratory pathway, which recycles C2 carbon molecules produced as a result of the oxidative reaction of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Consequently, these polypeptides accumulate to high concentrations in the mitochondria, where they may represent 30–50% of the mitochondrial matrix protein. In addition, *Arabidopsis* mutants that lack mitochondrial SHMT or glycine decarboxylase activity will grow quite normally in conditions under which photorespiration is repressed (Somerville and Orgen 1982a, b). This indicates that the only strict requirement for these enzymes in the mitochondria of green plants is as part of the photorespiratory pathway. Under conditions of high protein concentrations and low ionic strength it is possible to retain all four polypeptides of GDC on an XM–300 Diaflo membrane (Neuburger et al. 1986; Bourguignon et al. 1988), indicating that these proteins exist as a loosely bound complex. Oliver et al. (1990) have shown that the proteins have an elaborate but stable stoichiometry in this complex of 1 L protein dimer: 2 P protein dimers: 27 H protein monomers: 9 T protein monomers.

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Similar to other enzymes involved in the photorespiratory and photosynthetic pathways the four polypeptides of GDC appear to be light-induced (Day et al. 1985; Walker and Oliver 1986b). However while the expression of the light-induced genes encoding proteins involved in chloroplast metabolism, such as the Rubisco small subunit gene, has been well characterised (for review see Gilmartin et al. 1990), comparatively little work has been done on the light-induction of mitochondrial proteins. In addition, although the light-induced increase in the level of chloroplast protein is also accompanied by changes in the morphology of the chloroplast, the only obvious difference between mitochondria from etiolated and green tissue is the level of glycine decarboxylase (Day et al. 1985).

Although the five polypeptides involved in the glycine decarboxylase cleavage reaction are located in the mitochondria, evidence from cycloheximide inhibition experiments suggests that they are synthesized from nuclear-encoded genes (Walker and Oliver 1986b). More recently, cDNAs for the P (Turner et al. 1992a), L (Turner et al. 1992b) and H (Macherel et al. 1990; Kim and Oliver 1990) proteins of glycine decarboxylase and also SHMT (Turner et al. 1992c) from pea have been cloned and sequenced. The presence of polyA tails and mitochondrial targeting sequences also confirms that these genes are nuclear encoded. Although the mRNAs for the P, L and H proteins have been shown to be light-induced (Turner et al. 1992a, b; Kim and Oliver 1990; Macherel et al. 1990), no comparative study of the expression of all five polypeptides has been made. The aim of the present work is to describe the organisation of the genes that encode the polypeptides of GDC and SHMT and to carry out a comprehensive study of the expression of the polypeptides and their corresponding mRNAs.

Materials and methods

Plant material and nucleic acid extraction. Growth conditions for the plants, extraction of nucleic acids and measurement of RNA levels were as described previously (Turner et al. 1992a).

Restriction fragment length polymorphism (RFLP) mapping of genes. The segregation of RFLPs in pea DNA was scored in an F_8 population derived from the cross JI 281 × JI 399. The bands were detected following hybridisation at 65° C of nitrocellulose filter (Schleicher and Schuell BA85) in 25 ml of 4 × SET containing 5 × Denhardts solution, 250 μg/ml herring sperm DNA and 10% w/v dextran sulphate. The filters were washed in 0.5 × SCC (1 × SCC is 0.15 M NaCl, 15 mM sodium citrate), 0.5% sodium dodecyl sulphate (SDS) at 65° C. The polymorphism corresponding to the T, H and L proteins were scored on *Eco*RI digests, while those of the P protein and SHMT were scored on *Hin*dIII and *Eco*RV digests, respectively. The L protein genes were also scored on *Bam*HI digests. The L protein *Eco*RI polymorphism has also been scored in the F_8 progeny of the cross JI 15 × JI 61. A P protein *Bam*HI polymorphism has also been scored in the F_8 progeny of the cross JI 15 × JI 399. Linkage to other RFLP markers was calculated from the proportion of recombinant lines according to Haldane and Waddington (1931). All calculations were performed by simple Basic programs. More details of the linkage analysis and the deduction of linkage orders can be found in Ellis et al. (1992).

Identity of cDNA clones. The cDNA clones for P, H (Turner et al. 1992a), L (Turner et al. 1992b), and SHMT (Turner et al. 1992c) of glycine decarboxylase were as described reviously. Similar procedures were used to isolate a 1.4 kb clone for the T protein and the correct assignment of this clone has been confirmed by comparison with the sequence of the T protein from bovine liver. Over a 550 bp region of the T protein clone the deduced amino acid sequence shows 47% identity to the corresponding bovine liver sequence (Okamura-Ikeda et al. 1991). The cDNA clone encoding the chlorophyll *a/b* binding protein from pea was a gift from Dr.C. Domoney and the cosmid clone cDB107 containing pea rDNA genes was a gift from Dr.J. Gatehouse (University of Durham).

Western blots. Tissues were ground in 50 mM MOPS, pH 7.0, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), the extracts clarified by centrifugation at 10 000 *g* and proteins fractionated on SDS-polyacrylamide gels (Laemmli 1970). Proteins were then electroblotted onto nitrocellulose and developed essentially as described by Blake (1984) but including a 1 M salt wash after the incubations with the primary and secondary antibody.

Results

Restriction fragment length polymorphisms (RFLPs) have been identified for each of the cDNAs encoding the polypeptides that catalyse the glycine cleavage reaction of pea mitochondria (Fig. 1). When using the cDNAs for the P and T proteins and the L protein as probes in the cross JI 15 × JI 61, the hybridisation patterns in the parental lines are sufficiently different to allow scoring of a number of different RFLPs. In these cases, the different RFLPs identified with a single probe cosegregate, indicating that all fragments hybridising to a single cDNA probe map to a single locus. The two point linkages around the loci of these five cDNAs are shown in Table. 1.

Although the genes for SHMT and the H proteins are not detectably linked they both map to the same linkage segment. This segment carries the marker *i*, which determines cotyledon colour, and is assigned to linkage group I (Blixt 1974; Weeden and Wolko 1990). In the recombinant inbred progeny derived from the cross JI 281 × JI 399, we have detected the segregation of the 1/4 translocation; the linkage of SHMT and H protein to *i* assigns these markers to the group I portion of this translocation linkage group (Fig. 2). The locus corresponding to the P protein probe maps to a linkage seg-

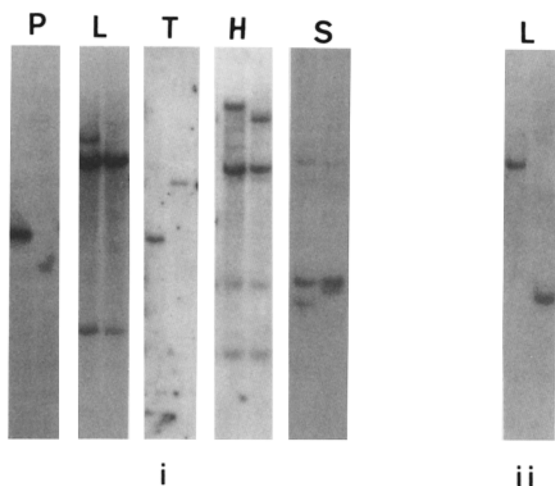

Fig. 1. Southern blot of pea DNA (~5 μg) cut with *Eco*RI (T, H and L), *Hin*dIII (P) or *Eco*RV. Probed with cDNAs encoding the four proteins of the glycine decarboxylase complex (GDC; P, H, T and L) and serine hydroxymethyltransferase (SHMT). The DNA is from the parents of the recombinant inbred lines either (i) JI 281 × JI 399 or (ii) JI 15 × JI 61

ment carrying the classical marker *a*, which governs anthocyanin pigmentation. The *a* locus is a classical linkage group I marker (Blixt 1974; Weeden and Wolko 1990); however, the P protein-containing segment does not show linkage to the SHMT, H protein-containing group I segment in the cross JI 281 × JI 399 (Fig. 2). The discrepancy between these results may be due to the translocation involving chromosomes 1 and 4 disrupting the classical linkage map; it is not known, however, whether some other additional rearrangement may be responsible. The loci corresponding to the L and T protein probes show linkage to one another, and to an rDNA-containing locus cDB107 (Fig. 2). This rDNA-containing locus corresponds to *Rrn2* (Weedon and Wolko 1990). The linkage of the L protein genes to *Rrn2* has been confirmed in the cross JI 15 × JI 61. The nucleolus organiser corresponding to *Rrn2* is carried on chromosome 7 and it seems highly probable that the small L and T protein-containing linkage group corresponds to the satellite of this chromosome.

The levels of mRNAs encoding the GDC proteins and SHMT were examined in RNA extracted from the leaves of pea seedlings grown in the dark and then exposed to light for various periods of time (Fig. 3). Different patterns of expression were seen for each of the different probes. The mRNA for the H protein appears to accumulate later than the others and its pattern of expression most closely resembles that of the mRNA for the chlorophyll *a*/*b* binding protein (Cab), which was used as a reference gene. The levels of both the Cab and H mRNAs were still increasing rapidly after 27 h in the light. In contrast the mRNA for the P protein accumulated to its highest level within 6 h and thereafter remained relatively constant. The patterns of increase of the mRNAs for the L and T proteins and SHMT are intermediate to those seen for the P and H proteins.

Table 1. Two point linkage around the cDNAs encoding the four subunits of glycine decarboxylase (GDC; P, L, T, and H) and serine hydroxymethyltransferase (SHMT)

T1/4

	GST-10/3	pST H	CHS-2/2	CHS-2/3	i	P7	cDNA 76	pPE 923/1	cDNA 206	c41	SHMT	cDNA 150/1
pST H	19.6											
CHS-2/2	32.5	6.13										
CHS-2/3	....	11.6	4									
i	....	10.1	2.29	3.12								
P7	....	17.3	18.2	24.8	15.3							
cDNA 76	....	13.3	20.2	26.9	22.3	2.6						
pPE 923/1	....	14.8	20.9	26.9	20.9	8.35	6.03					
cDNA 206	....	....	....	....	....	19.2	17	7.33				
c41	....	....	....	....	....	....	....	11.1	4.48			
SHMT	....	....	....	....	....	....	28.5	....	14	12.7		
cDNA 150/1	....	....	....	....	....	....	....	....	18.2	4.76	17.8	
DR 9	....	....	....	....	....	....	....	....	18.8	5.88	22.8	4.42

7

	cDNA 125/11	pST LL1	cDB 107(*Hin*dIII)	cDB 107(*Bgl*II)	pST T
pST LL1	13.9				
cDB 107(*Hin*dIII)	22.4	8.53			
cDB 107(*Bgl*II)	25.9	12.1	3.51		
pST T	24.6	12.7	7.22	6.67	
cDNA 289	23.7	15.7	8.01	7.02	1.66

1

	cDNA 125/04	cDNA 24	cDNA 56/1	cDNA 39	pST P
cDNA 24	6.98				
cDNA 56/1	9.78	1.58			
cDNA 39	17.8	8.93	7.85		
pST P	....	....	27.6	16.8	
shi/1	....	26.9	20.7	14	11.6

The data are tabulated for linkage groups 1 and 7 and for the 1/4 translocati segregating in the progeny of the cross JI 281 × JI 399 (see Results)

The pattern of expression of the GDC and SHMT polypeptides from the same plant material used for the mRNA study is shown if Fig. 4. There was very little change with time in the accumulation of the L protein, which appears to be present at reasonably high levels even in plants not exposed to light. The anti-L protein antibody also detected the presence of a second protein of slightly higher molecular weight. This upper band is specifically associated with the chloroplast fraction and may represent the chloroplast lipoamide dehydrogenase; its pattern of expression is in no way correlated with that of the mitochondrial protein (data not shown). In addition, the high levels of Rubisco large subunit in mature leaves interfere with the migration and transfer of the L protein making accurate measurement of L protein levels difficult. In contrast to the L protein the accumulation

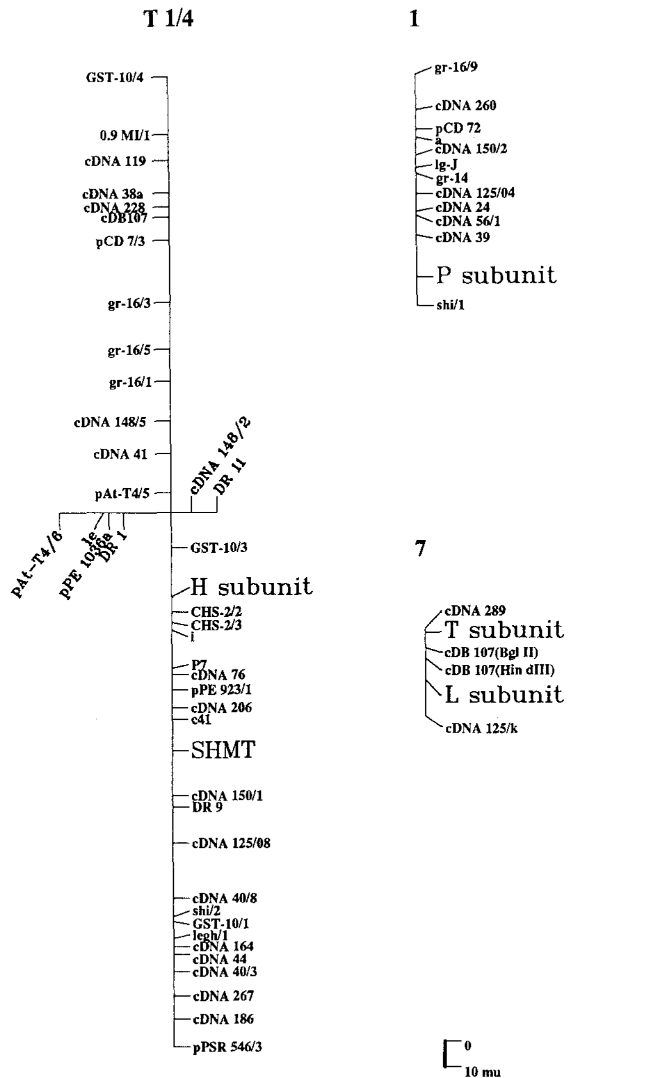

Fig. 2. Part of the pea restriction fragment length polymorphism (RFLP) map showing the linkage groups that contain genes for the proteins of GDC or SHMT

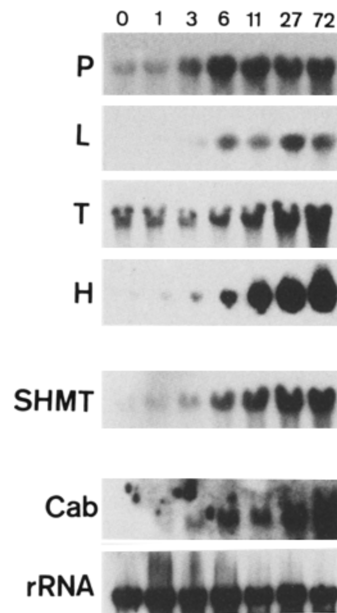

Fig. 3. Relative levels of mRNA in the leaves of etiolated seedlings exposed to light for various periods of time. Aliquots (~ 100 μg) of the same RNA sample were fractionated on denaturing agarose gels, blotted onto nitrocellulose filters and probed with cDNAs encoding the four proteins of GDC (P, L, T, and H) and SHMT. Chlorophyll *a*/*b* binding protein and ribosomal RNA genes were used as controls. *Numbers* along the top represent the time (h) after plants had been placed into the light

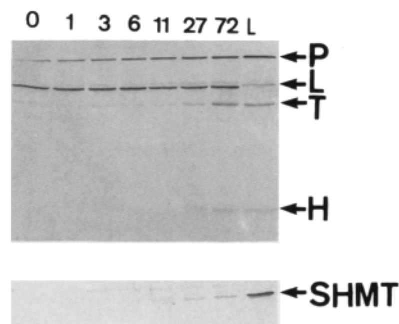

Fig. 4. Western blots of protein leaf extracts from the leaves of etiolated pea seedlings placed in the light for various periods of time were prepared as described in the Materials and methods. The blots were probed with antibodies for the four proteins of GDC (P, L, T and H) and SHMT. Loadings were adjusted to give equal amounts of total protein in each lane. *Numbers* along the top represent the time in the light (h), and the track labelled L is from mature leaves

of the other polypeptides showed a strong correlation with that of the corresponding mRNAs. Consequently, the P, T and H proteins show a very large increase on exposure to light and initially the P protein accumulates more rapidly than the H protein.

Pea seedlings grown in the dark do not develop normally and develop normal leaves only on exposure to light. In order to distinguish between effects due to light acting on the genes that encode the five polypeptides and effects due to leaf development, mature plants were placed in the dark for 48 h and then placed in continuous light for various periods of time. After 48 h in the dark the mRNAs encoding the P, T and H proteins and SHMT declined to almost undetectable levels. Although there was also a large decline in the level of mRNA encoding the L protein, it was less marked than for the other mRNAs. The levels of all five mRNAs increased rapidly on re-exposure to light (Fig. 5) and, with the exception of the mRNA for the H protein, after 48 h in the light the mRNA levels exceeded their levels before dark treatment. The pattern is similar to that previously seen when etiolated seedlings were exposed to light: the mRNA for the P protein appears to accumulate most rapidly and that for the H protein most slowly. Over the same time course, however, the change that occurred in the levels of the corresponding five polypeptides was relatively small (Fig. 6).

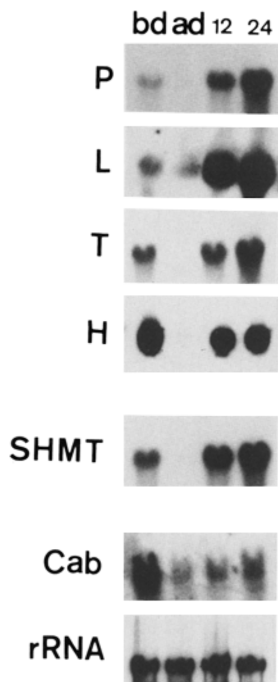

Fig. 5. Levels of mRNA in the leaves of mature plants (bd) placed in the dark for 48 h (ad) then re-exposed to light for various periods of time (12 or 24 h). Aliquots (~ 100 μg) of the same RNA sample were fractionated on denaturing agarose gels, blotted onto nitrocellulose filters and probed with cDNAs encoding the four proteins of GDC (P, L, T, and H) and SHMT. Chlorophyll *a*/*b* binding protein and ribosomal RNA genes were used as controls

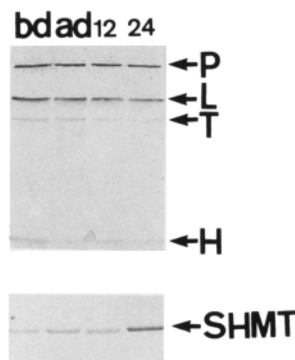

Fig. 6. Western blots of sodium dodecyl sulphate (SDS)-polyacrylamide gels containing total protein extracts from the leaves of mature plants (bd, before darkening), which had been placed in the dark for 48 h (ad) then re-exposed to light for various periods of time (12 or 24 h). The blots were probed with antibodies for the four proteins of GDC (P, L, T, and H) and SHMT. Loadings were adjusted to give 12 μg total protein in each lane

Discussion

RFLP analysis of the genes that encode the mitochondrial glycine cleavage complex and SHMT of pea clearly shows that they are nuclear. This is in agreement with the work of Walker and Oliver (1986b) who used transcriptional inhibitors to demonstrate that the proteins of GDC are nuclear encoded. There has been a report that mitochondrial SHMT is also mitochondrially encoded (Combette et al. 1991), and whereas we cannot exclude the possibility that there may be other forms of SHMT encoded by separate genes it is clear that the cDNA used in these experiments corresponds to a nuclear gene.

The five polypeptides of the glycine cleavage reaction each appear to be encoded by a small multigene family, $\sim$2–3 copies per haploid genome (Turner et al. 1992a, b, c). For the P, T and L proteins it is possible to score all of the restriction fragments detected by the cDNA and since these fragments always cosegregate, the different members of the multigene family must be tightly linked. Interpretation of the linkage analysis is complicated by the translocation that has occurred between chromosomes 1 and 4 in the cross JI 281 × JI 399, but since both P and H are linked to classic group I markers it is reasonable to assume that in a pea line not carrying the translocation, both these markers would be found on linkage group I. Consequently, the five gene families appear to be clustered into two groups with the genes for the T and L proteins linked together on chromosome 7, while the genes for the P and H proteins and SHMT are located on linkage group I. Whether or not this represents a random distribution is hard to establish. There is no relationship between genes that are closely linked genetically and genes that have similar expression patterns. For example in the experiments shown in Figs. 3 and 5 the patterns of expression of the mRNAs encoding the T protein and SHMT are almost identical, while the corresponding genes map on chromosome 7 and 1 respectively. Two other possible explanations for a clustering of genes relate to the symbiotic origin of mitochondria. Based on the widely held view that proteins located in the mitochondria but that are nuclear encoded were once encoded by mitochondrial DNA, which has subsequently transposed into the nucleus, it is possible that there may be preferential sites for the integration of these sequences in the pea genome. An alternative possibility is that the genes that encoded the glycine cleavage reaction may be grouped together in the prokaryotic genome (encoding a polycistronic RNA for example) and transposed as a gene cluster; though there is no functional relationship between these genes a semblance of their earlier linkage still exists. Whether either one of these possibilities is correct should become clearer as more nuclear genes encoding mitochondrial proteins are placed on the pea RFLP map and the prokaryotic organisation of the genes for the glycine cleavage complex is determined.

The induction of mRNAs encoding the P (Turner et al. 1992a), L (Turner et al. 1992b) and H (Kim and Oliver 1990; Macherel et al. 1990) proteins of GDC has already been demonstrated. The mRNA for the T protein is clearly induced in a similar fashion, in agrement with the work of Walker and Oliver (1986b) who showed light induction of the four polypeptides under similar conditions. In addition, SHMT and the mRNA that encodes it are also clearly light-induced (Figs. 3 and 4), consistent with the observation that the sole function of mitochondrial SHMT is in association with GDC as part of the photorespiratory cycle (Somerville and Ogren 1982b).

In the mitochondria from light-grown mature pea leaves the glycine cleavage complex has been estimated to represent 30–50% of the total matrix protein (Oliver et al. 1990). In addition, the vast majority of each of the proteins forms part of a loosely bound enzyme complex, with a consistent but elaborate stoichiometry (Bourguignon et al. 1988; Oliver et al. 1990). Only a small percentage of the GDC protein remains as individual proteins that are not integrated into the complex. How the accumulation of different proteins is controlled to give the correct ratios required for the complex represents a particularly intriguing problem: not only do the mRNAs accumulate with differing time courses, but the site of synthesis, the cytoplasm, is separated from the intact complex by the mitochondrial membranes. This presumably adds an additional problem to any possible feedback mechanism whereby the over-accumulation of a particular protein in the mitochondrial matrix causes a slowing down in its rate of synthesis.

The lipoamide dehydrogenase (L) protein of GDC is also a component of two other mitochondrial enzyme complexes: pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. Consequently, it is likely to be regulated independently of the other components of the GDC and of SHMT. Consistent with this suggestion is the finding that the L protein appears to be the only GDC polypeptide present in the leaves of etiolated pea seedlings (Fig. 4). The L protein is also notable since the level of L protein transcripts is relatively low in etiolated seedlings compared to the level of the polypeptide. This apparent anomaly is accounted for by the problems associated with trying to correlate mRNA levels with protein accumulation when protein turnover is slow (see below). If a high level of protein already exists compared to the relatively small changes occurring over the time-course described, the changes would be difficult to detect. More accurate determination of the correlation between mRNA levels and protein synthesis can only be obtained by measuring de novo protein synthesis directly. The presence of large amounts of the L proteins in the absence of any of the other components of GDC suggests that the stability or turnover of the L protein is not greatly influenced by the presence or absence of the other proteins. In addition, the mesophyll cells of the C_3–C_4 intermediate photosynthetic species *Moricandia arvensis* accumulate little or no P protein but have apparently normal amounts of the other proteins (Morgan et al. 1992). Experiments in which mature plants were placed in the dark show that while comparatively little mRNA for the five polypeptides is still present after 48 h in the dark there is virtually no change in the levels of the corresponding proteins. All the above observations suggest that the correct ratio of GDC proteins is not maintained by the increased turnover of excess uncomplexed proteins. In addition, the apparent slow turnover of the five polypeptides probably explains why under certain conditions (for example when plants were placed in the dark and then re-exposed to light) there is apparently no correlation between the level of the polypeptides and their corresponding mRNAs.

Kure et al. (1991) have shown that in the mammalian liver a strong coupling exists between the transcription of the mRNA for the H and P proteins. Similarly, Blackwell et al. (1990) have described a photorespiratory mutant of barley with greatly reduced levels of H protein, which also has decreased levels of the P protein. Unfortunately, it is not known whether this mutation is in the structural gene for the H protein or in a gene that regulates the expression of the two proteins. In pea, however, it is clear that the mRNAs for the different proteins accumulate in a consistent and co-ordinated pattern; since the rate of mRNA accumulation is correlated with the accumulation of the protein, the careful regulation of mRNA accumulation presumably contributes to the correct ratio of proteins required in the mitochondria.

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