Evidence for multiple forms of biotin holocarboxylase synthetase in pea (*Pisum sativum*) and in *Arabidopsis thaliana*: subcellular fractionation studies and isolation of a cDNA clone

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The intracellular compartmentation of biotin holocarboxylase synthetase has been investigated in pea (*Pisum sativum*) leaves, by isolation of organelles and fractionation of protoplasts. Enzyme activity was mainly located in cytosol (approx. 90% of total cellular activity). Significant activity was also identified in the soluble phase of both mitochondria and chloroplasts. Two enzyme forms were separated by anion-exchange chromatography. The major form was found to be specific for the cytosol compartment, whereas the minor form was present in mitochondria as well as in chloroplasts. We also report the isolation and DNA sequence of a cDNA encoding an *Arabidopsis thaliana* biotin holocarboxylase synthetase. This cDNA was isolated by functional complementation of a conditional lethal *Escherichia*

coli birA (biotin ligase gene, which regulates biotin synthesis) mutant. This indicated that the recombinant plant protein was able to biotinylate specifically an essential apoprotein substrate in the bacterial host, that is a subunit of acetyl-CoA carboxylase called biotin carboxyl carrier protein. The full-length nucleotide sequence (1534 bp) encodes a protein of 367 amino acid residues with a molecular mass of 41172 Da and shows specific regions of similarity to other biotin holocarboxylase synthetase genes as isolated from bacteria and yeast, and with cDNA species from human. A sequence downstream of the first translation initiation site encodes a putative peptide structurally similar to organelle-targeting pre-sequences, suggesting a mitochondrial or chloroplastic localization for this isoform.

INTRODUCTION

Biotin is a small coenzyme (vitamin H or B₈), synthesized by plants, most bacteria and some fungi, which occurs primarily in a protein-bound state within the cell. Biotinylated proteins use this prosthetic group as a carrier of activated carboxy groups during carboxylation and decarboxylation enzymic reactions. In all organisms, these carboxylases play housekeeping functions, such as acetyl-CoA carboxylase (EC 6.4.1.2; ACCase), which catalyses the first committed step in fatty acid biosynthesis (for a general review see [1]).

Escherichia coli contains only a single biotinylated protein called biotin carboxyl carrier protein (BCCP), which functions as a subunit of ACCase. Biotinylation of apo-BCCP occurs through the action of a biotin ligase (EC 6.3.4.10). This enzyme catalyses the post-translational attachment of D-biotin to a specific Lys residue of newly synthesized apo-BCCP, via an amide linkage between the biotin carboxyl group and a unique ε-amino group of a Lys residue [2]. This covalent attachment, essential for the enzymic activation of ACCase, occurs in two distinct steps as follows:

Biotin ligase has been purified from *E. coli* and its gene cloned [3,4]. This enzyme, also called BirA, is a 33.5 kDa protein [4] that also acts as a repressor of the biotin operon [5]. Its three-dimensional structure has recently been determined at 2.3 Å

resolution [6]. The complete nucleotide sequences of two other bacterial genes that encode proteins homologous with the *E. coli* biotin:apoprotein ligase have been reported from *Paracoccus denitrificans* [7] and *Bacillus subtilis* [8] respectively. Mammalian cells also contain biotin-dependent carboxylases that are localized in different cell compartments, i.e. ACCase in the cytosol, and 3-methylcrotonoyl-CoA carboxylase (EC 6.4.1.4), propionyl-CoA carboxylase (EC 6.4.1.3) and pyruvate carboxylase (EC 6.4.1.1) in mitochondria. As in bacteria, their activation from apo- to holo- (biotinylated) forms requires the action of a biotin ligase. Previous studies have demonstrated biotin ligase activity in both the cytosol and the mitochondria from mammalian cells [9,10], suggesting that biotinylation of biotin-dependent carboxylases occurs in their sites of enzymic activities. The corresponding enzymes from various mammalian species, which carry out the same reaction as bacterial biotin ligase, have been purified and were referred to as biotin holocarboxylase synthetase (HCS) [9,11]. Recently, clones encoding *Saccharomyces cerevisiae* HCS gene [12] and human HCS cDNA species have been obtained [13,14]. In plants, biotin and biotinylated proteins play a central role in metabolism. For example, a mutation in the biotin synthetic pathway is lethal for *Arabidopsis thaliana* [15], and plant ACCase is the target site of potent herbicides [16]. Biotin-dependent carboxylases are also present in different compartments of plant cells. As in bacteria and mammals, biotinylation of these enzymes is catalysed by HCS. Indeed, in a previous paper we provided the first direct evidence for the existence of HCS activity in plants [17]. In particular, we showed that the

Abbreviations used: ACCase, acetyl-CoA carboxylase (EC 6.4.1.2); BCCP, biotin carboxyl carrier protein; DTT, dithiothreitol; GraPDH, glyceraldehyde 3-phosphate dehydrogenase (NADP⁺-dependent) (EC 1.2.1.13); HCS, biotin holocarboxylase synthetase (EC 6.3.4.10); PFP, pyrophosphate:fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90); TTC, 2,3,5-triphenyltetrazolium hydroxychloride.

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The nucleotide sequence of *A. thaliana* biotin holocarboxylase synthetase cDNA will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U41369.

partly purified enzyme, as obtained from pea leaves, was able to biotinylate specifically bacterial apo-BCCP as a substrate [17]. Nevertheless it is unknown whether plant biotin-dependent carboxylases are biotinylated within the cytosol and then translocated into their final site of accumulation, or are targeted into organelles as apo-proteins and subsequently biotinylated.

In this study we analysed purified chloroplasts and mitochondria from pea leaves and also used protoplasts from pea leaves as a source of cytosol with low contamination. We conclude from these experiments that, in pea leaves, HCS activity can be detected in the cytosol, mitochondria and chloroplasts.

In contrast, on the basis of the observation that plant HCS was able to biotinylate a bacterial substrate, we set out to clone a plant HCS cDNA by a functional complementation approach using an *E. coli* strain carrying a temperature-sensitive *birA* mutation and a plant cDNA expression library prepared from *A. thaliana*. This approach proved successful for isolating human HCS cDNA clones and yeast HCS gene [12,14], thus demonstrating the capacity of HCS from eukaryotes in replacing the biotin ligase function of *E. coli* BirA protein. By this method we have, in the present study, cloned and characterized for the first time a cDNA encoding HCS from the plant kingdom.

MATERIALS AND METHODS

Materials

D-[8,9-³H]Biotin (42 Ci/mmol) and [³⁵S]methionine (1000 Ci/mmol) were purchased from Amersham. D-Biotin, carbenicillin, dithiothreitol (DTT), ATP, thiamin hydroxychloride and 2,3,5-triphenyltetrazolium hydroxychloride (TTC) were obtained from Sigma Chimie SARL. Isopropyl β -D-thiogalactoside was obtained from Bioprobe Systems. Casein hydrolysate for vitamin-free assay was from Difco, and trichloroacetic acid was from Merck.

Plant material

Pea (*Pisum sativum* L., var. Douce Provence) plants were grown from seeds in soil for 8–10 days under a 12 h photoperiod of white light from fluorescent tubes (10–40 μ E/s per m²) at 18 °C. The plants were watered each day with tap water.

Preparation of pea leaf crude extract

Pea leaves (2 g) were harvested and ground in liquid nitrogen with a mortar and pestle. The powder was then homogenized with 10 ml of 20 mM Tris/HCl (pH 7.8), 1 mM EDTA, 1 mM DTT, 5 mM ϵ -aminohexanoic acid and 1 mM benzamidine/HCl. The suspension was centrifuged at 72000 *g* for 30 min (50 Ti rotor, Beckman). The supernatant comprised the crude extract. All procedures were performed at 4 °C.

Preparation and fractionation of pea leaf protoplasts

Pea leaf protoplasts were purified from young leaves (10 days old) by the method of Baldet et al. [18]. Pea leaves (10–15 g) were cut into fine strips (1 mm) and placed in a medium containing 10 mM Mes/NaOH (pH 5.5)/0.5 M sorbitol/1 mM CaCl₂/0.05 % (w/v) PVP-25 (buffer A). After vacuum infiltration, leaf strips were washed twice in buffer A, then placed at 25 °C for 90 min in buffer A containing 2 % (w/v) cellulase Onozuka R10, 0.5 % (w/v) Macerozyme R10 and 0.2 % (w/v) Pectolyase Y-23 (Yakult Honsha Co., Shingikancho, Nishinomiya, Japan). All subsequent procedures were performed at 4 °C. Protoplasts were released from the digested tissues by gentle shaking and filtration through a 100 μ m nylon mesh. The filtrate was centrifuged at

100 *g* for 5 min (swinging-bucket rotor). The pelleted protoplasts were resuspended in 50 ml of 0.5 M sorbitol/1 mM CaCl₂/20 mM Tris/HCl (pH 7.8) (buffer B). Two additional washes were performed in buffer B before the protoplasts were finally resuspended in buffer B supplemented with 5 mM ϵ -aminohexanoic acid and 1 mM benzamidine/HCl, at a chlorophyll concentration of 0.1 mg/ml. Protoplasts were gently ruptured by being passed first through a 20 μ m nylon mesh and then through a 10 μ m nylon mesh. About one-tenth of the lysed protoplast suspension was used as the protoplast crude extract. The remaining protoplast lysate was centrifuged at 300 *g* for 5 min. The pelleted chloroplasts were suspended in buffer B containing 5 mM ϵ -aminohexanoic acid and 1 mM benzamidine/HCl. The supernatant was then centrifuged at 12000 *g* for 20 min, resulting in the mitochondrial (pellet) and cytosolic (supernatant) fractions. The mitochondrial pellet was added to the chloroplastic fraction to yield the organelle fraction.

Preparation of purified chloroplasts

Young pea leaves (9 days old) were homogenized in 330 mM sorbitol/50 mM Hepes/NaOH (pH 8)/1 mM EDTA/5 mM DTT with a Waring blender. Intact chloroplasts were rapidly prepared and purified with Percoll gradients as previously described [19]. The morphological integrity of purified chloroplasts was of the order of 95 % as judged by ferricyanide reduction by untreated and osmotically shocked organelles respectively [20]. Intact chloroplasts were lysed in a buffer containing 20 mM Tris/HCl (pH 7.8), 1 mM EDTA, 1 mM DTT, 5 mM ϵ -aminohexanoic acid and 1 mM benzamidine/HCl at a final protein concentration of 40 mg/ml after one freeze–thawing cycle (5 min in liquid nitrogen, thawing at 25 °C). The suspension of broken chloroplasts was centrifuged at 72000 *g* over a 0.6 M sucrose cushion for 20 min. The pellet and the supernatant comprised the chloroplast membranes (envelope membranes and thylakoids) and the soluble fraction (stroma) respectively. All procedures were performed at 4 °C.

Preparation of purified mitochondria

Mitochondria were isolated and purified from young pea leaves by using self-generating Percoll gradients as described by Douce et al. [21]. The morphological integrity of purified mitochondria was greater than 95 % as determined by the rate of KCN-sensitive cytochrome *c*-dependent O₂ uptake in untreated and osmotically shocked mitochondria respectively [21]. Total lysis of mitochondria was achieved by three freeze–thawing cycles (5 min in liquid nitrogen, thawing at 25 °C), performed in the same buffer as that used for the lysis of purified chloroplasts (see above), at a protein concentration of 40 mg/ml. The suspension of broken mitochondria was centrifuged at 72000 *g* over a 0.6 M sucrose cushion for 20 min. The pellet and the supernatant comprised the mitochondrial membranes and the soluble fraction (matrix) respectively. All procedures were performed at 4 °C.

Measurement of marker enzyme activities

Except where otherwise noted, enzymes were assayed spectrophotometrically at 340 nm by coupling to oxido-reduction of NADH (or NADPH) in 1 ml reaction volumes. Triton X-100 (0.05 %, w/v) was added to each reaction mixture. The activity of each enzyme in pea leaf extract was strictly dependent on the presence of all necessary substrates and cofactors, and linear with respect to the amount of extract assayed.

Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (NADP⁺-dependent) (EC 1.2.1.13; *GraPDH*) activity was measured by the conversion of 1,3-bisphosphoglycerate into glyceraldehyde 3-phosphate [22]. The assay mixture contained 50 mM Hepes/NaOH, pH 7.8, 1 mM MgCl₂, 4 mM EDTA, 5 mM DTT, 0.2 mM NADPH, 1 mM ATP, 10 units of phosphoglycerate kinase (EC 2.7.2.3), 10 units of triose-phosphate isomerase (EC 5.3.1.1) and 10 mM 3-phosphoglycerate.

Fumarase

Activity was determined by the method of Hill and Bradshaw [23], by following the appearance of fumarate spectrophotometrically at 250 nm from reaction assays containing 50 mM Hepes/NaOH, pH 8, and 50 mM malate.

Pyrophosphate:fructose-6-phosphate 1-phosphotransferase

Pyrophosphate:fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90; PFP) activity was measured as described by Weiner et al. [22]. The assay mixture contained 50 mM Tricine/NaOH, pH 7.8, 0.5 mM MgCl₂, 10 μ M fructose 2,6-bisphosphate, 5 mM fructose 6-phosphate, 10 units of triose-phosphate isomerase, 1 unit of glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), 0.1 unit of aldolase (EC 4.1.2.13), 0.15 mM NADH and 0.6 mM Na₄P₂O₇.

Thermolysin treatment of purified intact organelles

Proteolytic digestion was performed in accordance with a previously published procedure [24]. Intact chloroplasts or mitochondria (final concentration 10 mg/ml protein) were incubated for 1 h at 4 °C in the following medium: 0.3 M sucrose/10 mM Tricine/NaOH (pH 7.8)/1 mM CaCl₂/0.2 mg/ml thermolysin from *Bacillus thermoproteolyticus* (Boehringer). Thermolysin is active only in the presence of Ca²⁺ ions and its activity can be easily inhibited by the addition of 10 mM EGTA. Polypeptides localized in the inner envelope membrane or in the stroma of chloroplasts, and also in the inner membrane or the matrix space of mitochondria, are not hydrolysed during the incubation because thermolysin is unable to cross the outer envelope membrane of chloroplasts or the outer membrane of mitochondria. In addition, the integrity of organelles is maintained during incubations under mild conditions (at 4 °C, with low thermolysin concentration) [24]. Therefore after incubation for 1 h in the presence of thermolysin under these conditions, chloroplasts and mitochondria were re-purified on a Percoll gradient containing protease inhibitors (1 mM PMSF, 1 mM benzamidine/HCl and 5 mM ϵ -aminohexanoic acid) to remove the protease and the broken organelles. The treated, intact organelles were recovered, stored on ice and assayed for HCS activity; the soluble enzymes were then analysed by chromatography on a Mono Q anion-exchange column.

Mono Q anion-exchange chromatography

All chromatography experiments were performed at 4 °C with Mono Q HR 5/5 (Pharmacia) coupled to an FPLC system (Pharmacia) to obtain precise and repeatable elution patterns. Soluble protein extracts (crude leaf extract, chloroplast stroma and mitochondrial matrix) prepared as described above were desalted on a Sephadex G-25 (M) column in a medium containing 20 mM Tris/HCl, pH 8, 1 mM EDTA, 1 mM DTT, 1 mM benzamidine/HCl and 5 mM ϵ -aminohexanoic acid and then loaded (2–5 mg) on the anion-exchange column pre-equilibrated

with the same buffer. After loading, the column was washed with 5 ml of buffer and eluted with the following linear NaCl gradients: 0–0.3 M NaCl (30 ml), 0.3–0.5 M NaCl (10 ml), 1 M NaCl (5 ml) in the same buffer at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected and assayed for HCS activity.

Latency measurements

Corrections were made for extra-organellar activity by comparing the activities in ruptured and intact organelles. The organelles were kept intact by adding an osmoticum to the reaction medium (0.3 M sucrose) or ruptured by adding 0.05 % (w/v) Triton X-100. The percentage of latent activity is the ratio of organellar (ruptured minus intact) activity to the total (ruptured) activity. We verified that, under these conditions, enzymic activities were not affected by the presence of the detergent.

Protein and chlorophyll determinations

Protein was measured by the method of Bradford [25] using Bio-Rad protein assay reagent (Bio-Rad Laboratories) with bovine γ -globulin as a standard. Chlorophyll was measured by the method of Arnon [26].

Bacterial strains and growth conditions

A temperature-sensitive *E. coli birA215* mutant (strain BM4050) lacking HCS activity *in vitro* was generously provided by Dr. A. M. Campbell (Stanford University, Stanford, CA, U.S.A.) [27,28]. Mutations in the *birA* gene affect the biotin ligase function of the BirA protein, resulting in biotin auxotrophy. This strain grows normally at 30 °C on minimal medium M9 (48 mM Na₂HPO₄/22 mM KH₂PO₄/19 mM NH₄Cl/8.5 mM NaCl/1 mM MgSO₄/0.1 mM CaCl₂) supplemented with 0.2 % glucose, 2 nM biotin, 0.4 % casein hydrolysate, 100 μ g/ml TTC and 1 μ g/ml thiamin. In contrast, bacteria fail to grow under the same conditions (either in liquid medium or on plate) but at a temperature of 43 °C, since at this temperature the mutant biotin:apoprotein ligase has a greatly decreased affinity for biotin. When required, carbenicillin was added at 100 μ g/ml. Competent cells, grown in Luria–Bertani broth, were prepared according to the method of Dower et al. [29].

Measurement of HCS activity

HCS activity was determined by measuring the covalent attachment of D-[³H]biotin to bacterial apo-BCCP by a trichloroacetic acid precipitation assay as described previously [17]. The apo-BCCP protein substrate was prepared from the *E. coli birA215* mutant grown on glucose minimal medium supplemented with limiting amounts of D-biotin (0.4 nM) [17]. The reaction mixture contained the following components in a total volume of 200 μ l: 5 mM ATP, 5 mM MgCl₂, 0.2 mM DTT in 20 mM phosphate buffer (pH 7.5), 10–200 μ g of enzyme extract, 180 μ g of apo-BCCP extract and 250 nM D-[³H]biotin. The reaction was initiated by addition of the labelled D-biotin. Incubations were for 20–60 min at 37 °C. Then 125 μ l aliquots of the reaction mixture were collected on glass microfibre filters (Whatman GF/C) and proteins were precipitated by five washes of the filters in 10 % (w/v) trichloroacetic acid. The filters were washed once with ethanol and dried; radioactivity was counted in scintillation vials containing 8 ml of scintillation liquid (Ready protein; Beckman). Duplicate assays without apo-BCCP and/or HCS extract were run as controls.

Isolation of *A. thaliana* cDNA clones encoding HCS by functional complementation of *E. coli* *birA215*

A λ YES-R cDNA expression library prepared with mRNA species from *A. thaliana* plants was first converted into a plasmid library carrying ampicillin resistance, as described by Elledge et al. [30]. *E. coli* *birA215* competent cells were transformed by electroporation with this plasmid library by using a Bio-Rad Gene Pulser operating at 15 kV/cm pulse, 25 μ F and 200 Ω . Then bacteria were suspended in 1 ml of SOC broth [31] and incubated at 37 °C with shaking (250 rev./min) for 1 h. Cells were washed twice in M9 medium to eliminate free biotin and plated on M9 plates, supplemented with 2 nM biotin, 0.4 % casein hydrolysate, 100 μ g/ml TTC, 1 μ g/ml thiamin and 100 μ g/ml carbenicillin. Cell culture was conducted at 43 °C to select rescued clones. Transformation efficiency was determined by cell growth at 30 °C on identical M9 plates. Eight successive electroporations were performed as described above, with a transformation frequency of approx. 3×10^6 transformants per μ g of plasmid DNA. Plasmids were isolated [31] from survivor colonies growing at 43 °C on M9/carbenicillin plates, and the original *E. coli* *birA215* mutant was transformed again with the purified plasmids to confirm the functional complementation. Only transformants that survived the second round of selection were used for further analyses.

Preparation of bacterial crude extract

To assess the recombinant HCS activity, the biotin auxotroph bacterial strain *E. coli* *birA215* (strain BM4050) (untransformed and transformed) as well as the control wild-type strain *E. coli* *BirA*⁺ (strain BM2661) were grown on Luria–Bertani liquid medium (supplemented with carbenicillin in the case of transformed bacteria) at 37 °C, until A_{600} reached 0.5. After induction of the pYES lac promoter with 1 mM isopropyl β -D-thiogalactoside for 4 h, cells were collected by centrifugation at 3000 g for 15 min (JA 20 rotor; Beckman) and suspended in buffer A containing 20 mM phosphate buffer, pH 7.5, 1 mM Na₂EDTA, 1 mM DTT and a mixture of protease inhibitors (1 mM PMSF, 5 mM ϵ -aminohexanoic acid and 1 mM benzamidine/HCl). Cells were then disrupted by sonication at 0 °C and lysates were centrifuged for 15 min at 15000 g to remove cell debris (JA 20 rotor; Beckman). The supernatant was desalted by passage through a Sephadex G25 (M) column (Pharmacia) equilibrated in buffer A.

Subcloning and DNA sequencing

The cDNA inserts were subcloned into the *Eco*RI site of vector plasmid pBluescript II SK(–) (Stratagene). DNA sequence analysis was performed on both strands by using Prism Kit with fluorescent dideoxynucleotides, *Taq* DNA polymerase (Applied Biosystems) and T3 and T7 universal primers. In addition, specific oligonucleotide primers were used for further sequencing. Gene Works 2.4 and PCGENE (IntelliGenetics) software packages were used for sequence analyses.

Transcription–translation of pBluescript HCS *in vitro*

Transcription–translation *in vitro* of pBluescript HCS (which contains the entire region of *A. thaliana* HCS cDNA cloned under the control of the T7 promoter) was done with T7 RNA polymerase and a rabbit reticulocyte lysate from Novagen kit (Single Tube Protein[®] System 2, T7) and [³⁵S]methionine (Amersham) in accordance with the instructions of the manufacturer. For the reaction the DNA template (2 μ g of pBluescript

HCS-2) was transcribed at 30 °C for 15 min, followed by translation reaction (in the presence of 40 μ Ci of [³⁵S]methionine) at 30 °C for 60 min. A control reaction lacking a DNA template was performed under the same conditions. The translational products were precipitated with 10 % (w/v) trichloroacetic acid. After a 1 h incubation at 0 °C and centrifugation at 30000 g for 15 min at 4 °C (JA 20 rotor; Beckman), the precipitated protein pellets were washed with cold acetone, air-dried and resuspended in 50 mM Tris/HCl (pH 8)/1 mM PMSF/5 mM ϵ -aminohexanoic acid/1 mM benzamidine/HCl. Polypeptides labelled with [³⁵S]methionine were analysed by SDS/PAGE and fluorography. SDS/PAGE [10 % (w/v) gel] was performed at room temperature in slab gels (15 cm $\times$ 15 cm). The experimental conditions for gel preparation, sample solubilization, electrophoresis and gel staining were as detailed by Chua [32]. After staining, gels were soaked in Amplify solution (Amersham) and dried before fluorography for 1 week on X-ray-films.

Genomic Southern blot analysis

Total DNA was isolated from young *A. thaliana* plants. Approx. 2–5 μ g of DNA was digested overnight with appropriate restriction enzymes (New England Biolabs) and fragments were separated by 0.8 % agarose gel electrophoresis. After blotting to a Hybond-N⁺ membrane (Amersham), hybridization was performed with the entire *A. thaliana* HCS-2 cDNA or the *Eco*RI–*Eco*RV 5' end of the pBluescript HCS-2 cDNA probes that had been ³²P-labelled with a random priming kit (Pharmacia) [31]. The nucleic acid hybridization solution was composed of 6 $\times$ SSC (the stock solution was 20 $\times$ SSC, containing 175.3 g/l NaCl and 88.2 g/l sodium citrate, pH 7), 0.5 % SDS and 0.25 % low-fat dried milk. Hybridization proceeded overnight at 65 °C and membranes were washed at 65 °C for 30 min in 1 $\times$ SSC/0.1 % SDS and 0.1 $\times$ SSC/0.1 % SDS.

RESULTS

Intracellular localization of HCS activity in pea leaves

Protoplasts, isolated by enzymic digestion from young pea leaves (9 days old), were fractionated by gently rupturing through a fine nylon mesh followed by centrifugation to yield an organelle fraction (pellet) and a cytosolic fraction (supernatant). These two fractions were assayed for HCS activity, and their purity was assessed by measurement of selected subcellular marker enzyme activities. Marker enzymes used were fumarase for mitochondria, GraPDH for chloroplasts, and PFP for cytosol. As shown in Table 1, most of the chloroplast (83 %) and mitochondrial (87 %) marker activities were recovered in the pellet. Because it was difficult to rupture the small protoplasts quantitatively without affecting chloroplast integrity, we performed an incomplete rupture of the protoplasts to obtain only minimal chloroplastic contamination in the cytosolic fraction (supernatant). Thus approx. 31 % of the total cytosolic marker activity was recovered in the pellet. The supernatant obtained after differential centrifugation of lysed pea leaf protoplasts contained a substantial proportion of cytosolic marker enzymes (63 % of the total activity) and was only slightly contaminated by chloroplast and mitochondrial enzymes (Table 1). HCS activity was detected both in the supernatant (59 %) and in the pellet (38 %). As the activity of HCS in the organelle pellet was not very different from the cytosolic contamination level, it was difficult to conclude that chloroplasts and/or mitochondria contained an HCS form. From these results it is clear that, in higher plant cells, HCS activity is located mostly in the cytosol (probably up to 90 % of total cellular activity).

Table 1 Subcellular localization of HCS in pea leaf protoplasts

HCS activity was assayed in broken pea leaf protoplasts (protoplast extract, 12.5 mg of protein), organelles (pellet) and cytosol (supernatant) as described in the Materials and methods section in the presence of 0.05% (w/v) Triton X-100. Also indicated are the activities of marker enzymes to check for cross-contamination. The marker enzymes were as follows: cytosol, PFP; chloroplasts, GraPDH; mitochondria, fumarase. The results presented for distribution in the supernatant and pellet and for recovery are expressed as a percentage of the total activity recovered, and are means $\pm$ S.D. for four different experimental determinations.

	Enzyme activity			
	PFP	GraPDH	Fumarase	HCS
Activity in protoplast extract (mol/min)	154×10^{-9}	1108×10^{-9}	45×10^{-9}	0.61×10^{-12}
Distribution in pellet (%)	31 ± 5	83 ± 1	87 ± 3	38 ± 1
Distribution in supernatant (%)	63 ± 8	7 ± 2	3 ± 1	59 ± 9
Recovery (%)	94 ± 13	90 ± 3	90 ± 4	97 ± 10

Table 2 Distribution of activities of HCS and marker enzymes in Percoll-purified chloroplasts and mitochondria from pea leaves

Preparation of purified organelles, measurement of different enzymic activities in the presence of 0.05% (w/v) Triton X-100, and latent activity definition were as described in the Materials and methods section. The marker enzymes were identical with those used for the fractionation of purified pea protoplasts (Table 1). Results are from a representative experiment repeated three times. Abbreviation: n.d., not detectable.

Sample	Enzyme activity (mol/min per mg of protein)				HCS latency (%)
	PFP	GraPDH	Fumarase	HCS	
Chloroplasts	n.d.	342×10^{-9}	8×10^{-9}	0.026×10^{-12}	80
Mitochondria	n.d.	12×10^{-9}	870×10^{-9}	0.041×10^{-12}	92

With the aim of determining the possible occurrence of HCS activity in plant cell organelles, we conducted a large-scale purification of intact pea leaf chloroplasts and mitochondria on Percoll gradients. Assays of various selected marker enzymes for chloroplasts (GraPDH), mitochondria (fumarase) and cytosol (PFP) showed that this purification procedure completely eliminated cytosolic contamination. Furthermore, both the chloroplast and the mitochondrial fractions were essentially free from mitochondrial and chloroplast contamination, respectively (Table 2). HCS activity was found to be associated with both mitochondria and chloroplasts, with a specific activity of 0.041 and 0.026 pmol/min per mg of protein respectively (Table 2). On a protein basis, these values were one-quarter to one-sixth of those measured in the cytosolic fraction prepared from fractionated protoplasts (0.17 pmol/min per mg of protein). Nevertheless the total absence of cytosolic contamination and the high latency values of HCS activity measured in both purified chloroplasts and mitochondria demonstrated that the enzyme activity was present within the organelles (Table 2).

Separation of HCS activities by Mono Q anion-exchange chromatography

To determine whether HCS activities found in pea leaves represented different forms of the enzyme, extracts from pea leaves (crude leaf extract, soluble proteins from Percoll-purified chloroplasts and mitochondria, and cytosol from leaf proto-

Figure 1 Separation of multiple forms of HCS from pea leaves by ion-exchange chromatography

Pea leaf extracts were fractionated by chromatography on a Mono Q HR5/5 column in 20 mM Tris/HCl (pH 8)/1 mM EDTA/1 mM DTT/5 mM ϵ -aminohexanoic acid/1 mM benzamidine/HCl at 4 °C. After being loaded, the column was washed with 5 ml of buffer and eluted with a NaCl gradient at 0.5 ml/min. Fractions of 1 ml were collected and assayed for HCS activity. The samples loaded were: (A) leaf crude extract (5 mg); (B) cytosol (2 mg); (C) mitochondrial extract (2.3 mg); (D) chloroplast extract (4.9 mg); (E) extract from thermolysin-treated chloroplasts (3.8 mg).

plasts) were fractionated on a Mono Q HR 5/5 column. Recovery of HCS activity was in the range 85–90% for each of the chromatographies performed. The elution profile obtained for the leaf crude extract (Figure 1A) shows that HCS activity can be resolved into two peaks (A and B). The major peak (peak B),

Figure 3 HCS activity in crude protein extracts from *E. coli birA215* strain transformed or not with the four HCS cDNA clones and from *E. coli birA*⁺ wild-type strain

Total soluble proteins from the different strains were extracted after induction of the Lac promoter with isopropyl β -D-thiogalactoside, for an optimal time of 4 h, as described in the Materials and methods section. HCS activity was determined as described previously by measuring the specific incorporation of D-[³H]biotin into bacterial apo-BCCP [17]. D-Biotin (1 pmol) corresponds to 95 000 d.p.m.

amino acid sequence. HCS-2 is a 1534 bp cDNA including one large open reading frame of 1101 bp. The first in-frame ATG occurring at nucleotide 50 of the cDNA initiates the longest open reading frame present on this cDNA and encodes a predicted polypeptide of 367 residues with a molecular mass of 41172 Da. A second in-frame ATG is present 114 bp downstream from the first one. The nucleotide sequence around the first ATG codon, TTTAATGGA (positions 46–54), differs from the plant consensus translation initiation motif, AACAAUGGC [34]. In contrast, that for the second, AGCAATGGA (positions 160–168), more closely matches the plant consensus sequence. Finally, the presence of an in-frame nonsense codon TGA, located 27 bp upstream from the first ATG, confirms that HCS-2 is full length.

Functional characterization of HCS cDNA

To confirm that the four cDNA species encoded HCS, we measured this enzyme's activity in crude protein extracts obtained from the *E. coli birA215* mutant complemented with each of the four clones, by using bacterial apo-BCCP as the biotin acceptor substrate (Figure 3). All four clones had the same orientation in pYES vector and were under the control of the lac promoter. Whereas no HCS activity could be detected with the *birA215* mutant, the four complemented clones exhibited significant HCS activity (Figure 3). Thus levels of HCS activity from the recombinant clones were 13–14-fold higher than that from the wild-type *birA*⁺ (BM2661) strain (Figure 3).

Transcription–translation of pBluescript HCS-2 *in vitro*

Because the complete nucleotide sequence contains two in-frame ATG sequences, it is possible that isolated HCS cDNA species encode two distinct polypeptides. Coupled transcription–translation of HCS-2 cDNA *in vitro* subcloned in pBluescript under the control of the T7 promoter, using T7 RNA polymerase and a rabbit reticulocyte lysate, produced two major translation products of approx. 37 and 41 kDa, i.e. of the expected sizes for an initiation of translation at the two in-frame ATG sequences

Figure 4 Coupled transcription–translation of pBluescript HCS-2 *in vitro*

Polypeptides radioactively labelled with [³⁵S]methionine were subjected to SDS/PAGE and analysed by fluorography as described in the Materials and methods section. Lane 1, fluorography of radiolabelled polypeptides obtained from a control reaction lacking DNA template; lane 2, fluorography of translation products obtained with 2 μ g of pBluescript HCS-2. The positions of molecular mass markers (given in kDa) are indicated on the left.

(Figure 4, lane 2). The additional labelled polypeptide of molecular mass 52 kDa did not correspond to a translation product of the HCS-2 cDNA because it was also detected in the control experiment lacking the DNA template (Figure 4, lane 1). It seems unlikely that the production of the two polypeptides can be explained by a premature termination of translation because a similar result was obtained with wheat germ extract as the translation system *in vitro* (results not shown).

Southern blot analysis

Southern analysis was used to examine the number of genes encoding HCS in *A. thaliana*. Total DNA was digested with restriction enzymes that cut once (*EcoRV*) or do not cut (*EcoRI*, *HindIII*) within the cDNA, and the fragments were resolved by agarose gel electrophoresis. After transfer to a Hybond-N⁺ membrane, the resultant blot was probed with the ³²P-labelled complete HCS-2 insert as described in the Materials and methods section. As shown in Figure 5, digestion with *EcoRI* or *HindIII* produced two bands (7.1–4.6 and 8–3.4 kb), whereas digestion with *EcoRV* resulted in three hybridization bands of 13, 12 (arrowheads) and 1.7 kb. After double digestion of total DNA with *EcoRV* and *HindIII*, the probe detected three bands, a 1.7 kb band generated by *EcoRV*, a 3.4 kb band generated by *HindIII* and a 6.2 kb band. Hybridizations were also performed with a restriction fragment corresponding to the 501 bp 5' end (*EcoRI*–*EcoRV* fragment) of the pBluescript HCS-2 cDNA. After digestion with *EcoRI* or *HindIII* a similar pattern of hybridization was obtained, whereas after digestion with *EcoRV* or double digestion with *EcoRV* and *HindIII* the 5'-end probe revealed only two bands in both cases, that is the 13 and 1.7 kb bands and the 3.4 and 1.7 kb bands respectively (results not shown). Altogether, these results are consistent with the existence of two related genes encoding HCS in *A. thaliana*. However, such a banding pattern could also be generated by a single gene containing large introns. This last possibility cannot be completely ruled out.

DISCUSSION

Fractionation of pea leaf protoplasts and purification of chloroplasts and mitochondria from this tissue clearly indicate that HCS activity is associated with several subcellular compartments.

Figure 5 Southern blot hybridization of total *A. thaliana* DNA with the complete HCS cDNA as a probe

Total leaf DNA from *A. thaliana* was digested (2–5 μ g per reaction) with *Eco*RI (lane 2), *Hind*III (lane 3), *Eco*RV (lane 4) and *Hind*III–*Eco*RV (lane 5). Lane 1 corresponds to size markers (λ DNA *Hind*III digest). DNA restriction fragments were separated on a 0.8% agarose gel, transferred to Hybond-N⁺ membrane and then hybridized with the ³²P-labelled probe corresponding to the pBluescript HCS-2 cDNA excised with *Eco*RI.

HCS was resolved into two peaks by anion-exchange chromatography. The main peak (making up approx. 90% of the total activity) was located in the cytosol. The other peak was present in both chloroplasts and mitochondria. The great purity and the higher latency values of HCS activity measured in Percoll-purified chloroplasts and mitochondria, together with the protection of the enzyme activity in these organelles during thermolysin treatment, confirmed that HCS is a genuine constituent of chloroplasts and mitochondria.

Detailed characterization of the incorporation of D-biotin into plant biotin-dependent carboxylases requires large quantities of HCS isoenzymes. To overcome the very low abundance of these enzymes in plants, as judged by the low specific activities detected in each cell compartment, we attempted to obtain the cDNA species encoding these isoforms in order to over-express it. The biotinylation reaction is evolutionarily conserved such that biotin ligases function with various apocarboxylases across species boundaries [2]. We have therefore used the cross-species reactivity of biotin ligases to clone a cDNA encoding a complete *A. thaliana* HCS by functional complementation of a mutant *birA* strain of *E. coli*. Selection of plant cDNA species encoding HCS by this technique confirms our previous results showing that pea HCS efficiently biotinylates bacterial apo-BCCP *in vitro* [17].

Comparison of the predicted protein sequence of the isolated plant HCS cDNA with those compiled in the GenBank and EMBL databases showed low but significant similarity to biotin: apoprotein ligases from *E. coli* (17% identity; 33% similarity) [4], *B. subtilis* (18% identity; 31% similarity) [8], *P. denitrificans* (14% identity; 25% similarity) [7], *Homo sapiens* (24% identity; 40% similarity) [13,14] and *S. cerevisiae* (22% identity; 40% similarity) [12] (Figure 6). Specific areas of similarity are restricted to a region known in *E. coli* BirA to contain the biotin-binding site [6]. Thus, across a restricted 125-residue region (positions 122–246 of *A. thaliana* HCS), the plant enzyme shares 33% identity (51% similarity) with BirA protein from *E. coli*, 33% identity (47% similarity) with a putative homologue of BirA protein from *B. subtilis*, 30% identity (47% similarity) with the

candidate for BirA protein from *P. denitrificans*, 30% identity (49% similarity) with HCS from *H. sapiens* and 34% identity (51% similarity) with HCS from *S. cerevisiae*. Most importantly, the eight residues involved in direct contact with biotin in BirA from *E. coli*, as determined by X-ray crystallography [6], are strictly conserved in *A. thaliana* HCS. These residues are Ser¹²², Thr¹²³, Gln¹⁴⁵, Arg¹⁴⁹, Lys²²⁰, Gly²²³, Gly²⁴⁰ and Gly²⁴². Interestingly, similarity to chicken avidin, the protein with the highest known affinity for biotin [35] was also observed, particularly in the region located between Asp²⁰⁵ and Thr²²⁹ of the predicted amino acid sequence (Figure 6). The sequence identity in this zone was 36%, and increased to 60% when conservative substitutions were included. Altogether these results confirm the finding that this region is essential for biotin binding. Within this region, the sequence GRGRTK is present at positions 148–153 and partly matches that found in other biotin ligases (GRGRRG) (Figure 6). Although no crystallographic evidence for the ATP-binding site has been demonstrated [6], the structure GXGXXG has been associated with ATP binding in several enzymes [36,37]. However, it also seems to be involved in contact with biotin in BirA [6]. Presumably this reflects a requirement for ATP and biotin to be spatially close to permit the formation of biotinyl 5'-adenylate, an intermediate of the biotinylation reaction. Sequence comparisons within the helix–turn–helix DNA binding motif of *E. coli* and *B. subtilis* BirA proteins (residues 22–46 and 23–47 respectively) [4,8] revealed no region of similarity, suggesting that in contrast with what occurs in bacteria, plant HCS is not involved in the repression of biotin synthesis. This observation is in good agreement with previous findings showing that the free D-biotin concentration in the cytosol of plant cells is of the order of 11 μ M [17,18]. Indeed, this level, which is approx. 2000-fold that found in bacteria [38], was not compatible with the existence of a strong repressor of biotin synthesis in plants, comparable with the repressor function of BirA protein, which regulates the level of biotin in bacteria. Finally, there are sequences conserved between *A. thaliana* HCS and the two other eukaryotic proteins that are not found in BirA from bacteria (Figure 6). For example, in the C-terminal portion of the predicted *A. thaliana* HCS sequence (residues 301–367), the plant enzyme shares only 12% identity (19% similarity) with BirA from *B. subtilis* compared with 37% identity (58% similarity) with HCS from *S. cerevisiae*.

Comparison of the N-terminal portion of known HCS sequences indicates that the *A. thaliana* protein contains an N-terminal extension of approx. 30 amino acids compared with those from bacteria (Figure 6). This N-terminal extension is rich in hydrophobic, hydroxylated and positively charged amino acids, but poor in acidic residues. In addition, theoretical secondary-structure predictions indicate that this sequence would fold into an amphiphilic α -helix. Thus this extension has many characteristics in common with mitochondrial or chloroplast presequences. Furthermore, analysis of the primary sequence of the plant HCS sequence by the subroutine TRANSEP from the program PCGENE (IntelliGenetics) predicted that this protein might be targeted to an organelle, possibly the mitochondrion, and identified the sequence Arg²⁹-Leu/Ser-Phe as a putative cleavage-site motif [39]. However, the occurrence of an in-frame Met residue at position 39, which might act as a potential translation initiation site, as indicated by transcription–translation experiments *in vitro* (Figure 4), might indicate that the present clone encodes, in reality, a cytosolic HCS isoform. Therefore it is not possible from our data to determine the exact subcellular localization of the cloned *A. thaliana* HCS. Further studies on the uptake of the cloned plant HCS by mitochondria and/or chloroplasts, as well as *in situ* localization experiments on plant cells overexpressing this clone are in progress to make a

<i>A. thaliana</i>	MEAVRST	TTLNPHLLN	ILVLRSLKPL	HRLSFSFSA	AMESDAS--C	45	
<i>E. coli</i>				M-KDNTV	PLKILALLAN	16	
<i>B. subtilis</i>				MRSTLRK	DLIELFSQAG	17	
<i>P. denitrificans</i>							
<i>H. sapiens</i>	(351)	NLLKSSNFR	YEVLREILT	LGLSCDMKQV	PALTPIYLLS	AAEIRDPIM	400
<i>S. cerevisiae</i>	(269)	RAPNLTPLFM	ASAPNKRNL	QEMENLAHH	GMHANNVELC	SELNAETDSF	318
Consensus							
<i>A. thaliana</i>	SLVLOGKSSV	ETEVAGKLN	KNSIKLPDNT	KVSLILESEA	KNLVKDDNS	95	
<i>E. coli</i>	GEFHSGEQLG	ETLGMSSRAI	NKHIQTLRDW	GVDVFTVPGK	GYSILPEPQL	66	
<i>B. subtilis</i>	SEFISGGKIS	DALGCSRIV	WHIEELARKE	GVEEAVRRK	GYSILKPGK	67	
<i>P. denitrificans</i>							
<i>H. sapiens</i>	QWLGHVDSSE	GRKSGQLSL	RFVSSVYSEV	EITPSCPVPV	TNMEAFSSEH	450	
<i>S. cerevisiae</i>	QFYRGYRASV	DAASSSLHKK	EPDEVKPTVI	FPGVDEIPP	FQYTPNFDK	368	
Consensus							
<i>A. thaliana</i>	FNLSLFNNSI	ITHRFGRFLI	WSPRLST-H	DVSHNFSEL	PV-GSVCTD	143	
<i>E. coli</i>	LNKQILQGL	DGGSVA--	VLPVIDST-N	QYLLDRIGEL	KS-GDACIAE	110	
<i>B. subtilis</i>	LSESEIRFGL	KTEVMGQHLI	YQDVISST-Q	KTAHELANN	APEGTLVAD	116	
<i>P. denitrificans</i>							
<i>H. sapiens</i>	FNLEIYRQNL	QTKQLGVIL	FAEVTPTTMR	LLDGLMFQTP	QEMGLIVIAA	500	
<i>S. cerevisiae</i>	-EYFKYLNQV	NTIGSLLYV	EVVTSSTIL	NNKSLISSI	PESTLYHVT	417	
Consensus		T	ST		G VA		
<i>A. thaliana</i>	IQFQGRGRTK	NWVESGKGL	MYSETLEME-	DGRVVP--L	IQYVSVSLAVT	189	
<i>E. coli</i>	YQAGRGGRG	RKWFSPFGAN	LY--LSMFV	RLEQGP--A	AAIGLSLIV	154	
<i>B. subtilis</i>	KQTAGRGMS	RVHSGQEGN	IW-MSLILR	PD--IP--L	-KQTPQLTLL	158	
<i>P. denitrificans</i>	EQFAGRGGRG	REWVMPAGN-	-FAGTLVLR-	P-QGGA--L	AAQQLSFVA	71	
<i>H. sapiens</i>	RQTEGGRGGR	NWLSRGVCA	LSLLISLPL	RSGLQ--R	IFPVGHSV	547	
<i>S. cerevisiae</i>	ITPSGRGGRG	NWINKKVC	ASTAVTME	QSPVTNRNIS	VVPVQVLSML	467	
Consensus	Q GRGR G	N W P G	T L M L		L		
Avidin (29-59)			SLTG	KWINDL----	-----	GSNMT----	43
<i>A. thaliana</i>	EAVKDVCD-K	KGLPIYDVKI	KWINDLY----	-----	VN--G-IK	220	
<i>E. coli</i>	IYMAEV-L-R	K-LGADKRVV	KWINDLY----	-----	-LQ--D-RK	183	
<i>B. subtilis</i>	AAVAVQGG-I	EAAGITQDTI	KWINDLY----	-----	-LN--G-KK	189	
<i>P. denitrificans</i>	LALYDALG-L	ACGPAARLAI	KWINDLY----	-----	-LN--G-KK	102	
<i>H. sapiens</i>	AVVEAVRS-I	PEYQDILNRV	KWINDLY----	-----	-YS--DLMK	579	
<i>S. cerevisiae</i>	AYCKAIISSA	YCKAIISSA	KWINDLY----	-----	PTYKRKNLK	517	
Consensus	V V	L I	KWINDL		VN K		
Avidin							
	--IGAVNSRG	EF-----TGT					56
<i>A. thaliana</i>	--VGGI----	-----LCIST	YR-SKKFNVS	VGVGLNVNDG	OPT-TC-LNA	256	
<i>E. coli</i>	--LAGI----	-----LVELT	GKTGDAQIV	IGAGINMAMR	RVE-ESVNVQ	221	
<i>B. subtilis</i>	--TVGI----	-----LTEMQ	AEEDRVRSVI	IGIGINNVQO	SDDPFDELK	248	
<i>P. denitrificans</i>	--VAGI----	-----LLESS	GSGPQVQVAV	VGIGVNLAGA	PQAGAVEPGA	141	
<i>H. sapiens</i>	--IGGV----	-----LVNST	LMGF-ETFYIL	IGCGFVINS	NTPICINWLI	617	
<i>S. cerevisiae</i>	PLPGLDEPAY	LKIGSLGTLT	HFNINKCYLV	LGCGINLTSD	GPTTSLQTVI	567	
Consensus	VGGI	L T	V	IG GINV			
<i>A. thaliana</i>	V-LKGM-APE	SNLLKREELI	GAPFHKEFK	FDLFMDQ--G	FKSLEELY--	300	
<i>E. coli</i>	G-WITL-QEA	GINDRNTIA	AMILREIRAA	LELFEEQ--G	LAPYLSRWEK	267	
<i>B. subtilis</i>	I-ATSLQAS	GKIDRAGVI	QHLLCFEKR	YRDYMIH--G	FTPIKLLWES	275	
<i>P. denitrificans</i>	TPPVSVGGST	GHAVDEEPI	DILAPAFAM	QAQIDTY--G	FAPIRNMLA	189	
<i>H. sapiens</i>	TEYNQKRAE	LKPIRADYLI	ARVTVLEKL	IKFEQDK--G	PNSVLP--Y	663	
<i>S. cerevisiae</i>	DIINEERQQL	HIDLLPAIK	EKLQALYNN	LEVILKOFIN	VGAAEILPSY	617	
Consensus		L	L	G P			
<i>A. thaliana</i>	YRTWHSEQR	VIVEDKVEDO	VQNVVTIQ-	GLTSSGYLLA	VGDDN--CM	346	
<i>E. coli</i>	LDWFNRPVK	LIIQDR--E	IF--GIS-R	GIDKQALL	--EQD--GI	305	
<i>B. subtilis</i>	YALGEGTNR	ARTING--T	FY--GKA-L	GIDDEGVALL	--ETQ--EG	313	
<i>P. denitrificans</i>	RAARLGEPII	ARTGTA--E	SH--GIF-E	GIDDSGALLI	--RGP--AG	227	
<i>H. sapiens</i>	YRYWHSQGI	VHLGSA--E	GP--KVSIV	GLDSSGLQV	HOEGG--EV	704	
<i>S. cerevisiae</i>	YELWLHSGQI	VTLPDHGTQ	AMITGITEY	GLLIAKELVS	GSSTQGTGNV	667	
Consensus		L	I	GL G LL			
<i>A. thaliana</i>	YELHPDGNF	DFFGKGVRRK	I			367	
<i>E. coli</i>	IKPVMGGEI-	-----SLRSAE	K			321	
<i>B. subtilis</i>	IKKIYSADI-	-----ELG				325	
<i>P. denitrificans</i>	ROVIPAEEV-	-----FFG--G				240	
<i>H. sapiens</i>	VTVHPDGNF	DMLRNILPK	R			725	
<i>S. cerevisiae</i>	YNLPQDGNF	DIFKSLIAKK	VQS			690	
Consensus		G F D L					

Figure 6 Amino acid sequence comparison between biotin ligases and chicken avidin

Sequences are shown for *A. thaliana* HCS, *E. coli* BirA protein [4], *B. subtilis* BirA protein [8], *P. denitrificans* putative biotin ligase [7], *H. sapiens* HCS [13,14], *S. cerevisiae* HCS [12] and chicken avidin [35]. The sequences are aligned with gaps (–) to maximize identity. Conserved amino acids represented in bold are reported in the deduced consensus line (Consensus). Conservative amino acid substitutions were determined in accordance with the following grouping: I-L-M-V, N-Q, R-K-H, A-S-P-T-G, Y-F-W and D-E. The amino acid positions for *H. sapiens* and *S. cerevisiae* HCS and chicken avidin are shown at the right.

definitive assignment of the cellular localization of this clone to a specific compartment. It is interesting to note a recent proposal that human cytosolic and mitochondrial HCS isoforms are synthesized from a single species of mRNA either by alternative translational initiation at two in-frame AUGs or by a splicing mechanism [14].

Finally, the presence of HCS isoforms in different cell compartments, as determined biochemically in pea leaves, raises the question of their physiological significance in plants. In mammals, two isoforms of the same HCS targeted to cytosol and mitochondria respectively have been characterized, catalysing the biotinylation of one cytosolic biotin-dependent carboxylase (ACCase) for the former and three mitochondrial biotin-dependent carboxylases (3-methylcrotonoyl-CoA carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase) for the latter [9–11,14]. In plant cells, we and others have characterized different biotin-dependent carboxylases localized in three cell

compartments, namely a mitochondrial 3-methylcrotonoyl-CoA carboxylase, a chloroplastic ACCase (of eukaryotic type in Gramineae and of prokaryotic type in other plants) and a putative cytosolic ACCase [40–45]. Thus the existence of HCS isoforms in different cell compartments suggests that the different biotin-dependent carboxylases in plants are biotinylated in the cell compartment within which they are localized. In a previous publication we showed that pea leaf cells contain a pool of free D-biotin localized in the cytosol, and no detectable levels of this vitamin in the organelles [18]. This therefore raises the question of how chloroplast and mitochondrial HCS forms might function *in vivo*. More recently we demonstrated that plant HCS displayed a very low K_m for D-biotin of 28 nM [17]. As the detection limit for the biological assay used to determine free D-biotin levels in pea leaf cell compartments was of the order of 0.05 ng [18], it is possible that chloroplasts and mitochondria actually contain very low D-biotin concentrations that are sufficient to allow the biotinylation of biotin-dependent carboxylases present in these organelles by HCS isoenzymes. Further characterization of these enzymes, and particularly the identification of a possible structurally distinct HCS isoform, will be necessary for understanding the mechanism of biotinylation of apocarboxylases in plants and to elucidate the question of why HCS activity is compartmentalized in plant cells.

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