Biotin holocarboxylase synthetase was partially purified from pea leaves by a sequence of ammonium sulphate fractionation and DEAE 52-cellulose chromatography. Enzyme activity was assayed using apo-(biotin carboxyl carrier protein) from an *Escherichia coli* bir A mutant affected in biotin holocarboxylase synthetase activity. Conditions for optimal catalytic activity and biochemical parameters of the plant enzyme were determined. This is the first direct evidence of the existence of biotin holocarboxylase synthetase activity in plants.

INTRODUCTION

Biotin (vitamin H or B8) is an essential coenzyme synthesized by plants, most bacteria, and some fungi that occurs primarily in a protein-bound state within the cell. Biotinylated proteins use this prosthetic group as a carrier of activated carboxyl groups during carboxylation and decarboxylation enzymic reactions. In all organisms, these carboxylases play housekeeping functions, as for example acetyl-CoA carboxylase (ACCase), which catalyses the first committed step in fatty acid biosynthesis.

Biotinylated proteins are extremely rare in Nature. For example, the only biotin-dependent carboxylase in *Escherichia coli* is ACCase, a multisubunit enzyme in which one of the subunits is biotinylated and corresponds to the biotin carboxyl carrier protein (BCCP). Other bacteria contain one to three biotinylated proteins [1]. Eukaryotic cells appear to contain a slightly greater number of biotinylated protein species. Thus *Saccharomyces cerevisiae* contains four or five biotinylated proteins depending on growth conditions [2], whereas both mammals [3,4] and plants [5] are reported to contain four biotinylated proteins. Analyses of amino acid sequences for biotin-dependent enzymes have shown that almost all these enzymes conserve the tetrapeptide sequence (Ala/Val)-Met-Bct-Met within the biotinyl site, where the biotinylated Lys residue (Bct) is generally located 34 or 35 residues from the C-terminal amino acid [6]. However, two biotinylated proteins, the rat [7] and avian [8] ACCases, have the coenzyme attached at a site far removed from the C-terminus. Recently, Schatz [9] reported the isolation from a peptide library of synthetic peptides that did not contain the consensus sequence found in natural biotinylated proteins but were efficiently biotinylated in *E. coli*, suggesting that these peptides somehow mimic the folded structure formed by the natural substrates.

In *E. coli*, the biotinylation of apo-ACCase is realized by biotin holocarboxylase synthetase (HCS; EC 6.3.4.10). The enzyme catalyses the post-translational incorporation of d-biotin to a specific Lys residue of newly synthesized apoenzyme, via an amide linkage between the biotin carboxyl group and a unique ε-amino group of the Lys residue [6]. This covalent attachment, essential for enzymic activation of apo-ACCase into holo-ACCase, occurs in two steps (1) and (2) as follows:

\[
\begin{align*}
\text{d-Biotin} + \text{ATP} & \rightarrow \text{d-biotinyl 5'-AMP + PP}_i \\
\text{d-Biotinyl 5'-AMP + apo-BCCP} & \rightarrow \text{holo-BCCP + AMP}
\end{align*}
\]

The first step is the activation of d-biotin by ATP, which yields d-biotinyl 5'-AMP. This is then followed by the covalent attachment of the biotinyl group to the ε-amino group of a specific Lys residue of the apo-BCCP, with release of AMP [10].

Interestingly, HCS acts across species barriers, i.e. HCS from eukaryotic organisms can biotinylate bacterial apo-carboxylases [6]. This cross-species activity reveals a molecular mechanism common to all of these enzymes.

HCS has been purified from *E. coli* and its gene cloned [11,12]. This enzyme is also called Bir A and acts as a repressor of the biotin operon [13]. Also, mammalian HCS have been purified from different species [14,15]. Recently, cDNA clones encoding human HCS have been obtained [16,17]. However, for the Plant Kingdom nothing is known about the mechanisms of protein biotinylation.

In this report, we provide the first direct evidence for the existence of HCS activity in pea leaves. The requirements of the reaction catalyzed by the plant synthetase have been determined and the conditions for optimal catalytic activity of the incorporated biotin have been determined. In accordance with the cross-specificity previously noted among eukaryotic and prokaryotic systems, we show that the plant HCS catalyzes the incorporation of radiolabeled d-biotin in bacterial apo-BCCP.

MATERIALS AND METHODS

Media, bacterial strains and chemicals

Adenine, AMP, ADP, ATP[S], ATTP, CTP, GTP, UTP, d-biotin, biocytin, desthiobiotin, diaminobiotin, iminobiotin and...
peroxidase-labelled streptavidin were obtained from Sigma Chemic SARL. Quinaldof ((R,S)-2-[4-(6-chloro-quinoxalin-2-yl)phenoxyl]proionic acid) was from Nissan Chemical Industries. D-[carboxyl-14C]Biotin (57 mCi/mmol), D-[8,9-3H]biotin (42 Ci/mmol) and NaH14CO3 (53,1 mCi/mmoll) were purchased from Amersham.

Temperature-sensitive E. coli bir A mutants (strain BM 4050) were generously provided by A. M. Campbell [18]. Mutations in the bir A gene affect the HCS function of the Bir A protein, resulting in biotin auxotrophy. This strain was grown at 30 °C on minimal medium M9 [19], supplemented with glucose and 43 mM D-biotin. Unlike the situation in wild-type E. coli extracts, measurements of labelled biotin incorporation into protein in sonicated extracts reveal no activity in vitro [20]. Solutions of D-biotin for bacterial growth were sterilized by filtration.

**Plant material**

Pea (Pisum sativum L., var Douce Provence) plants were grown from seeds in soil under a 12 h photoperiod of white light from fluorescent tubes (10–40 µE m−2 s−1) at 18 °C. The plants were watered every day with tap water. Plant material was harvested at different times and stored at −80 °C until use.

**Preparation of plant extracts**

Plant leaves were frozen in liquid nitrogen, and ground to a fine powder using a mortar and pestle. The powder was homogenized with 0.1 M NaCl in buffer B. Active fractions were pooled and after extensive washing with buffer B, the enzyme was eluted with 0.1 M NaCl. Buffer B was equilibrated in buffer B. The precipitate was collected by centrifugation at 40000 g and 1 mM benzamidine hydrochloride), filtered and centrifuged in 2 vol. of buffer A (20 mM phosphate buffer, pH 7.5, 1 mM EDTA, 1 mM DTT, 5 mM 6-aminohexanoic acid and 1 mM benzamidine hydrochloride), filtered and centrifuged at 40000 g for 30 min (JA 20 rotor, Beckman). The supernatant was then fractionated by addition of crystalline ammonium sulphate with slow stirring between 30% and 55% saturation. The precipitate was collected by centrifugation at 40000 g for 20 min (JA 20 rotor, Beckman) and resuspended in a minimal volume of buffer B (Tris-HCl, pH 8, 1 mM EDTA, 1 mM DTT, 5 mM 6-aminohexanoic acid and 1 mM benzamidine hydrochloride). The suspension was used immediately for the different analyses or stored at −80 °C until use.

**Preparation of HCS extracts**

The above extract (200 ml) was desalted by passage through a Sephadex G25 column (Pharmacia) equilibrated in buffer B. The clear eluate (300 ml) was loaded on to a DEAE 52-cellulose column (Serva) equilibrated with buffer B. The column was connected to a HiLoad chromatography system (Pharmacia). After extensive washing with buffer B, the enzyme was eluted with 0.1 M NaCl in buffer B. Active fractions were pooled and desalted as described above.

**Preparation of apoprotein substrate**

The apoprotein used as a substrate was the apo-BCCP from an E. coli Bir A mutant lacking HCS activity in vitro [20]. Bacteria were grown at 30 °C for 15 h in 500 ml of glucose minimal medium M9 supplemented with 0.4% casein hydrolysat (Difco) and limiting D-biotin at a concentration of 0.4 nM. Under these conditions, the mutated HCS is not able to biotinylate all the available endogenous apo-BCCP. That is, crude extracts of cells grown under these conditions contain sufficient amounts of apo-BCCP to serve as substrate for exogenous HCS. The culture was centrifuged at 3000 g and the cell pellet resuspended in 50 ml of buffer A. All subsequent manipulations were carried out at 0–4 °C. The cells were disrupted by sonication (10 min pulses at setting 5 with a Vibra Cell sonifier; Sonic and Materials, Danbury, CT, U.S.A.). After centrifugation of the disrupted bacteria at 17000 g for 20 min, the supernatant was retained and proteins precipitated with solid ammonium sulphate (0.361 g/ml of supernatant). After 1 h, the precipitate was collected by centrifugation at 17000 g for 20 min, then dissolved in a minimal volume of buffer A, desalted by passage through a Sephadex G 25 column (Pharmacia) and stored in portions at −80 °C. Under these conditions, the apo-BCCP extract was stable for at least 1 year.

**Protein determination and electrophoresis**

Protein quantification was determined by the method of Bradford [21] with γ-globulin as standard. Polypeptides labelled with D-[14C]biotin were analysed by SDS/PAGE and fluorography. SDS/PAGE was performed at room temperature in slab gels (15 cm × 15 cm) containing 12% (w/v) acrylamide. The experimental conditions for gel preparation, sample solubilization, electrophoresis and gel staining were as detailed by Chua [22]. After staining, gels were soaked in Amplify solution (Amersham) and dried prior to fluorography for one week on X-ray films.

**Assay for ACCase, methylcrotonoyl-CoA carboxylase (MCCase) and HCS**

All assays were optimized with respect to the concentration of each reaction components and to the pH of the reaction mixture. The activities of ACCase and MCCase were measured as the incorporation of radioactivity from NaH14CO3 into an acid-stable product as described previously [23,24]. The HCS assay was performed by measuring the attachment of D-[3H]biotin to apo-BCCP. The reaction mixture contained the following components in a total volume of 200 µl: 5 mM ATP, 5 mM MgCl2, 0.2 mM DTT in 20 mM phosphate buffer (pH 7.5), 10–200 µg of plant HCS extract, 0.2 mg of bacterial apoprotein (apo-BCCP) extract and 250 nM D-[8,9-3H]biotin (42 Ci/mmol). The reaction was initiated by addition of the labelled D-biotin. Incubations were for 120 min at 37 °C. Then, 125 µl 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% trichloroacetic acid. The filters were washed once with ethanol, dried, and radioactivity was counted in scintillation vials containing 8 ml of scintillation liquid (Ready protein; Beckman). Duplicate assays without apo-BCCP and/or plant HCS extract were run as controls. By this technique, the background values were of the order of 1/200 of the input radioactivity. The radioactivity incorporated from D-[3H]biotin by the plant enzyme present in cell-free crude extracts was partially quenched by remaining chlorophyll (Chl) pigments. In this case, this quenching effect could be eliminated by long exposures to light of the scintillation vials before counting. Kinetics of product formation were linear and rates varied linearly with enzyme concentration, demonstrating adherence to steady-state conditions. Kinetic data were fitted to the appropriate theoretical equations by non-linear regression analyses using the KaleidaGraph® software (Abelbeck Software) on a Macintosh Icx computer.

**RESULTS AND DISCUSSION**

**Preparation of plant HCS extract**

HCS activity measured in crude soluble extracts from 10-day-old pea leaves, using saturating amounts of bacterial apo-BCCP and
\( 13 \text{kDa} \) was also labelled by biotin, although this labelling was not noted [10]. Figure 1 clearly shows that biotin is incorporated into prokaryotes, where a strict dependence for a bivalent cation was verified that the extracts from the reaction demonstrate that the plant HCS is responsible for containing bacterial apo-BCCP as substrate. Several features of accounting for about 3 cation, a low but significant biotin incorporation was observed, in source for apo-BCCP did not contain any HCS activity.

Characterization of HCS

The assay we have used for pea-leaf HCS measures the specific attachment of \( \beta\text{-}[\text{H}] \) biotin to protein in a reaction mixture containing bacterial apo-BCCP as substrate. Several features of the reaction demonstrate that the plant HCS is responsible for the observed \( \beta\text{-}[\text{H}] \) biotin incorporation (Table 1). First, we have verified that the extracts from the \( E. \text{coli} \) \( \beta\text{ir} \ A \) mutant used as a source for apo-BCCP did not contain any HCS activity \textit{in vitro} (Table 1; Figure 1). On the other hand, the reaction catalysed by the plant HCS was dependent upon the presence of ATP and apo-BCCP extract in the reaction medium. The reaction was stimulated by \( \text{MgCl}_2 \). However, in the absence of this bivalent cation, a low but significant biotin incorporation was observed, accounting for about 3\% of the maximum level obtained with 5 mM \( \text{MgCl}_2 \). This result is different from the situation in prokaryotes, where a strict dependence for a bivalent cation was noted [10]. Figure 1 clearly shows that biotin is incorporated into apo-BCCP to form holo-BCCP. Thus a biotinylated polypeptide of the expected size (about 21 kDa) was detected from the complete assay medium. Another polypeptide of molecular mass 13 kDa was also labelled by biotin, although this labelling was rather faint compared with that of BCCP. This polypeptide is possibly a degradation product of BCCP or a non-identified biotinylated protein of \( E. \text{coli} \) but not from the plant, since it was not detected in controls lacking apo-BCCP extracts. It is interesting to notice at this level that HCS acts across species barriers, since pea plant HCS biotinylates bacterial apocarboxylase. This feature was previously observed by various authors with bacterial or eukaryotic HCS [17,27–29]. Table 1 and Figure 1 also indicate that 10-day-old pea leaves do not contain detectable levels of endogenous apo-carboxylases, since very little biotin was incorporated in the absence of bacterial apo-BCCP in the reaction medium and no plant labelled polypeptide was detected under these conditions.

Catalytic properties of plants HCS

To optimize the assay conditions for HCS activity, the pH optima for activity, the linearity of each assay with respect to incubation time, enzyme and bacterial apo-BCCP concentrations and the kinetic parameters for \( \beta\text{-} \) biotin and ATP substrates were established. For prepurified plant HCS activity, the accumulation of the radioactive trichloroacetic acid-precipitable product increased linearly with time of incubation up to 200 min. Furthermore, the enzyme activity was directly proportional to the amount of plant HCS extract in the assay up to 400 \( \mu \)g of protein. Finally, the minimal amount of bacterial apo-BCCP extract required to ensure substrate saturation of plant HCS was determined to be about 200 \( \mu \)g. Therefore all subsequent assays routinely contained 10–200 \( \mu \)g of prepurified plant HCS and 200 \( \mu \)g of apo-BCCP extract, and they were incubated for 120 min.

HCS was active over a wide range of pH with an optimum between pH 7.5 and 8.5. A similar pH optimum has been reported for HCS from human fibroblast extracts when analysed with apo-propionyl-CoA carboxylase as the acceptor substrate [30] and for rat liver HCS with apo-pyruvate carboxylase and apo-
propionyl-CoA carboxylase as acceptor substrates [14]. In contrast, prokaryotic HCSs were reported to have optimum pH values ranging from 7 to 7.5 [10,11,31].

The influence of substrate concentrations on enzyme activity was assessed by determination of apparent $K_m$ values. When one substrate was varied at fixed (saturating) levels of the other substrates, Michaelis–Menten kinetics were observed. The apparent $K_m$ value for D-biotin was 28 nM. This value is very similar to those reported for HCS from other organisms, as for example bovine liver (13 nM) [15] or rat liver (50–55 nM) [14], but it is much lower than that reported for Propionibacterium shermanii HCS (0.9 µM) [31]. In a previous paper, we reported that the concentration of free D-biotin in the cytosol of pea leaf cells was of the order of 11 µM [33]. Thus, compared with its apparent $K_m$ for D-biotin, the plant HCS apparently operates at full substrate saturation in vivo. The apparent $K_m$ value for ATP was about 1 mM. A similar high apparent $K_m$ for ATP was also found for E. coli HCS (3 µM) [34], a value that is consistent with intracellular ATP concentration [35]. In plant cells, ATP concentration varies from 0.3 to 1.4 mM in chloroplast stroma in the dark and in the light respectively, and is of the order of 3 mM in the cytosol [36]. Thus the apparent $K_m$ of plant HCS for ATP matches the estimated concentration of this nucleotide in various plant cell compartments. In contrast, bovine liver and rat liver HCS exhibit much lower apparent $K_m$ values for ATP, being in the range of 20–200 µM [14,15].

Specificity for metal ions

Mg$^{2+}$ was the most efficient bivalent metal ion supporting the plant HCS activity. However, substitution of Mg$^{2+}$ by equimolar Ni$^{2+}$, Mn$^{2+}$ and Co$^{2+}$ resulted in substantial HCS activity (relative rates of 50, 45 and 35 % respectively). Other bivalent cations tested (Zn$^{2+}$, Ca$^{2+}$ and Cu$^{2+}$) were unable to substitute for Mg$^{2+}$. Bacterial HCS shows comparable bivalent cation requirements, since Mn$^{2+}$ as well as Mg$^{2+}$ could satisfy the cation requirement [10]. However, in this case, Mn$^{2+}$ was as efficient as Mg$^{2+}$ in supporting HCS activity.

Specificity for biotin analogues

Of the different biotin analogues tested (biocytin, desthiobiotin, diaminobiotin and iminobiotin), none had any inhibitory effect on the plant HCS activity, at least in the concentration range of 10 nM–1 µM. Such behaviour was also observed in the case of rat liver HCS using biocytin as a biotin analogue [15]. This feature reflects the very high specificity of HCS for its substrate, D-biotin.

Specificity for nucleotides

Various nucleotides and their derivatives were assayed for plant HCS activity. Adenine and AMP were found to be ineffective substrates for HCS, in agreement with expected requirements from reaction (1). On the other hand, ADP, ATP[S], ATTP, UTP, CTP and GTP were able to substitute for ATP to various extents. Thus HCS activity observed with ADP and ATP[S] was estimated to be 65 % of the control value, while ATTP, UTP, CTP and GTP supported HCS activity to only 20–30 % of the control value. This behaviour is quite similar to that reported for prokaryotic HCS [10,31] but differs from that for with bovine liver HCS, where CTP is the best substrate (relative rate of 117 %), GTP can replace substantially ATP (relative rate of 46 %) and no activity is detected with UTP [15].

**Figure 2** Evaluation of HCS, ACCase and MCCase activities during pea-leaf development

Crude extracts were prepared as described in the Materials and methods section. Assays were carried out as described in the Materials and methods section using 100 µg of plant protein extracts. (A) Evaluation of HCS activity measured in the presence (●) or absence (○) of 180 µg of bacterial apo-ApoBCCP extract. (B) Evaluation of ACCase activity, including total ACCase activity (●). The two pea-leaf ACCases can be differentiated by the inhibition by quizalofop, a herbicide of the aryloxyphenoxypropionate class. The prokaryotic form (●) is insensitive and the eukaryotic form (○) is totally inhibited by 100 µM quizalofop [23]. (C) Evaluation of MCCase activity (●).

Evaluation of HCS activity during pea-leaf development

During leaf development, HCS activity was high in young leaves with a maximum at day 6 after planting and then decreased in older leaves to reach a plateau at day 8–14 (Figure 2A). This activity pattern followed those for the two pea-leaf ACCase isoforms respectively (Figure 2B; [23]). Recent studies have shown the existence, in pea leaves, of a prokaryotic form of ACCase, composed of different subunits, that is localized in chloroplasts of the mesophyll cells and a eukaryotic form of ACCase that is present in epidermal cells, probably in the cytosol [37]. The former enzyme is insensitive to quizalofop, a herbicide of the aryloxyphenoxypropionate class, while the latter is totally inhibited by 100 µM quizalofop [24,37]. The prokaryotic form of pea-leaf ACCase was found to be very active in young leaves, and then its activity decreased to low levels in mature leaves, while the activity of the eukaryotic form of ACCase was relatively constant during leaf development. A second peak of HCS activity was detected in developing leaves with a maximum at day 25 after planting. This high level of activity could be correlated to MCCase activity, another biotin enzyme present in mitochondria and for which activity is high in fully developed and in senescent pea leaves (Figure 2C; [24]). On the other hand, the level of HCS activity measured in the absence of added apo-BCCP in the reaction medium remained at background values in pea-leaf extracts at any stage of the plant development, indicating that the level of apo-carboxylases in pea leaves is negligible (Figure 2A).
These results and those described in Figure 1 and Table 1 contrast markedly with those obtained recently by Wang and co-workers [38], who suggested from labelling experiments with [125]I-streptavidin that tomato leaves contain a pool of non-biotinylated MCCase.

In conclusion, we have developed an efficient procedure that allows the measurement of HCS activity in pea-leaf extracts. The assay is based upon the use of radiolabelled biotin and bacterial apo-BCCP as an acceptor substrate. The present work demonstrates for the first time that pea leaves contain an active HCS activity able to biotinylate a bacterial apo-carboxylase, thus revealing a molecular mechanism common to the enzyme in both Kingdoms. The evaluation of plant HCS in developing leaves was correlated to that of biotin enzymes, and no pool of apo-carboxylase was detected in the leaf at any stage of its development, indicating that HCS activity in the leaf is not limiting. Nevertheless, the central role of plant HCS in converting inactive apoenzymes into their active holo-forms makes it a key enzyme in the maintenance of active plant metabolism. Finally, the occurrence of various peaks of HCS activity during leaf development is possibly linked to the existence of HCS isoforms that are, presumably, involved in the formation of the different compartments of pea leaves, as was recently suggested for human HCS [17]. Indeed, we recently obtained evidence that pea leaves also contain different HCS isoforms. Characterization of these enzymes is currently in progress.

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