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Isolation and Characterization of Biotin Carboxylase from Pea Chloroplasts[1]

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Pea (*Pisum sativum* L.) leaf acetyl-coenzyme A carboxylase (ACCase) exists as two structurally different forms: a major, chloroplastic, dissociable form and a minor, multifunctional enzyme form located in the leaf epidermis. The dissociable form is able to carboxylate free D-biotin as an alternate substrate in place of the natural substrate, biotin carboxyl carrier protein. Here we report the purification of the biotin carboxylase component of the chloroplastic pea leaf ACCase. The purified enzyme, free from carboxyltransferase activity, is composed of two firmly bound polypeptides, one of which (38 kD) is biotinylated. In contrast to bacterial biotin carboxylase, which retains full activity upon removal of the biotin carboxyl carrier component, attempts to dissociate the two subunits of the plant complex led to a complete loss of biotin carboxylase activity. Steady-state kinetic studies of the biotin carboxylase reaction reveal that addition of all substrates on the enzyme is sequential and that no product release is possible until all three substrates (MgATP, D-biotin, bicarbonate) are bound to the enzyme and all chemical processes at the active site are completed. In agreement with this mechanism, bicarbonate-dependent ATP hydrolysis by the enzyme is found to be strictly dependent on the presence of exogenous D-biotin in the reaction medium.

ACCase (acetyl-CoA:carbon-dioxide ligase [ADP forming], EC 6.4.1.2) is a biotin-containing enzyme that catalyzes the following overall reaction (reaction 1):

$$MgATP + HCO_3^- + \text{acetyl-CoA} \rightarrow MgADP + Pi + \text{malonyl-CoA} \quad (1)$$

This carboxylation reaction, which is the first committed step in de novo fatty acid biosynthesis, proceeds in two distinct half-reactions (Knowles, 1989). In the first reaction, the biotin moiety that is covalently attached to the enzyme via a specific Lys residue is carboxylated in a MgATP-dependent manner (reaction 2). In the second, the carboxyl group is transferred from carboxybiotin to acetyl-CoA to form malonyl-CoA (reaction 3) (Knowles, 1989).

$$\text{E-biotin} + MgATP + HCO_3^- \rightarrow \text{E-biotin-}CO_2^- + MgADP + Pi \quad (2)$$

$$\text{E-biotin-}CO_2^- + \text{acetyl-CoA} \rightarrow \text{E-biotin} + \text{malonyl-CoA} \quad (3)$$

In *Escherichia coli*, ACCase consists of three distinct, separable protein components, including a 17-kD biotin carboxyl carrier protein, a 51-kD biotin carboxylase, and two nonidentical subunits of the carboxyltransferase of molecular mass 33 and 35 kD (Guchhait et al., 1974a; Li and Cronan, 1992a, 1992b). In contrast, in animal, fungi, and yeast cells, ACCase is composed of multifunctional and identical subunits, each of approximately 220 to 280 kD and containing the biotin carboxylase, the carboxyltransferase, and the biotinylated domains (Mishina et al., 1976; Samols et al., 1988; Iverson et al., 1990; Al-Feel et al., 1992). In plants, both structurally distinct forms of ACCase exist. For example, pea (*Pisum sativum* L.), a dicotyledonous plant, contains a dissociable form of ACCase comparable to that found in prokaryotes. This enzyme is localized in plastids and, as such, is involved in the synthesis of fatty acids required for lipid biogenesis (Sasaki et al., 1993; Alban et al., 1994; Konishi and Sasaki, 1994). Leaves also contain a high-molecular-mass form of ACCase comparable to that described in other eukaryotes. This enzyme was detected exclusively in leaf epidermis and its exact role is unknown at present (Alban et al., 1994). This latter enzyme, but not the dissociable form of ACCase, was also detected in pea seeds (Duval et al., 1993; Dehaye et al., 1994). In contrast, monocotyledonous plants such as maize and wheat contain two eukaryotic isozymes of ACCase, the major one being present in mesophyll chloroplasts and the other one probably in the cytosol (Egli et al., 1993; Gornicki and Haselkorn, 1993; Ashton et al., 1994; Elborough et al., 1994). It is interesting that the selective grass herbicides of the aryloxyphenoxypropionate and cyclohexanedione types strongly inhibit the plastidic eukaryotic form of ACCase from maize or wheat but do not affect the activity of the plastidic prokaryotic form of ACCase from pea, indicating that the tolerance of pea plants toward these compounds relies on the insensitivity of the prokaryotic form of the enzyme (Egli et al., 1993; Alban et al., 1994; Dehaye et al., 1994; Konishi and Sasaki, 1994).

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Abbreviations: ACCase, acetyl-CoA carboxylase; AMP-PNP, 5′-adenylylimidodiphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ATTP, adenosine 5′-tetraphosphate.

In this paper we describe the isolation of the biotin carboxylase component of the chloroplastic form of pea leaf ACCase, free from the carboxyltransferase activity. The minimal structure of the active biotin carboxylase component of ACCase corresponds to a complex of two polypeptides in tight interaction, one of which (38 kD) is biotinylated. Steady-state kinetic results are compatible with an ordered mechanism in which MgATP binds first, followed by free biotin, and then bicarbonate. Consistent with this mechanism, bicarbonate-dependent ATP hydrolysis by the enzyme can be observed only in the presence of added D-biotin. Our structural and functional data suggest that some differences may exist between the multisubunit isoform of ACCase found in plants and that in bacteria.

MATERIALS AND METHODS

Reagents

Adenine, AMP, AMP-PNP, ADP, ATPγS, ATTP, dATP, dideoxyATP, CTP, GTP, UTP, D-biotin, biocytin, desthiobiotin, diaminobiotin, iminobiotin, acetyl-CoA, and avidin were obtained from Sigma. $NaH^{14}CO_3$ (53.1 Ci/mol) was purchased from Amersham. [α-^{32}P]ATP (25 Ci/mmol) was purchased from ICN. Horseradish peroxidase color development reagent, peroxidase-labeled streptavidin, and hydroxylapatite gel (Bio-Gel HTP) were purchased from Bio-Rad. Sepharose CL-4B, Sephacryl S-300 High Resolution, and prepacked columns of Superdex-200, Q-Sepharose (Hi-Load 16/10), and phenyl-Superose were obtained from Pharmacia. All other chemicals were of analytical grade.

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 μmol m^{-2} s^{-1} PPFD) at 18°C. The plants were watered every day with tap water.

Preparation and Fractionation of Purified Chloroplasts

Young pea leaves (12 d; 2–3 kg) were homogenized in 330 mM sorbitol, 50 mM Hepes-KOH, pH 8.0, 1 mM EDTA, 1 mM DTT, and 0.1% (w/v) BSA using a Waring blender for 3 to 5 s at low speed. Intact chloroplasts were rapidly prepared and purified as described by Douce and Joyard (1982) using discontinuous Percoll gradients. Intact chloroplasts (2 g of total protein; 100 mg of Chl) were rapidly washed in the same buffer without BSA and then lysed with lysis buffer containing 50 mM Hepes-KOH, pH 8.0, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 5 mM 6-aminocaproic acid, and 1 mM benzamidine-HCl. After mixing, chloroplasts were left on ice for 10 min to ensure complete lysis. The suspension of broken chloroplasts was centrifuged at 72,000*g* for 30 min on 5-mL 0.6 M Suc layers. The pellet and the supernatant fractions comprised the chloroplast membranes (envelope membranes and thylakoids) and the soluble fraction (stroma), respectively. All procedures were carried out at 4°C.

Purification of Biotin Carboxylase

The enzyme was isolated from pea leaf chloroplast stroma. All operations were carried out at 4°C. Stroma (up to 2 g of protein) was subjected to ammonium sulfate fractionation by addition of solid $(NH_4)_2SO_4$ to 20% saturation. After 20 min of stirring, the mixture was centrifuged at 39,000*g* for 20 min. The supernatant fluid was recovered and brought to 60% saturation with solid $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation, resuspended in a minimum volume of buffer A (20 mM Hepes-KOH [pH 8], 10% [v/v] glycerol, 1 mM DTT, 1 mM EDTA, 1 mM benzamidine-HCl, 5 mM 6-aminocaproic acid), and then loaded onto a 2.6 × 35 cm Sephacryl S-300 column equilibrated in the same buffer at a flow rate of 1 mL/min. The column was connected to a fast-protein liquid chromatography system (Pharmacia). Fractions containing biotin carboxylase activity were pooled and concentrated by precipitation with solid $(NH_4)_2SO_4$ at 70% saturation. The pellet was resuspended in a small volume of buffer A containing 0.5 M KCl and applied onto a monomeric avidin-Sepharose CL-4B column (1 × 3 cm) prepared as described previously (Kohansky and Lane, 1990; Alban et al., 1993) and equilibrated with the same buffer. After a 16-h exposure of the protein fraction, the column was washed with 20 mL of the above buffer (flow rate 0.2 mL/min). Biotin carboxylase activity was eluted (together with the biotinylated subunit of ACCase) with 5 mL of the equilibration buffer containing 2 mM D-biotin. Active fractions were pooled, concentrated, and desalted by successive filtrations with Macrosep-10 tubes (Filtron, Northborough, MA) to a final concentration of about 0.5 mg/mL and stored at −80°C until use.

Determination of Native Biotin Carboxylase Molecular Mass

The molecular mass of native biotin carboxylase was estimated by gel-filtration chromatography on a prepacked Superdex-200 column (1.6 × 60 cm, 120 mL of gel [Pharmacia]) equilibrated with a buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.2 M KCl. The column, connected to a fast protein liquid chromatography system, was eluted at a flow rate of 1 mL/min. Fractions of 1.5 mL were collected. The molecular mass was deduced by comparing the elution volume of biotin carboxylase to the elution volume of standard proteins.

Enzymatic Assays

All assays were optimized with respect to the concentration of each reaction component and to the pH of the reaction mixture. ACCase activity was measured as the incorporation of radioactivity from $NaH^{14}CO_3$ into an acid-stable product (Baldet et al., 1993).

Biotin carboxylase activity was assayed by two protocols. The first protocol is the radiometric assay developed by Guchhait et al. (1974a), which is based on the detection of [^{14}C]carboxybiotin formed during the enzymic reaction from ATP, $MgCl_2$, $NaH^{14}CO_3$, and biotin. Except where

otherwise noted, assays contained 100 mM Hepes-KOH (pH 8), 1 mM ATP, 4 mM $MgCl_2$, 5 mM $NaH^{14}CO_3$ (2000 dpm/nmol), 50 mM D-biotin, 3 mM DTT, 0.6 mg/mL BSA, 5% (v/v) ethanol, and 1 to 200 μg of protein in a final volume of 0.5 mL. The reaction was initiated with enzyme and incubated for 5 to 20 min at 30°C, and carboxylation was terminated by rapid transfer of a 0.4-mL aliquot to 1 mL of ice-cold water containing two drops of 1-octanol in a scintillation-counting vial. To remove the remaining $H^{14}CO_3^-$, the solution was bubbled for 30 min with CO_2 and maintained at 2 to 4°C. After gassing, 0.1 mL of 0.1 M NaOH was added and the radioactivity was quantified in a liquid-scintillation counter (model LS 1801, Beckman). Duplicate assays, without D-biotin, were conducted as controls. The rates of product formation were linear and varied linearly with enzyme concentration, documenting adherence to steady-state conditions. Kinetic data were fitted to the appropriate theoretical equations by nonlinear regression analyses using the KaleidaGraph software (Abelbeck Software, Reading, PA) on a Macintosh IIcx computer. Data concerning substrate and cofactor specificity and effects of metabolites on biotin carboxylase activity were mean values obtained from three separate experiments. One unit of biotin carboxylase activity corresponds to the amount of enzyme that catalyzed the incorporation of 1 μmol bicarbonate per min.

The second protocol is based on detection of [^{32}P]ADP formed during the enzyme reaction (reaction 2). Reactions were carried out at 30°C in the same medium as for the ^{14}C radiometric assay described above, except that nonradioactive $NaHCO_3$ and [α-^{32}P]ATP (0.02 Ci/nmol) were used. The final volume was 50 μL. After a 20-min incubation, 4-μL aliquots were spotted onto poly(ethyleneimine)-cellulose sheets (Polygram PEI-300, Macherey and Nagel, Dueren, Germany) and analyzed by TLC (de Mercoyrol et al., 1992). Separation of mononucleotides (ATP, ADP, and AMP) was achieved by a 0.42 M HCOOH/0.32 M LiCl solvent system. Labeled products were visualized after autoradiography of the sheets on Kodak X-Omat films at −80°C with an intensifying screen. Radioactive areas were cut from plates and transferred to scintillation vials, scintillator was added, and radioactivity was quantified in a liquid-scintillation counter. Nonradioactive nucleotides were used as markers and detected under incident short-wave UV light.

Electrophoretic Analyses of Proteins

SDS-PAGE was performed at room temperature in slabs (15 × 15 cm) containing a 7.5 to 15% (w/v) acrylamide gradient. The experimental conditions for gel preparation, sample solubilization, electrophoresis, and gel staining were as detailed by Chua (1980). In some experiments, polypeptides were also transferred electrophoretically onto nitrocellulose sheets (Gelman Sciences, Ann Arbor, MI), essentially according to Towbin et al. (1979). Biotin-containing polypeptides were detected with a system analogous to western blotting using streptavidin conjugated with horseradish peroxidase (Baldet et al., 1992).

Protein Determination

Protein was measured by the method of Bradford (1976) using a Bio-Rad protein assay reagent, with γ-globulin as standard.

RESULTS AND DISCUSSION

Isolation of the Biotin Carboxylase Component of ACCase from Pea Leaf Chloroplasts

The purification of a eukaryotic and prokaryotic form of ACCase from young pea leaves has previously been reported (Alban et al., 1994). In contrast to the former, the prokaryotic type of ACCase was detected in the chloroplast-stromal fraction and consisted of several nonidentical subunits. In the course of enzyme purification, the different subunits of this prokaryotic type of ACCase readily dissociated, resulting in complete loss of ACCase activity. Yet, ACCase activity could be fully restored by a combination of the separate constituents. However, by this approach it was not possible to identify the different resolved subunits except for a 38-kD polypeptide, which could be detected by western blotting and probing with peroxidase-labeled streptavidin. Thus, this biotin-containing polypeptide might correspond to the biotin carboxyl carrier component analogous to that of bacterial ACCase. By using the ^{14}C radiometric assay described in "Materials and Methods," we observed in preliminary experiments that the chloroplastic ACCase was able to carboxylate free D-biotin as an alternate substrate in lieu of biotin carboxyl carrier protein and in the absence of acetyl-CoA. In contrast, the eukaryotic form of ACCase was unable to support this reaction. This specific property of the chloroplastic ACCase was used to purify the biotin carboxylase component of the enzyme.

Results of the purification protocol outlined in "Materials and Methods" are shown in Table I. Ammonium sulfate fractionation concentrated the biotin carboxylase activity, which was then directly loaded onto a Sephacryl S-300 gel-filtration column without prior desalting. During this chromatography, the enzyme eluted in a fraction with an apparent molecular mass of about 600 kD, indicating that, at this stage of the purification, the distinct subunits of ACCase remained associated in the high-molecular-mass complex described previously (Alban et al., 1994). Indeed, ACCase activity was recovered in this fraction (Table I; see also Alban et al., 1994). Upon subsequent chromatography on the monomeric avidin-Sepharose column, the overall ACCase activity was lost because of the dissociation of the multisubunit complex, as shown before (Alban et al., 1994). However, the biotin carboxylase activity was retained on this column and was specifically eluted with free D-biotin. It is worth noting that only about 25% of the biotin carboxylase activity was recovered from the avidin-Sepharose column. This finding suggests that, as in *E. coli*, biotin carboxylase activity is higher in the presence of the carboxyltransferase subunits (Guchhait et al., 1974a), indicating the existence of a ternary complex between the different protein components of ACCase. After elimination of free D-biotin by desalting and concentration with

Table I. *Purification of the biotin carboxylase component of pea chloroplast ACCase*

Biotin carboxylase and ACCase activities were measured as the incorporation of radioactivity from $H^{14}CO_3^-$ into carboxybiotin and malonyl-CoA, respectively (see "Materials and Methods").

Purification Step	Protein	Biotin Carboxylase		Yield	Purification	Whole ACCase Specific Activity
		Total activity	Specific activity			
	mg	*munits*	*munits/mg*	*%*	*-fold*	*munits/mg*
Stromal fraction	1325	2067	1.56	100	1	1.9
$(NH_4)_2SO_4$ fraction	1216	1314	1.08	64	0.7	1.5
Sephacryl S-300	82.7	789	9.54	38	6.1	7.8
Monomeric avidin-Sepharose						
Void	51	5	0.1	0.2	0.06	0.1
Eluted	0.38	192	506	9.3	324	0.3

Macrosep-10 tubes, biotin carboxylase could be stored at −80°C for up to 12 months with essentially no loss of activity. Starting from the chloroplast-stromal fraction, the procedure resulted in an approximately 320-fold purification of biotin carboxylase with a yield of about 9%.

Structural Properties of the Enzyme

The fraction containing peak biotin carboxylase activity, as eluted from avidin-Sepharose, gave two polypeptide bands of near equal intensity on SDS-PAGE (Fig. 1). Densitometric scans of the Coomassie blue-stained gels of purified biotin carboxylase preparations indicated that the molar stoichiometry of the two polypeptides is 1:1 (data not shown). In agreement with previous findings (Alban et al., 1994; Dehaye et al., 1994), these two polypeptides are components of chloroplastic ACCase: the larger polypeptide, molecular mass 38 kD, is biotinylated, whereas the other one, molecular mass 32 kD, is biotin free (Fig. 1). Because the medium used for monomeric avidin-Sepharose chromatography contained 0.5 M KCl, electrostatic interactions between the two polypeptides seem unlikely. Therefore, hydrophobic interactions or linkage by disulfide bridges most probably account for the formation of the tight complex between these two polypeptides. However, when KCl was omitted in the elution buffer for the monomeric avidin-affinity column, both polypeptides remained associated and the complex exhibited full biotin carboxylase activity. We also chromatographed the enzyme fraction isolated from the affinity column on a phenyl-Superose column in the presence of $(NH_4)_2SO_4$ or ethanediol. In both cases, the two polypeptides were eluted by decreasing the concentration of salt or solvent. However, both polypeptides always co-eluted, and the biotin carboxylase activity was greatly reduced (data not shown).

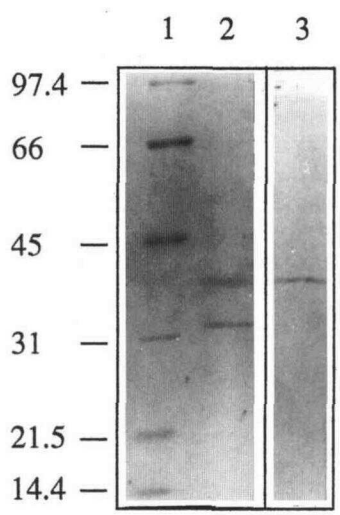

Figure 1. Analysis by SDS-PAGE of the purified biotin carboxylase component of pea leaf chloroplast ACCase. Polypeptides were separated on a 7.5 to 15% (w/v) gradient polyacrylamide/SDS slab gel and stained with Coomassie brilliant blue R-250 (lanes 1 and 2) or labeled with horseradish-peroxidase-conjugated streptavidin and revealed with the color development reagent 4-chloro-1-naphthol after electrotransfer onto a nitrocellulose sheet (lane 3) (see "Materials and Methods"). Lane 1, Molecular mass markers, 10 μg each; lanes 2 and 3, enzyme after chromatography on the monomeric avidin-Sepharose column, 5 μg. Molecular masses on the left are given in kD.

In another experiment, we attempted to dissociate the two polypeptides by employing a method used to separate the two subunits of *Achromobacter* methylcrotonoyl-CoA carboxylase (Schiele et al., 1975). This method is based on the reduction of intermolecular disulfide bridges during dialysis of the protein fraction against a buffer containing 10 mM Gly/10 mM Cys at pH 9.8 under a nitrogen atmosphere. After chromatography of the dialyzed fraction on the avidin-Sepharose column, we observed a partial dissociation of the two polypeptides. However, under these conditions, biotin carboxylase activity could be detected neither in the "void" fraction nor in the biotin-eluted fraction, even after neutralization with phosphate buffer. Furthermore, combination of the separated fractions did not restore biotin carboxylase activity (not shown). Finally, chromatography of the enzyme fraction on hydroxylapatite or ion-exchange columns such as Q-Sepharose also failed to separate the 32- and the 38-kD polypeptides.

Thus, it seems that any modification of the interaction between the two components results in loss of biotin carboxylase activity, and we conclude that, in contrast to bacterial ACCase (Guchhait et al., 1974a), the minimal structure of chloroplastic biotin carboxylase is a complex of two major polypeptides, one of which contains biotin. Biotin carboxylases from *E. coli*, *Anabaena* sp., *Pseudomonas*

aeruginosa, and tobacco have molecular masses ranging from 49 to 51 kD (Li and Cronan, 1992a; Best and Knauf, 1993; Gornicki et al., 1993; Shorrosh et al., 1995). These values are markedly higher than those found for the two pea chloroplast biotin carboxylase subunits. That the 32-kD polypeptide corresponds to a degradation product of the 38-kD polypeptide is unlikely, since it does not contain biotin (Fig. 1) and, as such, it would not bind to the avidin-Sepharose column. On the other hand, all chromatographic media used during biotin carboxylase isolation contained protease inhibitors, and the purified biotin carboxylase fraction obtained was very active. In contrast, the fact that both polypeptides co-elute from the avidin-Sepharose column provides direct evidence that they physically interact. Finally, it is clear from our data that the molecular mass of the biotinyl subunit from pea chloroplast ACCase is very different from that of *E. coli* (38 compared to 17 kD), thus demonstrating that no direct correlation can be made concerning the molecular masses of the different ACCase subunits from various organisms. Nevertheless, the possibility that the 32-kD polypeptide is derived from a higher-molecular-mass polypeptide by proteolytic cleavage cannot be excluded, because it was recently reported that an antibody raised to the C-terminal 124 amino acids of a putative biotin carboxylase cDNA from castor cross-reacts with a 47-kD soluble protein from pea chloroplasts (Shorrosh et al., 1995). This type of cross-reactivity may also suggest the existence of isoforms of the "prokaryote-like ACCase" in plants.

The apparent molecular mass of the native biotin carboxylase purified from the monomeric avidin-affinity column was estimated by gel-filtration chromatography on a Superdex-200 column and was found to be about 135 kD. Again, the active fraction contained both the biotinyl and the biotin-free polypeptides (not shown). These results are consistent with an $\alpha_2\beta_2$ organization of these polypeptides in the native structure.

Catalytic Properties of the Purified Biotin Carboxylase

The biotin carboxylase-catalyzed reaction was measured by following $H^{14}CO_3^-$ incorporation into carboxybiotin. The reaction was strictly dependent on the presence of D-biotin, ATP, and divalent metal ion. In contrast to the bacterial enzyme (Kondo et al., 1984), the plant biotin carboxylase was only slightly stimulated by 5% (v/v) ethanol and was severely inhibited by higher alcohol concentrations (data not shown). This result was in agreement with previous observations concerning the overall ACCase reaction (Alban et al., 1994). Biotin carboxylase activity was markedly pH sensitive between pH 6.5 and the optimum pH (8–8.2). Thus, at pH 7.5, the activity was only about 15% of that at pH 8.0. Above pH 8, the enzyme was active over a broad pH range, with 80% of maximum activity occurring at pH 9 and 50% occurring at pH 9.5. When one substrate was varied at fixed (saturating) levels of the other substrates, Michaelis-Menten kinetics were observed. Apparent K_m values for D-biotin, bicarbonate, and ATP were: 42 ± 12 mM (ATP = 1 mM; HCO_3^- = 5 mM), 1.53 ± 0.13 mM (D-biotin = 50 mM; ATP = 1 mM), and 47 ± 3 μM (D-biotin = 50 mM; HCO_3^- = 5 mM), respectively. Similar values have been reported for biotin carboxylase isolated from *E. coli* (Polakis et al., 1974). In both cases, the high K_m value for D-biotin (42 mM for the pea leaf enzyme; 167–214 mM for the bacterial enzyme [Polakis et al., 1974; Kondo et al., 1984]) probably reflects the fact that free D-biotin is not the natural substrate for biotin carboxylase and that the protein part of the biotin carboxyl carrier component allows considerable enhancement in biotin binding to biotin carboxylase. In support of this suggestion, it was shown that a synthetic peptide corresponding to the biotin-binding sequence of the *E. coli* biotin carboxyl carrier protein (Ala-Met-Lys-Met) lowered the K_m for free D-biotin considerably, from 214 to 12 mM (Kondo et al., 1984).

Specificity for Divalent Metal Ions

Mg^{2+} was the most efficient divalent metal ion supporting biotin carboxylase activity. However, substitution of Mg^{2+} by equimolar Co^{2+}, Mn^{2+}, and, to a lesser extent, Fe^{2+} resulted in substantial biotin carboxylase activity (relative rates of 81 ± 4, 60 ± 2, and 51 ± 1%, respectively). Other divalent cations tested (Zn^{2+}, Cu^{2+}, and Ca^{2+}) were unable to substitute for Mg^{2+}. Bacterial biotin carboxylase shows comparable divalent cation requirements, since Co^{2+} and Mn^{2+} could also substitute efficiently for Mg^{2+} (Tipton and Cleland, 1988). Furthermore, such a feature was also observed for other biotin-dependent enzymes such as methylcrotonoyl-CoA carboxylase (Diez et al., 1994). When the ATP concentration was fixed at 0.1 or 1 mM, increasing the concentration of Mg^{2+} resulted in a hyperbolic or a sigmoidal increase in bicarbonate incorporation by biotin carboxylase, respectively. In both cases, the maximal activity occurred at about 4 mM Mg^{2+} but was higher when the ATP concentration was maintained at 1 mM (data not shown). These results indicate that the true substrate for biotin carboxylase activity is MgATP, which is also the case for all of the biotin enzymes studied to date (Knowles, 1989). On the other hand, the initial sigmoidal response of biotin carboxylase activity to increasing Mg^{2+} concentrations, at a fixed 1 mM ATP concentration, also indicated that activity was inhibited in a competitive manner by the free ATP that prevailed in the assay when the total Mg^{2+} concentration was less than that of the total ATP concentration (Segel, 1975). Such behavior was previously observed for the overall reaction catalyzed by pea leaf and pea seed ACCase (Dehaye et al., 1994).

Specificity for Biotin Analogs

Of the different biotin analogs tested (10 mM biocytin, iminobiotin, desthiobiotin, or diaminobiotin), only biocytin supported biotin carboxylase activity, although at a much lower rate compared to D-biotin (relative rate of 29 ± 3%). On the other hand, none of these compounds had an inhibitory effect on biotin carboxylase activity, at least up to 50 mM (data not shown). The fact that substitution at the 1′-*N* position of the ureido ring (which is the case for diamino- and iminobiotin) completely abolished the biotin carboxylase-catalyzed HCO_3^- fixation reaction was in

agreement with the proposal that carboxylation by bacterial biotin carboxylase occurs at this level (Guchhait et al., 1974b; Knowles, 1989). Desthiobiotin, which differs from D-biotin only by the absence of the sulfur atom from the thiophane ring, was not recognized by biotin carboxylase, emphasizing the great specificity of the active site for D-biotin.

Specificity for Nucleotides

Various nucleotides and their derivatives were assayed for biotin carboxylase activity. UTP, CTP, and GTP were found to be ineffective substrates for biotin carboxylase. On the other hand, none of these compounds had inhibitory effects on enzyme activity, indicating that they were not recognized by the ATP binding site. Thus, the enzyme is highly specific for the nucleotidic base.

Adenine, AMP, ADP, ATPγS, ATTP, and AMP-PNP were unable to substitute for ATP. However, some of these compounds, when included at a concentration of 0.5 mM, were strong inhibitors of biotin carboxylase activity. This was the case for ADP, a product of the enzyme-catalyzed reaction (83 ± 3% inhibition), adenine (59 ± 2% inhibition), and two nonhydrolyzable analogs of ATP, AMP-PNP (51 ± 3% inhibition) and ATPγS (85 ± 4% inhibition). The most efficient inhibitors of biotin carboxylase activity, namely ADP and ATPγS, were found to behave as competitive inhibitors relative to ATP (Fig. 2). The inhibition constant values were 100 (ADP) and 25 μM (ATPγS). Altogether, these results demonstrate that the various ATP derivatives were well recognized by the ATP binding site and point out the role of the triphosphate chain for binding and catalysis.

It is surprising that 1 mM dATP supported biotin carboxylation to a higher extent than equimolar ATP (relative rate of 168 ± 5%). This difference was accounted for by a difference in V_{max} values, since the value with dATP was about 70% higher than that with ATP as a substrate. Likewise, the K_m for dATP was found to be 38 ± 4 μM, a value very similar to that measured for ATP. On the other hand, 1 mM dideoxyATP was not a substrate for biotin carboxylase activity, but inhibited it significantly at 0.1 mM (35 ± 3% inhibition). Thus, contrary to the 2′-OH group of ATP, the 3′-OH group of the nucleotide substrate is absolutely required for catalysis and/or productive binding to the enzyme.

Thus, chloroplastic biotin carboxylase is highly specific for ATP, owing to specific interactions of the enzyme with the triphosphate chain, the adenine base, and the 3′-OH group on the sugar unit.

Influence of Quizalofop

The aryloxyphenoxypropionic acid herbicide quizalofop did not affect biotin carboxylase activity at concentrations up to 100 μM, confirming previous data concerning the overall ACCase activity catalyzed by the prokaryotic form of pea leaf ACCase (Alban et al., 1994; Dehaye et al., 1994).

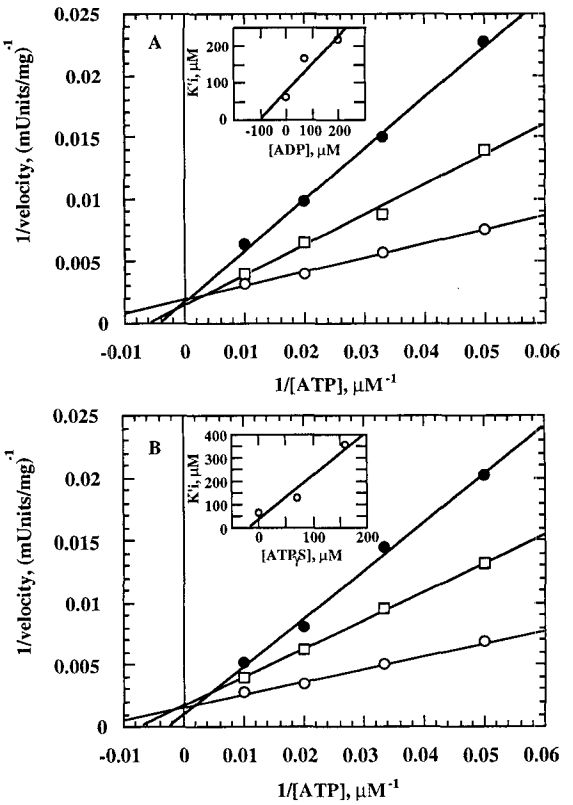

Figure 2. Lineweaver-Burk analysis of the inhibition of pea chloroplast biotin carboxylase activity by ADP (A) and ATPγS (B). Enzyme activity was measured as the incorporation of radioactivity from $H^{14}CO_3^-$ into carboxybiotin in the presence of 1.75 μg of enzyme. A, Effect of increasing ATP concentration in the presence of 0 (○), 70 (□), or 200 μM (●) ADP. B, Effect of increasing ATP concentration in the presence of 0 (○), 70 (□), or 160 μM (●) ATPγS. The insets show the secondary plots of $-1/x$ intercept (K'i) of the Lineweaver-Burk plots versus the ADP (A) or ATPγS (B) concentration. Other conditions were as described in "Materials and Methods."

Influence of Substrate and Cofactor Concentrations

The kinetic mechanism of biotin carboxylase was investigated by assaying the enzyme at a fixed saturating concentration of Mg^{2+} (4 mM). This reduced the complexity of the reaction mechanism to a terreactant system, including MgATP, HCO_3^-, and D-biotin. Under these conditions, two substrates were varied at fixed values of the third. Double-reciprocal plots of initial velocity data obtained by sequential variation of ATP, bicarbonate, and D-biotin gave rise to intersecting patterns in all cases, which excluded any ping-pong mechanism. Thus, as for the *E. coli* biotin carboxylase (Knowles, 1989), in the reaction catalyzed by the stromal enzyme there are no products released until all three substrates are bound to the enzyme and all chemical processes at the active site are complete.

When the ATP concentration was varied at different fixed levels of D-biotin or HCO_3^-, the lines intercepted off the 1/velocity axis (data not shown). This suggests either a random binding of ATP and D-biotin and of ATP and HCO_3^- or an equilibrium-ordered mechanism in which ATP binds before D-biotin and HCO_3^- in agreement with

the kinetic mechanism reported for the *E. coli* biotin carboxylase (Tipton and Cleland, 1988). On the other hand, when HCO_3^- concentration was varied at different fixed levels of D-biotin, the lines intercepted on the 1/velocity axis (Fig. 3), suggesting a rapid, equilibrium-ordered mechanism in which D-biotin binds first, followed by bicarbonate (Segel, 1975). This behavior differs from that seen with the *E. coli* biotin carboxylase, for which the same double-reciprocal plots intercept off the 1/velocity axis (Tipton and Cleland, 1988; eq. 5). For the *E. coli* enzyme, the steady-state kinetic results have been interpreted in terms of a mechanism in which HCO_3^- is activated by MgATP prior to reaction with D-biotin, leading to the formation of a carboxyphosphate intermediate (Tipton and Cleland, 1988). This mechanism received strong experimental support following the discovery by Climent and Rubio (1986) and Tipton and Cleland (1988) that *E. coli* biotin carboxylase possesses a low ATPase activity in the absence of D-biotin, an activity that appears to be an intrinsic property of the enzyme. Thus, this ATPase activity is dependent on the presence of bicarbonate, its rate is increased by the presence of ethanol in the same proportion as in the normal biotin carboxylase reaction, it shows the same Mg^{2+} and K^+ dependence, it is independent of the presence of avidin, and the thermal stabilities of the two activities are identical (Climent and Rubio, 1986). Thus, investigation of the exact requirements for ATP hydrolysis in reaction 1 is critical for assessment of the catalytic mechanism of biotin carboxylase.

By using the protocol described under "Materials and Methods" that allows the direct detection of [^{32}P]ADP formation, it was shown that the plant enzyme catalyzed an efficient ATP hydrolysis in the presence of MgATP, bicarbonate, and D-biotin (Fig. 4; Table II). This reaction exhibited the same properties as those determined by following the formation of [^{14}C]carboxybiotin. Thus, the reaction was strictly dependent on the presence of enzyme and bicarbonate. Furthermore, ATP hydrolysis was inhibited in the presence of ATPγS, ADP, and, to a lesser extent, adenine.

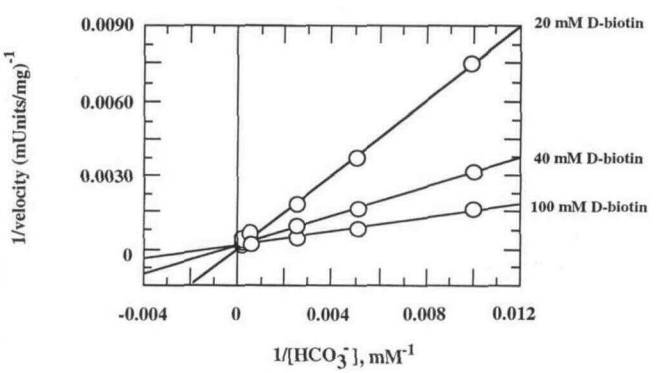

Figure 3. Steady-state kinetic analysis of purified pea chloroplast biotin carboxylase. Double-reciprocal plots of initial velocities with variable $H^{14}CO_3^-$ concentrations and different fixed concentrations of D-biotin. Each assay was initiated by addition of 1.75 μg of purified enzyme to 500 μL of the reaction mixture described in "Materials and Methods."

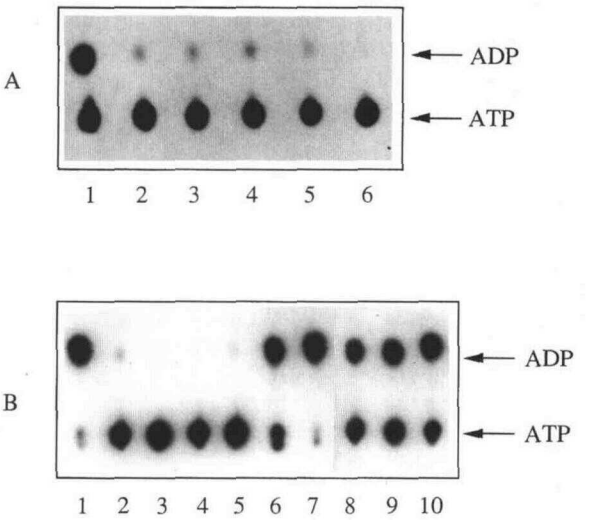

Figure 4. Requirements for ATP hydrolysis by biotin carboxylase purified from pea chloroplasts. Reaction assays were conducted by measuring [α-^{32}P]ADP formation from [α-^{32}P]ATP as described in "Materials and Methods." Composition of the complete reaction medium was as in Table II. After a 30-min incubation at 30°C, 4-μL aliquots were analyzed by TLC and autoradiographed as described in "Materials and Methods." A, Lane 1, Complete reaction medium; lane 2, minus enzyme; lane 3, minus exogenous HCO_3^-; lane 4, minus D-biotin; lane 5, minus D-biotin, plus 20 μg streptavidin; lane 6, minus Mg^{2+}. B, Lane 1, Complete reaction medium; lane 2, minus D-biotin; lane 3, minus enzyme; lane 4, minus Mg^{2+}; lane 5, minus Mg^{2+}, plus 4 mM Cu^{2+}; lane 6, minus Mg^{2+}, plus 4 mM Co^{2+}; lane 7, minus Mg^{2+}, plus 4 mM Mn^{2+}; lane 8, plus 1 mM ATPγS; lane 9, plus 1 mM ADP; lane 10, plus 1 mM adenine. Experiments in A and B were performed with two distinct enzyme preparations.

Also, Mn^{2+} or Co^{2+} substituted for Mg^{2+}. Low ATPase activity (about 3%) was observed with the complete assay lacking D-biotin. It is clear that the activity detected in the absence of D-biotin and in the presence of streptavidin was not different from that measured in controls lacking exogenous HCO_3^- or enzyme. Comparison of control levels obtained in the absence of enzyme or Mg^{2+} indicated that a substantial portion of the biotin-independent ATPase activity was due in fact to the bivalent metal itself. From these data and critical evaluation of the various controls, it is clear that the biotin-independent, bicarbonate-dependent ATPase activity catalyzed by the plant enzyme did not account for more than 0.4% of the maximal activity determined in the presence of D-biotin. Thus, given the background values of about 2%, this level of specific ATPase activity was not significant. Therefore, if the biotin-independent ATPase activity were to exist for the plant enzyme, it is, indeed, a very slow and inefficient enzyme-catalyzed reaction. We conclude that for the plant enzyme, ATPase activity is strictly dependent on added D-biotin. Such a strict dependence was also observed by Attwood and Graneri (1992) for ATP cleavage catalyzed by chicken liver pyruvate carboxylase.

What might be the reason for this apparent difference in mechanism between the *E. coli* and plant biotin carboxylases? Given the structural properties of the plant enzyme

Table II. *Requirements for ATPase activity of biotin carboxylase purified from pea chloroplasts*

The activity was followed by measuring [α-^{32}P]ADP formation from [α-^{32}P]ATP as described in "Materials and Methods." Incubations were for 30 min. The data represent the mean ± SD from five measurements (also see Fig. 4).

Reaction Medium	[α-^{32}P]ADP Formed
	nmol/assay
Complete[a]	5.16 ± 0.18
Minus enzyme	0.10 ± 0.01
Minus exogenous HCO_3^-[b]	0.11 ± 0.01
Minus biotin	0.14 ± 0.02
Minus biotin, plus 20 μg of streptavidin[c]	0.12 ± 0.01
Minus Mg^{2+}	0.043 ± 0.005
Minus Mg^{2+}, plus Cu^{2+} (4 mM)	0.040 ± 0.004
Minus Mg^{2+}, plus Co^{2+} (4 mM)	4.05 ± 0.15
Minus Mg^{2+}, plus Mn^{2+} (4 mM)	5.20 ± 0.20
Plus ATPγS (1 mM)	1.70 ± 0.08
Plus ADP (1 mM)	2.55 ± 0.10
Plus adenine (1 mM)	3.02 ± 0.11

[a] The complete reaction medium (50 μL) contained 110 μM [α-^{32}P]ATP, 4 mM $MgCl_2$, 8 mM $NaHCO_3$, 50 mM D-biotin, 3 mM DTT, 0.6 mg/mL BSA, 5% (v/v) ethanol, and 1.75 μg of enzyme in 100 mM Hepes-KOH, pH 8. [b] The reaction medium was previously degassed under a N_2 flux for 2 min. [c] Streptavidin was added to block the biotin linked to the biotinyl subunit of ACCase present in the purified fraction and any remaining traces of free D-biotin.

(biotin carboxylase activity requires the presence of the biotin-carrier protein), one possibility could be that the biotin covalently bound to the biotin carboxyl carrier component is poised in the carboxylation active site, presumably because of the absence of carboxyltransferase activity that has been removed during purification. Thus, in the absence of exogenous D-biotin, HCO_3^- binding would be impeded because this anion presumably binds close to biotin in the biotin carboxylase active site. It follows that for HCO_3^- binding and biotin carboxylase activity to occur, this biotin bound to the carboxyl carrier protein must first be removed by free D-biotin. This possibility, which might account for the steady-state kinetic results and the observed requirements for ATPase activity, is appealing because it would allow one to interpret the reaction of the plant biotin carboxylase in terms of the same mechanism as that established for the *E. coli* enzyme (Climent and Rubio, 1986; Tipton and Cleland, 1988; Knowles, 1989), i.e. via the formation of a carboxyphosphate intermediate, with the restriction that because of the structural difference between the bacterial and chloroplastic enzymes (the bacterial biotin carboxylase readily dissociates from the biotin-carrier protein, whereas for the chloroplastic enzyme the biotin carboxylase component is in tight association with the biotin-carrier protein), the formation of the carboxyphosphate intermediate requires the presence of D-biotin.

In conclusion, we have isolated the biotin carboxylase component of a chloroplast ACCase. This enzyme activity exhibits some differences, both at the structural and functional levels, with its counterpart from bacteria. The availability of this purified plant enzyme should allow the isolation and characterization of the other component involved in the overall ACCase reaction, i.e. the carboxyltransferase activity. This will also allow investigations of the structure of the active sites of the prokaryotic form of ACCase present in dicotyledonous plants and possibly the rational design of new inhibitor families targeting this enzyme class. Indeed, such compounds are at present unknown.

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