Purification and characterization of acetohydroxyacid reductoisomerase from spinach chloroplasts

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Acetohydroxyacid reductoisomerase was purified over 400-fold to a specific activity of 62 nkat · mg⁻¹, with 2-aceto-2-hydroxybutyrate as substrate, from the stroma of spinach leaf chloroplasts. The enzyme was not intrinsically membrane bound. The native enzyme was a tetramer with a subunit M_r of 59 000. The activity was optimum between pH 7.5 and 8.5. The apparent K_m for 2-acetolactate was 25 μ M and for 2-aceto-2-hydroxybutyrate was 37 μ M. The enzyme required Mg²⁺ and the V_{max} was attained at physiological Mg²⁺ concentrations. NADP⁺ competitively inhibited the reaction when NADPH was the varied substrate. The native enzyme eluted from Mono-Q ion-exchange resins as three distinct peaks of activity. This elution pattern was preserved when the peaks were combined, dialysed and re-chromatographed. Each form exhibited identical M_r of 59 000 after SDS/polyacrylamide gel electrophoresis (PAGE), whereas they were easily distinguishable from each other after PAGE under non-denaturing conditions. These results provide evidence for the existence of multiple forms of acetohydroxyacid reductoisomerase in chloroplasts isolated from spinach leaves.

INTRODUCTION

The biosyntheses of valine and isoleucine in a variety of micro-organisms and plants proceed by a similar sequence of reactions, three of which are catalysed by enzymes (acetohydroxyacid synthase, EC 4.1.3.18; acetohydroxyacid reductoisomerase, EC 1.1.1.86; dihydroxyacid dehydratase, EC 4.2.1.9) common to both sequences. In other words, the three enzymes which catalyse the three key steps in assembly of the carbon skeleton of valine also catalyse the synthesis of the carbon structure of isoleucine (Bender, 1985; Bryan, 1980).

We initiated a thorough examination of acetohydroxyacid reductoisomerase in higher plants because it catalyses an unusual two-step reaction in which the substrate, either 2-acetolactate or 2-aceto-2-hydroxybutyrate, is converted via an alkyl migration and a NADPH-dependent reduction to either 2,3-dihydroxyisovalerate or 2,3-dihydroxy-3-methylvalerate (Fig. 1) and because there is little information available on the plant enzyme (Satyanarayana & Radhakrishnan, 1962, 1965; Schultz *et al.*, 1988). Interestingly, the reductoisomerase is able to catalyse the reduction of oxopantoate to produce pantoate, the intermediate in CoA synthesis (Primerano & Burns, 1983). In this study we have purified the chloroplastic acetohydroxyacid reductoisomerase to homogeneity and characterized the steady-state kinetics of the enzyme as obtained from spinach leaf chloroplasts.

MATERIALS AND METHODS

Isolation of intact and purified chloroplasts

Chloroplasts were isolated from 2–3 kg of spinach leaves and purified as described by Douce & Joyard

(1982), using discontinuous Percoll gradients formed by two layers of 50 and 20 ml of 40 and 80 % (v/v) Percoll solutions, respectively. After centrifugation at 6000 g (maximum) for 20 min (HS4 rotor, Sorvall), the intact chloroplasts were found in a dark-green band near the bottom of the tube, whereas the thylakoids remained near the top of the tube. The intact and purified chloroplasts were subsequently concentrated by differential centrifugation. The chloroplasts were suspended in a medium containing 0.3 M-mannitol and 10 mM-Mops/NaOH buffer, pH 7.8, at approx. 15–20 mg of chlorophyll/ml (final volume 9–10 ml).

Preparation of soluble (stroma) proteins from spinach leaf chloroplasts

All steps were performed at 4 °C unless otherwise indicated. Purified and intact spinach chloroplasts (about

Fig. 1. Reaction catalysed by acetohydroxyacid reductoisomerase

Note that the two natural substrates for the enzyme differ in the identity of the R group that undergoes migration.

150–200 mg of chlorophyll, 2.5–4 g of protein) were diluted in 90 ml of lysis buffer containing 10 mM-Mops/NaOH buffer, pH 7.8, and 4 mM-MgCl₂. The addition of 4 μ M-leupeptin to the lysis buffer provided further protection for the enzyme against endogenous proteinases. The suspension of broken chloroplasts was centrifuged at 72 000 *g* for 60 min (20 000 rev./min, Beckman SW-27 rotor) to remove all the chloroplast membranes (envelope membranes and thylakoids). The amber-coloured supernatant (stromal extract: approx. 1.5–2.0 g of protein in about 80 ml of lysis buffer) contained acetohydroxyacid reductoisomerase activity. Stromal extracts were stored at –80 °C and pooled for further purification.

Enzyme assay

Acetohydroxyacid reductoisomerase activity was assayed in 50 mM-phosphate buffer, pH 8.0, 2 mM-MgCl₂, 250 μ M-NADPH in a final volume of 1 ml. Reactions were initiated by adding either 2 mM-2-aceto-2-hydroxybutyrate or 2-acetolactate and the progress of the reaction monitored by the decrease in absorbance of NADPH at 340 nm (Uvikon 860 spectrophotometer, Kontron) (Arfin & Umbarger, 1969). Acetohydroxyacid reductoisomerase activity was expressed as nkat of NADPH oxidized $\cdot$ mg of protein^{–1}.

2-Acetolactate and 2-aceto-2-hydroxybutyrate, unavailable commercially, were prepared by the method of Krampitz (1948). Under the conditions of our assay, no significant decomposition of 2-acetolactate and 2-aceto-2-hydroxybutyrate occurred.

Purification of acetohydroxyacid reductoisomerase

The soluble proteins from spinach leaf chloroplasts (200 ml, 3.6 g of protein) in lysis buffer were applied to a 2 cm $\times$ 18 cm column of Trisacryl M-DEAE (Industrie Biologique Française) equilibrated in 25 mM-potassium phosphate (pH 7.5) and 0.5 mM-dithiothreitol (buffer A) and connected to a Pharmacia f.p.l.c. system. The enzyme was eluted after washing with 55 ml of buffer A at 4 °C (flow rate 0.5 ml/min; fraction size 2 ml). Fractions containing acetohydroxyacid reductoisomerase activity were dialysed and concentrated to 2 ml by ultrafiltration (PM30 membrane; Amicon) in buffer A. This extract (30 mg of protein) was loaded on to a Red Sepharose (Pharmacia) column (1 cm $\times$ 18 cm) designed to fit into Pharmacia's f.p.l.c. system and previously equilibrated in buffer A. The column was then washed with 20 ml of buffer A and developed with a 20 ml linear gradient of KCl (0–150 mM) in the same buffer (flow rate 0.2 ml/min; fraction size 1 ml). The fractions that contained most of the acetohydroxyacid reductoisomerase activity were pooled and concentrated to 2 ml by ultrafiltration (PM30 membrane; Amicon) in buffer B (10 mM-potassium phosphate, pH 7.5, 0.5 mM-dithiothreitol). This extract (4 mg of protein) was then applied to a Mono-Q HR 5/5 column (Pharmacia) previously equilibrated with buffer B. The column connected to a Pharmacia f.p.l.c. system was then washed with 5 ml of buffer B followed by a 35 ml linear gradient of 10–50 mM-potassium phosphate (flow rate 0.33 ml/min; fraction size 0.5 ml). Acetohydroxyacid reductoisomerase was eluted as three distinct peaks at 25 mM-potassium phosphate. Purified acetohydroxyacid reductoisomerase was stored at –80 °C in the elution buffer.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis (PAGE). Electrophoresis was carried out at room temperature in SDS/polyacrylamide slab gels containing a 7.5–15% (w/v) linear acrylamide gradient. The experimental conditions for gel preparation, sample solubilization, electrophoresis and gel staining were as described by Chua (1980).

Native gels. Gels consisting of acrylamide [5–10% (w/v) linear gradient] were prepared as described by Laemmli (1970), except that detergent was omitted from the different buffers. Electrophoresis was performed at 10 mA/gel at 4 °C for 12 h.

Isoelectric focusing

Isoelectric focusing was performed as described by Righetti (1982). The gel contained acrylamide (8%, w/v), methylenebisacrylamide (0.2%, w/v), *NNN'*-tetramethylethylenediamine (0.06%, v/v) and 6% (v/v) of total ampholytes [Pharmalyte 3–10 or 4–6.5 (carrier ampholyte for isoelectric focusing), Pharmacia]. Acetohydroxyacid reductoisomerase (10 μ g) was applied, and acid (pI 6.85) or basic (pI 7.35) horse myoglobin was used to see the progress of the run, which was carried out at 4 °C. The gel was first run at constant current (5 mA), then the power supply was switched to constant voltage when the voltage reached 500 mV. After the pH gradient was fully developed, the gel was sliced into 5 mm sections for pH measurement. For the latter, the gel slices were each soaked in 1 ml of water for 12 h, and the pH of the resulting solution was then measured with a small combination electrode.

Protein determination. Protein was determined with the Bio-Rad protein assay (Bradford method) using bovine γ -globulin as the standard.

RESULTS

Isolation of acetohydroxyacid reductoisomerase from chloroplasts

Spinach chloroplasts were purified on Percoll gradients (92–97% intact). The amount of cytoplasmic contamination in the Percoll-purified chloroplast fraction was determined by measuring the activity of the cytoplasmic enzyme alcohol dehydrogenase (not shown). This analysis showed that cytoplasmic contamination is negligible after purification of chloroplasts on Percoll gradients. The isolated intact chloroplasts were lysed and membranes were separated from soluble proteins by centrifugation. Each fraction was assayed for acetohydroxyacid reductoisomerase activity. Over 94% of the enzyme activity was found in the soluble protein fraction.

The purification procedure described under Materials and methods provides the reductoisomerase in good yield and at a high level of purity in 2 working days. Table 1 shows a representative purification of the reductoisomerase from spinach chloroplasts using ion-exchange and Red Sepharose chromatography. The most important step is chromatography on Trisacryl M-DEAE. Thereafter, chromatography on Red Sepharose and ion-exchange chromatography completed the purification. The procedure resulted in a 350–400-fold purification. The yield obtained from 3600 mg of chloroplast stromal

Table 1. Purification of acetohydroxyacid reductoisomerase from spinach leaf chloroplasts

Purification stage	Total protein (mg)	Total activity* (nkat)	Specific activity (nkat·mg ⁻¹)	Yield (%)
Stroma	3660	650	0.18	100
Trisacryl M-DEAE pool	26	550	21	85
Red Sepharose pool	4	316	77	49
Mono-Q HR5/5 pool	2.85	175	62	27
Iso 1	0.72	43	61	—
Iso 2	0.98	60	62	—
Iso 3	1.15	72	63	—

* Enzyme assays were carried out at 30 °C in 50 mM-phosphate buffer (pH 8) with 2 mM-2-aceto-2-hydroxybutyrate.

Fig. 2. Documentation of purification procedure for acetohydroxyacid reductoisomerase by SDS/PAGE

Proteins were separated on an SDS/7.5–15% (w/v) gradient polyacrylamide slab gel stained with Coomassie Brilliant Blue R-250. Lane 1, stromal extract, 25 µg; lane 2, Trisacryl M-DEAE pool (see Table 1), 25 µg; lane 3, Red Sepharose pool (see Table 1), 15 µg; lane 4, purified acetohydroxyacid reductoisomerase (Iso 1, see Table 1 and Fig. 3), 6 µg; lane 5, purified acetohydroxyacid reductoisomerase (Iso 2, see Table 1 and Fig. 3), 4 µg; lane 6, purified acetohydroxyacid reductoisomerase (Iso 3, see Table 1 and Fig. 3), 4 µg. M_r standards as indicated.

protein was about 2.8 mg and the overall yield of activity was about 25–30% owing in large part to losses during Red Sepharose and Mono-Q HR chromatography. The proteins at different stages of the purification are shown in Fig. 2. On the basis of SDS/PAGE, the reductoisomerase gave a single band corresponding to an M_r of 59000 ± 2000 . Interestingly, multiple peaks of acetohydroxyacid reductoisomerase activity were clearly resolved (Iso 1, Iso 2, Iso 3; Fig. 3) from chloroplasts by ion-exchange (Mono-Q HR) f.p.l.c. These forms were not artefacts of chromatography, because re-chromatography of the three separated forms on the anion-exchange column gave a single peak of reductoisomerase activity at their original elution volumes. Furthermore, in other experiments, the distribution of acetohydroxyacid reductoisomerase between the three peaks was not affected by the inclusion of protein inhibitors (5 mM- ϵ -aminohexanoic acid, 1 mM-benzamidine, 1 mM-

Fig. 3. Purification of acetohydroxyacid reductoisomerase by f.p.l.c. Mono-Q HR5/5 chromatography

Approx. 4 mg of the Red-Sepharose pool (see Table 1) was applied to the Mono-Q HR5/5 column equilibrated with buffer B (see text). A linear gradient of 10–50 mM-potassium phosphate caused the elution of three protein peaks which were subsequently identified as Iso 1 (peak 1), Iso 2 (peak 2) and Iso 3 (peak 3), based on their respective electrophoretic mobilities on PAGE under non-denaturing conditions (Fig. 4). The continuous line represents protein concentration measured by A_{280} . The broken line represents the salt gradient from 10 to 50 mM-potassium phosphate; the dashed line with triangles represents acetohydroxyacid reductoisomerase activity. We have not yet found a correct explanation for the splitting of each peak.

phenylmethanesulphonyl fluoride) in the lysis buffer or by the amount of protein loaded on to the column (not shown). Collectively, these results indicated that the occurrence of three peaks of acetohydroxyacid reductoisomerase activity during anion-exchange (Mono-Q HR) chromatography was not an artefact of the experimental protocol, and that the three forms were not interconvertible. The distribution of activity between the three forms was very stable from one experiment to another. About 25% of the total recovered activity was eluted with peak 1 (Iso 1), 35% was eluted with peak 2

Fig. 4. PAGE under non-denaturing conditions of purified aceto-hydroxyacid reductoisomerase

Proteins were separated on a 5–10 % (w/v) acrylamide gel (see Materials and methods), stained with Coomassie Brilliant Blue R-250. Lane 1, Mono-Q HR pool (see Table 1), 8 μ g; lane 2, left side of peak 1 identified as Iso 1 (see Fig. 3), 8 μ g; lane 3, right side of peak 1 identified as Iso 1 (see Fig. 3), 8 μ g; lane 4, left side of peak 2 identified as Iso 2 (see Fig. 3), 7 μ g; lane 5, right side of peak 2 identified as Iso 2 (see Fig. 3), 7 μ g; lane 6, left side of peak 3 identified as Iso 3 (see Fig. 3), 9 μ g; lane 7, right side of peak 3 identified as Iso 3 (see Fig. 3), 9 μ g. M_r standards as indicated.

(Iso 2) and 40 % was eluted with peak 3 (Iso 3) (Fig. 3). No additional activity was eluted when the column was washed with 200 mM-potassium phosphate. Interestingly, each form exhibited an identical M_r of 59000 ± 2000 after SDS/PAGE (Fig. 2), whereas they were easily distinguishable from each other after PAGE under non-denaturing conditions (Fig. 4). Under these conditions the M_r values of each form were estimated to be 235000 (Iso 1), 220000 (Iso 2) and 205000 (Iso 3), respectively. This would therefore suggest that under our experimental conditions the native aceto-hydroxyacid reductoisomerase was a tetramer composed of four subunits with identical M_r , 59000. A tetrameric native form of aceto-hydroxyacid reductoisomerase has also been reported for *Salmonella typhimurium* (Hofler *et al.*, 1974). In addition, the elution of multiple peaks of this enzyme activity upon ion-exchange chromatography and the separation of three distinct protein bands after native PAGE strongly suggest that the chloroplast aceto-hydroxyacid reductoisomerase contained at least three isoforms differing from each other by their pI. In support of this suggestion, the pI values of the three bands were estimated to be 5.15, 5.05 and 4.95, respectively (not shown).

Properties

Isoenzyme 1 was used for kinetic experiments. Apparent K_m values for the substrates 2-acetolactate and 2-aceto-2-hydroxybutyrate and for NADPH were calculated to be 25, 37 and 5.7 μ M, respectively, at saturating concentrations of either substrates (2 mM) or nucleotide (250 μ M). The enzyme also utilized NADH as electron donor. However, the K_m for NADPH was much lower than that for NADH. In addition, in the presence of NADPH, and at pH 8, the V_{max} obtained with 2-aceto-

Fig. 5. Initial-velocity (V_i) patterns of NADPH oxidation by purified aceto-hydroxyacid reductoisomerase (Iso 1) isolated from spinach leaf chloroplasts

The reaction media and the preparation of aceto-hydroxyacid reductoisomerase were as described in the text. NADPH was the variable substrate and $[NADP^+]$ was fixed at zero (control) or at various concentrations as indicated. NADPH oxidation was performed at 30 °C in 50 mM-potassium phosphate using 2-aceto-2-hydroxybutyrate as substrate. The K_m for NADPH was $5.7 \pm 0.7 \mu$ M; the K_i for $NADP^+$ was $7.5 \pm 2 \mu$ M.

2-hydroxybutyrate (62 nkat $\cdot$ mg $^{-1}$) was five times higher than that obtained with 2-acetolactate. Interestingly, the product of the reaction catalysed by aceto-hydroxyacid reductoisomerase, namely $NADP^+$, competitively inhibited the reaction when NADPH was the varied substrate at saturating concentration of 2-aceto-2-hydroxybutyrate and Mg^{2+} (Fig. 5).

The activity of the isoform 1 was determined as a function of pH by buffering the reaction mixture with either phosphate or Tris/HCl from 6.5 to 9. The results shown in Fig. 6 indicate that with 2-acetolactate the optimum pH range was quite broad from 7 to 8.5. On the other hand, with 2-aceto-2-hydroxybutyrate, the optimum pH was shifted towards more alkaline region.

In contrast with previous work carried out on plants (Satyanarayana & Radhakrishnan, 1965), aceto-hydroxyacid reductoisomerase requires Mg^{2+} for full activity and no residual activity was found in the absence of Mg^{2+} . Incubation of the enzyme with a range of Mg^{2+} concentrations yielded a hyperbolic curve. Under these conditions, the apparent K_m for Mg^{2+} measured with 2-acetolactate as substrate was three times lower than that measured with 2-aceto-2-hydroxybutyrate (30 versus 100 μ M). In bacteria (Arfin & Umbarger, 1969), and *Neurospora crassa* (Kiritani *et al.*, 1966), Mg^{2+} is also required for the reductoisomerase activity. Interestingly, the affinity of the enzyme for Mg^{2+} was independent of NADPH concentration (not shown) suggesting, in agree-

Fig. 6. Effect of pH on the activity of purified acetohydroxyacid reductoisomerase (Iso 1) isolated from spinach leaves

Enzyme assays were carried out at 30 °C in 50 mM-potassium phosphate using either 2-acetolactate or 2-aceto-2-hydroxybutyrate as substrates (see the text).

ment with Chunduru *et al.* (1989) for the enzyme from *Escherichia coli*, that it is the uncomplexed metal and nucleotide that are the true substrates for the reaction.

Some biochemical properties of isoforms 2 and 3 were also examined. It appeared that isoforms 1, 2 and 3 were not differentiated either by their substrate or Mg^{2+} saturation curves or by the pattern of pH dependence (not shown). Each form of acetohydroxyacid reductoisomerase as assayed in the presence of valine, leucine and isoleucine: none of these amino acids had any significant effect on the activity of either isoform 1 or isoforms 2 and 3 at concentrations up to 1 mM.

DISCUSSION

The purification procedure described in the Materials and methods section provides chloroplastic acetohydroxyacid reductoisomerase from spinach leaves in good yield and at high level of purity in 2 working days. Based on SDS/PAGE, the reductoisomerase subunit has an M_r of 59000 in agreement with values of 57000 published by Hofler *et al.* (1974) and Shematek *et al.* (1973) for the *Salmonella typhimurium* reductoisomerase. The results presented here indicate that reductoisomerase from spinach leaf chloroplasts has a native M_r of approx. 220000 based on PAGE under non-denaturing conditions (Fig. 4). This would therefore suggest that the native enzyme was a tetramer composed of four identical subunits.

This report presents the first evidence for the existence of three forms of acetohydroxyacid reductoisomerase in chloroplasts. The origin of these forms can be attributed to combination of similar, but non-identical, polypeptide chains. In this case, the multiplicity could be a result of the synthesis of different types of subunit coded by distinct structural genes or the occurrence of secondary modified forms (post-translational modifications). To resolve this problem, detailed analysis of the amino acid

sequences of these polypeptides or of the nucleotide sequences coding for them will be required. Likewise, the existence of several forms opens up a great number of theoretical possibilities for regulation of either acetohydroxyacid reductoisomerase activity itself or branched-chain amino acid synthesis in general. Interestingly, multiple forms of acetohydroxyacid synthase have been reported in micro-organisms (for review, see Bender, 1985) and plants (Singh *et al.*, 1988; Durner & Böger, 1988; Muhitch, 1988), which have different sensitivity to feedback inhibition by amino acids.

Two points of interest emerge from these studies with respect to the properties of acetohydroxyacid reductoisomerase. First, the enzyme requires Mg^{2+} and the V_{max} is attained at physiological Mg^{2+} concentrations, i.e. the light-induced movement of Mg^{2+} from the thylakoid compartment to the stromal compartment (for review, see Walker, 1976) would appear to be sufficient to bring about the change in Mg^{2+} concentration necessary for full reductoisomerase activity. Secondly, the enzyme is regulated by the NADPH/NADP⁺ ratio, i.e. the product of reductoisomerase activity, namely NADP⁺, competitively inhibited the reaction when NADPH was the varied substrate. Consequently, increasing the ratio of NADP⁺ to NADPH in the stromal space after a light-dark transition would result in a logarithmic increase in inhibition. Since Mg^{2+} and NADP⁺ regulate reductoisomerase activity, we are therefore forced to imagine that, *in vivo*, its activity is strongly stimulated under light conditions (high Mg^{2+} , high NADPH/NADP⁺ ratio), whereas it becomes inhibited under dark conditions (low Mg^{2+} , low NADPH/NADP⁺ ratio).

Finally, the results presented here demonstrate that all the protein components involved in the overall synthesis of valine from pyruvate or isoleucine from 2-oxobutyrate become separable non-associated proteins upon lysis of chloroplasts.

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