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Oligomeric Forms of Plant Acetolactate Synthase Depend on Flavin Adenine Dinucleotide[1]

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ABSTRACT

Acetolactate synthase (ALS, EC 4.1.3.18) has been extracted and partially purified from etiolated barley shoots (*Hordeum vulgare* L.). Multiple forms of this enzyme were separated by gel filtration and/or anion-exchange chromatography using fast protein liquid chromatography. It could be demonstrated that these two species are in equilibrium, which strongly depends on the structural role of flavin adenine dinucleotide and pyruvate. With 50 micromolar of flavin adenine dinucleotide in the medium most of the ALS aggregates as a high molecular weight form (M_r = 440,000), while 50 millimolar pyruvate facilitates dissociation into the smaller form (M_r = 200,000). Data are presented to show that two enzymatically active forms are not isozymes but different oligomeric species or aggregates of the basic 58-kilodalton subunit of ALS. These different ALS species exhibit little difference in feedback inhibition by valine, leucine and isoleucine or in inhibition by the sulfonylurea herbicide chlorsulfuron. Both aggregation forms show a broad pH-optimum between 6.5 and 7. Furthermore, the affinity for pyruvate and the amount of directly-formed acetoin indicate similar properties of these separated ALS forms.

Acetolactate synthase (ALS, EC 4.1.3.18; also known as acetohydroxy acid synthase, AHAS) is the first common enzyme in the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine. The enzyme has been found in enteric bacteria (5, 6, 14), fungi (12), and in chloroplasts of higher plants (7, 17). The catalyzed reaction is either the formation of acetohydroxy butyrate from pyruvate and α-oxobutyrate or the synthesis of acetolactate from two molecules of pyruvate. As was established with the ALS form II of *Salmonella typhimurium,* cofactors are thiamine (TPP[2]), Mg^{2+}, or Mn^{2+}, and FAD. The requirement for flavin is an unusual feature of ALS, because the catalyzed reaction involves no redox step (16). The enzyme is under cooperative feedback control by valine, leucine, and isoleucine (9).

Interest in acetolactate synthase has increased since the development of three structurally unrelated classes of modern herbicides, the sulfonylureas (16), the imidazolinones (18), and the triazolo pyrimidines (20) which have been shown to specifically inhibit ALS.

The enzyme of microorganisms has been well studied. In *Escherichia coli* and *Salmonella typhimurium,* three isozymes (ALS I, II, III) have been identified, each of them encoded by a particular gene (5, 6, 14). Bacterial ALS is a tetramer composed of two large (59–60 kD) and two small (9–17 kD) subunits. Little is known about the enzyme from higher plants. Most recently ALS has been purified from barley (3). A polypeptide with a molecular mass of 58 kD could be identified as the basic subunit of ALS. No smaller subunit could be found.

Controversy remains about the role of FAD. This paper presents findings which indicate that occurrence of enzyme aggregates depends on FAD. When dealing with aggregates the question whether aggregates or isozymes exist in higher plants becomes relevant. Although in *Arabidopsis thaliana* only one gene coding for ALS could be identified, tobacco (*Nicotiana tabacum*) was shown to have two ALS genes (8). At the protein level several reports described different activity peaks of ALS from corn (*Zea mays*) during chromatographic steps with different sensitivity to feedback inhibition by valine (11, 19). With tobacco two activity peaks of ALS have been found after anion-exchange enrichment (2), while Relton *et al.* (13) reported a single ALS fraction only.

Most of the findings reported, however, refer to crude extracts. In this paper, we present evidence that several activity peaks of highly enriched barley ALS obtained by elaborate column chromatography are due to different polymeric states of a basic ALS subunit. These different forms of ALS were characterized with respect to feedback inhibition, their affinity to pyruvate, pH optima and sensitivity to chlorsulfuron.

MATERIALS AND METHODS

Plant Cultivation

Barley seeds (*Hordeum vulgare* L.) were placed onto a nylon net, covered with filter tissue which had been soaked with distilled water, and allowed to germinate in a dark chamber at 23 °C. The roots were immersed into a medium containing 2 g/L KNO_3, 34 mg/L $CaSO_4$ and 0.25 mg/L Na_2MoO_4 (pH 7.0). Three d after germination the etiolated shoots were harvested (*i.e.* the lower half was used, which contained more than 75% of the total ALS activity), washed in distilled water, and either homogenized or stored at 70° K for later use.

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[2] Abbreviations: TPP, thiamine pyrophosphate; FAD, flavin adenine dinucleotide; FPLC, fast protein liquid chromatography.

Enzyme Assays

The enzyme was assayed as described previously (3). The different forms of ALS were characterized by a modified assay: for the 'L'-form FAD was present with 50 μM and pyruvate was reduced to 10 mM; the assay for the 'S'-form contained 5 μM FAD and either 20 mM or 10 mM pyruvate in the absence or presence of chlorsulfuron, respectively.

Protein Determination

For the enzyme assays protein was determined according to Bradford (1) and to the manual of the manufacturer (Bio-Rad Laboratories). The protein patterns obtained by column chromatography were measured by absorbance at 280 nm.

Mol Wt Determination

The apparent native mol wt were estimated by FPLC using Superose 6 and 12 columns (60 cm × 16 mm i.d.) as described previously (3). Additionally, the mol wt were determined by an analytical 6.0 μm HPLC column (Zorbax Bio-Series GF-450, 25 cm × 9.4 mm i.d.). In this case the amount of glycerol in the mobile phase was reduced to 7.5% (v/v). The flow was 1 mL/min and pressure between 28 and 35 bar (equivalent to 420 and 525 psi). Mol wt markers from M_r 29,000 to 700,000 were purchased from Sigma.

Enzyme Preparation

The enzyme was prepared by fractionated ammonium-sulfate precipitation, hydrophobic-interaction chromatography, and anion-exchange chromatography. This procedure was carried out as described previously (3) except for the anion-exchange step where the DEAE-Fractogel column was replaced by a semipreparative Mono-Q (10 cm × 10 mm i.d., FPLC). Fractions containing ALS activity were pooled and concentrated by ammonium sulfate precipitation (0–45% saturation, *i.e.* 2.76 g were added to 10 mL). The precipitate with a specific activity of 50 to 60 nmol min^{-1} mg^{-1} protein was resuspended in gel filtration buffer and stored at 70 °K for later use.

Gel filtration described in this paper was performed on a Superose 12 prep grade column (60 cm × 16 mm i.d., FPLC), which was equilibrated with 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 50 mM NaCl, and 10% (v/v) glycerol. FAD and pyruvate were added as described in "Results" and as indicated in Figure 1, A and B. Specific activities after gel filtration were 0.39 to 0.47 μmol min^{-1} mg^{-1} protein for the 'L'-form and 0.58 to 0.71 μmol min^{-1} mg^{-1} protein for the 'S'-form.

Analytical anion-exchange runs were performed with a resin-based HPLC column (Bio-Gel TSK DEAE-5-PW, 75 × 7.5 mm) using the HPLC equipment described previously (3). Neither FAD nor pyruvate were added to the mobile phases.

RESULTS

Aggregation of ALS depending on FAD and/or pyruvate was examined by gel filtration on Superose 12 prep grade.

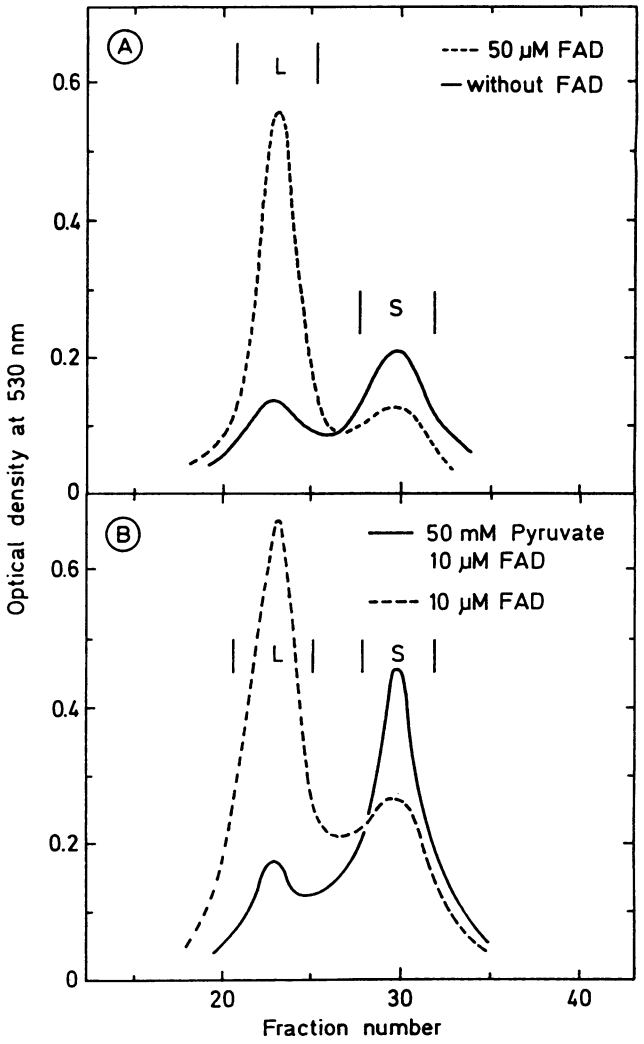

Figure 1. A, Effect of FAD on enzyme aggregation. Partially purified barley ALS was preincubated for 2 h in absence or in presence of 50 μM FAD. Subsequent gel filtration was performed with Superose 12 prep grade with elution buffer identical to incubation media. B, Effect of pyruvate on enzyme aggregation. Procedure as described for (A). ALS activity is plotted as absorbance at 530 nm (Westerfeld test, standard assay). For specific activities of the activity peaks obtained see legend of Figure 4.

Partially purified ALS was preincubated for 90 min in the gel filtration buffer with or without 50 μM FAD. After this treatment the specific activity of the samples incubated with FAD was found between 48 and 62 nmol min^{-1} mg^{-1} protein, while in the samples without the flavin it was between 37 and 41 nmol min^{-1} mg^{-1} protein. This indicates a 30% increase of activity due to the stabilizing presence of FAD during incubation (see "Discussion"). Subsequently, an aliquot of 1.25 mL from each sample was loaded onto the column. Each fraction was assayed for ALS activity using the standard assay. Typical elution profiles of ALS activity of samples incubated and chromatographed with or without FAD are illustrated in Figure 1A. A strong shift toward the high mol wt form (M_r = 440,000) with FAD present was observed, while in the absence

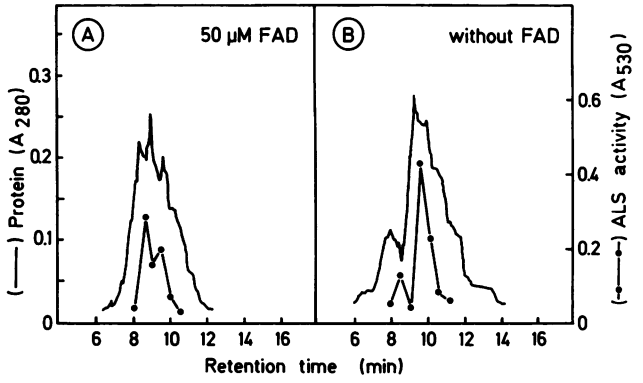

Figure 2. A, Reversibility of dissociation of large ALS oligomers. 'S'-fractions of a foregoing semipreparative gel filtration without FAD (see Fig. 1A) were pooled, concentrated and incubated in the presence of 50 μM FAD. Subsequently, this sample was loaded onto a HPLC column (Zorbax Bio-Series GF-450, 25 cm × 9.4 mm i.d.) preequilibrated with gel filtration buffer containing 50 μM FAD. B, Control run of a sample not preincubated with FAD. ALS activity is plotted as absorbance at 530 nm (Westerfeld test, standard assay).

of FAD the smaller form (M_r = 200,000) was dominant. Recovery of activity of the loaded samples was more than 90% for the run with FAD and 75% in case FAD was not added. Specific activities after gel filtration were 0.39 to 0.47 for the 'L'- and 0.58 to 0.71 μmol min^{-1} mg^{-1} protein for the 'S'-form representing a 120- to 150-fold purification relative to the first protein precipitation by ammonium sulfate or a more than 600-fold purification relative to the crude homogenate, respectively. The protein elution pattern is not shown in Figure 1.

An opposite effect could be observed in the presence of pyruvate. A 90-min preincubation was carried out with or without pyruvate (50 mM). The specific activities after this period were 46 to 52 and 47 to 56 nmol min^{-1} mg^{-1} protein for the samples incubated either in presence and in absence of pyruvate, respectively. Subsequent gel filtration led to a strong shift to the smaller form of ALS in presence of pyruvate (Fig. 1B). Without pyruvate the activity recovered was 88% and 72% in the presence of pyruvate. In both cases preincubation and gel filtration runs were carried out in presence of a 10 μM FAD. This was necessary because a substantial conversion of ALS into the smaller form due to pyruvate could only be demonstrated when both peaks of both forms showed up in the activity-elution profile (solid line, Fig. 1B). For that purpose 10 μM FAD was added, and 50 mM pyruvate shifted the 'L'-peak to the 'S'-peak. Concurrent gel runs with various concentrations of $MgCl_2$ or TPP did not show any influence of these cofactors on the apparent mol wt of ALS. A combination of valine and leucine (2 mM each) slightly enhanced the effect of pyruvate (data not shown). No effect on the activity pattern as described above could be observed by a moderate change of salt concentration (30–60 mM NaCl), or pH (6.5–8.0) of the elution buffer.

Reversibility of dissociation, *i.e.* the association of the small ALS oligomers ('S'-form) resulting in the high mol wt aggregate ('L'-form) is shown by Figure 2. Fractions containing the smaller ALS species obtained by a foregoing semipreparative gel filtration (as presented in Fig. 1A) have been pooled, concentrated and incubated for 90 min in the gel filtration buffer including 50 μM FAD. Subsequent chromatography using the analytical Zorbax column in presence of the flavin yielded most of the ALS as the large aggregate ('L'-form) with an apparent mol wt of 440,000 (Fig. 2A). Figure 2B serves as a control run and shows a chromatography of 'S'-fractions not incubated with FAD (M_r = 200,000).

Separation of the two ALS forms was possible using HPLC anion-exchange columns. Figure 3, A and B, shows the elution profiles of the 'L'- and the 'S'-fractions of a foregoing gel filtration run. In both runs, a minor peak became apparent probably due to incomplete separation by the gel filtration. Separate rechromatography of the main peak fractions of both runs yielded only one peak exhibiting the original retention times for the 'L'- and the 'S'-form, respectively.

The influence of branched-chain amino acids on ALS activity is shown in Figure 4. The degree of inhibition of both forms obtained by gel filtration was compared at various pH with 1 mM of each amino acid. For both forms highest inhibition was observed at pH 6.5. The 'S'-fraction appeared to be slightly more sensitive to feedback inhibition by valine, leucine and isoleucine than the 'L'-form. The same holds true for cooperative inhibition or synergism by a mixture of valine plus leucine or leucine plus isoleucine (data not shown).

Inhibition of ALS by various concentrations of chlorsulfuron was examined in a 30 min fixed-time assay. Again, there is little difference in degree of inhibition of both the large and

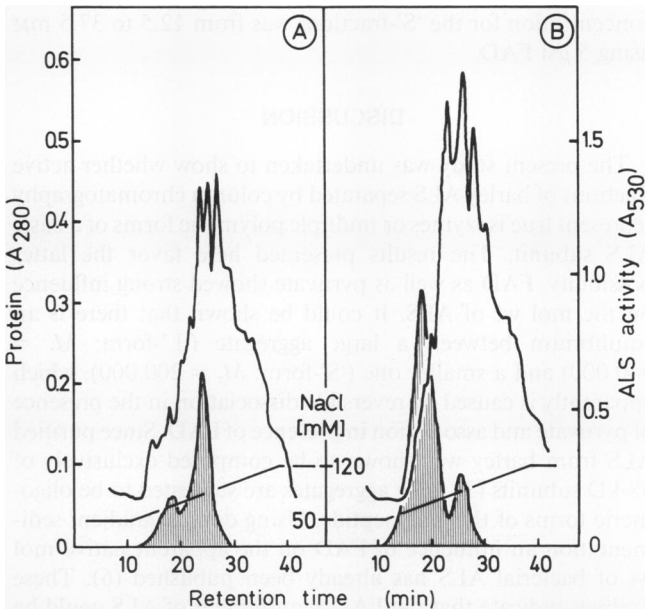

Figure 3. A, Elution profile of DEAE-HPLC runs (Bio-Gel TSK DEAE-5-PW). Chromatography of concentrated 'L'-fractions of a preparative gel filtration on Bio-Gel A-1.5 M. B, Chromatography of the 'S'-fractions of the gel filtration run. ALS activity is plotted as absorbance at 530 nm (standard assay, right ordinate) resulting in the hatched peaks. The NaCl-gradient was from 50 to 120 mM.

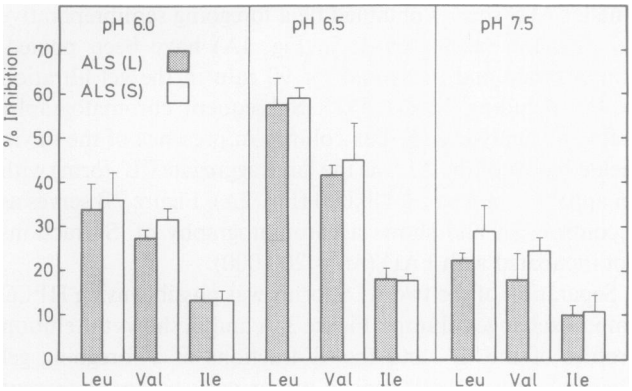

Figure 4. Percent inhibition *versus* control of the different forms of barley ALS by branched-chain amino acids at various pH. The concentration of the amino acids was 1 mM; specific activities of the controls were 0.39 to 0.47 ('L'-form) and 0.58 to 0.71 μmol acetoin min^{-1} mg^{-1} protein ('S'-form).

the small form of ALS (Fig. 5). The same holds true for inhibition by imazaquin (data not shown).

As additional characterization the pH-optimum was determined which showed little or no difference (6.5–7.0). The acetoin formed directly by the enzymic reaction was found less than 5% and the K_m for pyruvate was measured as 4.1 mM for the large and 5.4 mM for the small aggregate. Both values are based on Michaelis-Menten kinetics with saturation between 50 and 65 mM pyruvate. To avoid a possible conversion of a ALS form into the complementary form, the 'L'-fractions have been assayed between 0.5 and 10 mM pyruvate in the presence of 50 μM FAD, while the range of pyruvate concentration for the 'S'-fractions was from 12.5 to 37.5 mM using 5 μM FAD.

DISCUSSION

The present study was undertaken to show whether active fractions of barley ALS separated by column chromatography represent true isozymes or multiple polymeric forms of a basic ALS subunit. The results presented here favor the latter possibility. FAD as well as pyruvate showed strong influence on the mol wt of ALS. It could be shown that there is an equilibrium between a large aggregate ('L'-form; M_r = 440,000) and a smaller one ('S'-form; M_r = 200,000), which apparently is caused by reversible dissociation in the presence of pyruvate and association in presence of FAD. Since purified ALS from barley was shown to be composed exclusively of 58-kD subunits (3) these aggregates are suggested to be oligomeric forms of this polypeptide. Using density gradient sedimentation an influence of FAD on the apparent native mol wt of bacterial ALS has already been published (6). These findings indicate that the FAD requirement of ALS could be due to a structural role of the flavin. Obviously, FAD is involved in stabilization of the quarternary structure of the protein and does not have a redox function (15, 16). When the main part of the ALS is present as the large oligomer, the total activity (after gel filtration) is significantly higher. Stabilization of ALS and increased activity due to FAD (14) can now be explained by the conversion of ALS to a larger and more active aggregate.

A puzzling question is whether these different fractions obtained by column chromatography represent different forms of ALS present in the plant cell or are aggregates induced by protein extraction or chromatography conditions applied here. The effect of FAD may indicate that the larger form is probably the predominant species. On the other hand an equilibrium between both the large and the small oligomers in the chloroplast seems to be possible but presumably not exclusively due to FAD or pyruvate. Especially the pyruvate concentration (50 mM) necessary to manipulate the aggregation of ALS on the column is far away from physiological concentration in the chloroplast which has been reported as about 0.1 mM (17).

Various physiological parameters showed little differences between the separated forms of ALS. In contrast to findings with corn ALS (19) both forms of the enzyme exhibited sensitivity to branched-chain amino acids, although the 'S'-fraction appeared to be slightly more sensitive to feedback

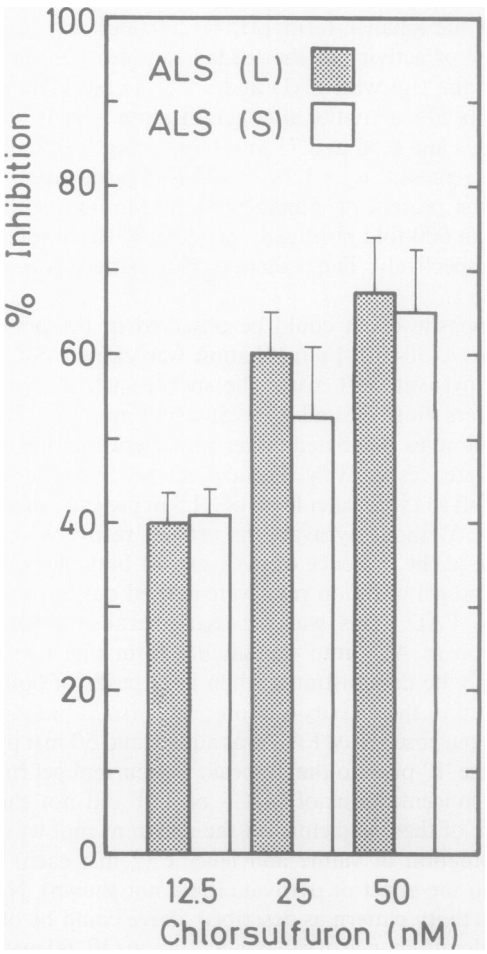

Figure 5. Inhibition of the two different forms of barley ALS by chlorsulfuron. In a 30 min assay the specific activities of the controls were 0.39 to 0.47 ('L'-form) and 0.58 to 0.71 μmol acetoin min^{-1} mg^{-1} protein ('S'-form).

inhibition (Fig. 4). Chlorsulfuron from 12.5 to 50 nM led to inhibition of the enriched ALS preparation between 40 and 70%. Again this holds true for both the 'L'- and the 'S'-fractions, although in this case the larger aggregate was found to be somewhat more sensitive. Inhibition by chlorsulfuron was stronger than reported previously with purified ALS from barley (4). Apparently this is caused by the reduction of pyruvate from 32 to 10 mM in the assay applied here.

Furthermore, little difference was observed in pH-optima between the two forms of the enzyme. For both forms there was a broad optimum between 6.5 and 7, although the larger aggregate showed a more distinctive pH-dependence (data not shown).

The K_m for pyruvate were found to be 4.1 mM ('L'-form) and 5.4 mM ('S'-form), and do not agree with the reported K_m of 1.68 for partially purified ALS from tobacco (13), and for barley ALS assayed in crude extracts (K_m = 14 mM; see ref. 10). For both the large and the small aggregates a strict Michaelis-Menten kinetics was observed. An allosteric feature, which may possibly be expected for the large oligomer, could not be shown.

Lack of difference in feedback inhibition, in affinity for pyruvate, in amount of directly formed acetoin, and in inhibition by specific inhibitors suggests the separated aggregates of ALS are not instrumental as isozymes in the plant cell but are formed by conditions during chromatography.

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