

# The oxygenase reaction of acetolactate synthase detected by chemiluminescence

Jörg Durner, Valérie Gailus\*, Peter Böger\*\*

Lehrstuhl für Physiologie und Biochemie der Pflanzen Universität Konstanz, D-78434 Konstanz, Germany

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**Abstract** In addition to the synthesis of ketolacids the enzyme acetolactate synthase shows an oxygen-consuming side reaction. Partially purified acetolactate synthase from corn (*Zea mays* L.) and barley (*Hordeum vulgare* L.) exhibits chemiluminescence in the presence of oxygen,  $Mn^{2+}$  and low concentrations of pyruvate. Light emission is inhibited by azide, but not by catalase or superoxide dismutase. The data suggest the formation of singlet oxygen during the catalytic cycle, and provides a basis for a highly sensitive assay for the oxygenase reaction of acetolactate synthase. Both synthase activity and chemiluminescence are inhibited by sulfonylurea herbicides. The results add a new aspect to the irreversible inhibition of acetolactate synthase by these herbicides which may be enhanced by the presence of reactive oxygen species.

**Key words:** Acetolactate synthase; Acetohydroxy-acid synthase; Oxygenase; Chemiluminescence; Liquid scintillation counting

## 1. Introduction

Acetolactate synthase (ALS, EC 4.1.3.18, also commonly referred to as acetohydroxy acid synthase, AHAS) catalyzes the first committed step in the biosynthesis of branched-chain amino acids [1]. The enzyme has been found in enteric bacteria, fungi and in chloroplasts of higher plants. Strong interest in the biosynthesis of branched-chain amino acids has raised with the identification of ALS as the target site of new and exceptionally potent classes of inhibitors used as modern weed control agents [2]. For valine and leucine biosynthesis, two molecules of pyruvate are condensed to form  $\alpha$ -acetolactate, while the condensation of pyruvate and  $\alpha$ -ketobutyrate leads to isoleucine biosynthesis. Both reactions are accompanied by the release of  $CO_2$  from pyruvate [3]. As was established with the ALS II isozyme from *Salmonella typhimurium*, cofactors are thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD) and di- or trivalent metal ions [4]. The various bacterial ALS isozymes and the enzyme from higher plants differ considerably with respect to feedback inhibition, subunit composition and size [5]. The need for FAD (there is neither an oxidation nor a reduction in the catalytic cycle of ALS) has been attributed to a common ancestry of pyruvate oxidase and ALS and seems to be a vestigial remnant [6,7]. In the plant enzyme FAD has a structural function and maintains the oligomeric enzyme structure [8].

Interestingly, *Escherichia coli* pyruvate oxidase (EC 1.2.2.2) has been proven to possess ALS-activity [6]. Furthermore, sequence homologies between ALS and the mechanistically related  $O_2$ -dependent pyruvate oxidase (EC 1.2.3.3) have been reported [9]. Consequently, it has been found that also ALS supports an oxygen-consuming reaction with either pyruvate,  $\alpha$ -ketobutyrate or acetolactate as substrate [10,11]. Either ALS

and other carbanion-forming enzymes like pyruvate decarboxylase (EC 4.1.1.1) catalyze a previously undetected oxygenase side reaction as has been reported for ribulosebiphosphate-decarboxylase (RuBisCO; [12]). Recently it has been shown, that the oxygenase activity of manganese-activated RuBisCO can be measured by chemiluminescence [13]. In this communication we show the detection of the oxygenase reaction of ALS by chemiluminescence with a liquid scintillation counter. The data suggest that during turnover reactive oxygen species are produced. In addition the involvement of radicals in the irreversible damage of ALS by herbicides is discussed.

## 2. Materials and methods

### 2.1. Purification of ALS from *Hordeum vulgare* and *Zea mays*

Seeds of barley (*Hordeum vulgare* L.) and corn (*Zea mays* L.) were cultivated as described [5,14]. The purification of ALS from etiolated barley shoots was slightly modified [5]. For hydrophobic-interaction chromatography the Butyl-Sepharose was replaced by Fractogel TSK Butyl-650 M. The final purification step was carried out with an Alkyl-Superose HR 5/5 column (hydrophobic interaction). The enzyme preparations had specific activities of 2.4 to 3.1  $\mu\text{mol}$  acetolactate  $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein. The corn enzyme was partially purified by ammonium sulfate precipitation, the first hydrophobic interaction chromatography (Fractogel TSK Butyl-650M) and an anion-exchange step (Fractogel TSK DEAE-650 S) as described for the barley enzyme. The specific activity was 70 to 100  $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein. ALS could be stored at 70K with negligible loss of activity.

### 2.2. Assay of ALS activity

Unless otherwise indicated, the enzyme assay (final volume 0.5 ml) contained 40 mM Na-phosphate buffer (pH 6.5), 0.1 mM Na-pyruvate, 1 mM  $MnCl_2$ , 0.5 mM TPP and 50  $\mu\text{M}$  FAD plus ALS (25  $\mu\text{g}$  of the barley enzyme, 100  $\mu\text{g}$  of the corn preparation, respectively). For the synthase reaction incubation of the samples and detection of acetoin was as described previously [5]. The reaction was stopped with 0.25 ml 3 M  $H_2SO_4$  and heated at 60°C for 10 min. Acetoin (formed by decarboxylation of the produced acetolactate) was determined by the method of Westerfeld [15]. Under these conditions 100 nmol/1.3 ml acetoin were equivalent to an absorbance of 1.55 at 530 nm. Protein was determined according to Bradford [16].

### 2.3. Chemiluminescence measurements

A Rack-Beta II liquid scintillation counter (LKB-Pharmacia) was used. The reactions were initiated by adding the enzyme preparation to the pre-mixed and tempered (25°C) assay mixture (section 2.2), which has been bubbled with air for 5 min. Chemiluminescence assays

\*Corresponding author. Fax: (49) (7531) 883042.

\*\*Present address: Arbeitsgruppe Biochemie, Fakultät für Biologie, Universität Konstanz, D-78434 Konstanz, Germany.

**Abbreviations:** ALS, acetolactate synthase; TPP, thiamine pyrophosphate; FAD, flavin adenine dinucleotide; SOD, superoxide dismutase; cpm, counts per minute.

were performed as described by Mogel and McFadden [13]. Counts were recorded manually every 2 min starting 3 min after initiation. Luminescence rates (cpm) were determined from the slope of the straight line obtained by plotting counts vs. time.

### 3. Results and discussion

Recently it had been shown that ALS II from *S. typhimurium* supports an oxygen-consuming side reaction [11]. To examine whether this holds true for the enzyme from higher plants we carried out similar experiments with highly purified ALS from barley (the specific activity was  $3.1 \mu\text{mol acetoin} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein). However, because the oxygenase activity is only 1 to 2% compared with the turnover of the synthase reaction and because of the low sensitivity of this method an amount of 25  $\mu\text{g}$  purified enzyme per assay is necessary. Using a Clark-type oxygen electrode and with 0.1 mM pyruvate as substrate, the specific oxygenase activity of ALS of could be estimated as  $75 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  (which is about 30% of the bacterial ALS II; data not shown). Considering the very low abundance of ALS in higher plants and the difficulties in purifying sufficient quantities of enzyme [5] we have been interested whether the oxygenase assay by chemiluminescence as described for Ru-BisCO [13] was applicable for ALS. Although not widely recognized oxygenase reactions are potential sources of reactive oxygen species. First evidence for activated oxygen species involved in ALS turnover was obtained by experiments on inhibition of corn ALS [17]. The oxygenase reaction of bacterial ALS II was accompanied by the formation of peracetate and hydrogen peroxide [10].

Fig. 1 illustrates the effect of various substances on the observed chemiluminescence. Superoxide dismutase had no effect on the rate of light emission. This indicates the absence of free superoxide. On the other hand, newly formed superoxide would result in the formation of hydrogen peroxide and singlet oxygen [18]. Catalase did not show any influence (data not shown).

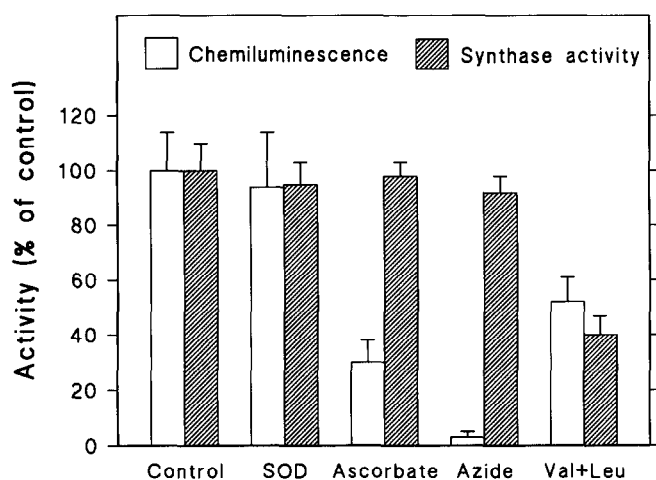


Fig. 1. Chemiluminescence and synthase activity of acetolactate synthase (ALS) in the presence of various substances. ALS from barley (25  $\mu\text{g}/\text{assay}$ ) was incubated with 0.5 mM pyruvate and the indicated substances present. Concentrations were 5 mM ascorbate, 2 mM azide, 50 units  $\text{ml}^{-1}$  SOD, 0.1 mM Val and 0.1 mM Leu. Standard deviation was from three experiments. The control rates were 1090 cpm per assay (chemiluminescence) and 120  $\text{nmol acetoin} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein (synthase activity), respectively.

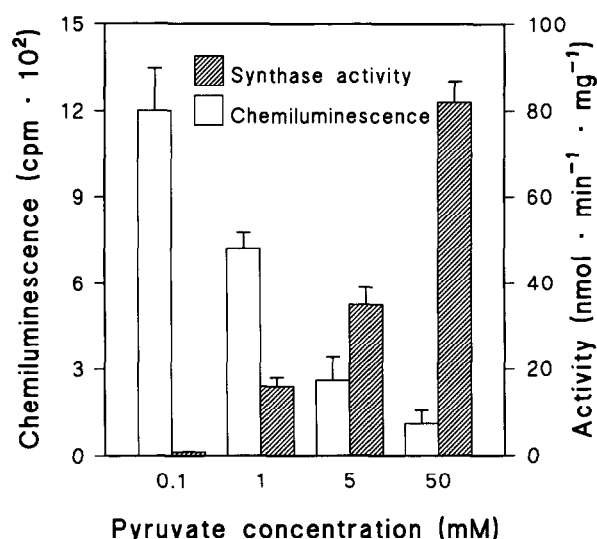


Fig. 2. Chemiluminescence and acetolactate-producing reaction of acetolactate synthase (ALS) dependent on pyruvate concentration. Corn ALS (100  $\mu\text{g}$  protein/assay) was incubated with pyruvate present as indicated. Acetolactate ( $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein) and chemiluminescence (cpm) were detected as described. Standard deviation was from three experiments.

Interestingly, the addition of ascorbate decreased the light emission by about 60%. Complete inhibition of luminescence was observed in presence of azide, a quencher of singlet oxygen [19]. As expected, the physiological inhibitors of ALS, valine and leucine, reduced both acetolactate synthesis and oxygenase activity. Complete absence of chemiluminescence was achieved after bubbling the media with argon (not shown). Although we did not determine the exact emission maximum of light (which is between 620 and 660 nm) and also the generation of carbon radicals or alkyl peroxides should be considered [20] these results suggest the formation of singlet oxygen as a result of the oxygenase reaction of ALS. If this is an inherent property of ALS, the oxygen inactivation of the enzyme from different sources [4,21] may be due to a radical reaction.

Additional experiments have been carried out with respect to the role of the flavin and metal. Unlike RuBisCO [13] and similar to the bacterial ALS both the synthase and the oxygenase activities of the plant enzyme did not show an absolute dependence on the presence of  $\text{Mn}^{2+}$  but a high degree of promiscuity with respect to other metals [11]. Both with the corn and the barley enzyme replacement of  $\text{Mn}^{2+}$  by  $\text{Mg}^{2+}$  reduced light emission only by 20%, respectively. Similar results were obtained with a flavin-stripped enzyme, whereby chemiluminescence was reduced from 950 cpm (control) to 880 cpm ( $-\text{FAD}$ ; data not shown). This implicates that the flavin is not involved in the interaction with molecular oxygen. Until now the only reported function of FAD seems to be the stabilization of the quaternary structure of the ALS-octamer [8]. Substitution of FAD with 5-deaza-FAD (a non-functional analogue) had no effect on the oxygenase activity of *S. typhimurium* [10]. On the other hand the presence of flavin drastically increases the sensitivity of the oxygen-sensitive ALS from *Methanococcus aeolicus* [21].

Fig. 2 shows the influence of pyruvate concentration on the relation between oxygenase activity as detected by chemilumi-

Table 1  
Inhibition of acetolactate synthase (ALS) from corn by chlorsulfuron

Chlorsulfuron (nM)	ALS-activity <sup>a</sup> (% of control)	Chemiluminescence <sup>b</sup> (% of control)
0	(100)	(100)
10	85	92
25	48	60
75	24	37
100	21	28

Inhibition of the synthase activity (as detected by acetolactate production) is compared with chemiluminescence of the corresponding assays.

<sup>a</sup>ALS-activity was determined according to Westerfeld (1945) after an incubation of 30 min in presence of the indicated concentrations of chlorsulfuron (comp. ref. [14]). Control activity (100%) was 70 nmol·min<sup>-1</sup>·mg<sup>-1</sup> protein.

<sup>b</sup>Chemiluminescence was measured after pre-incubation with chlorsulfuron present over a period of 5 min. The control rate was 950 cpm. Protein was 100 µg/assay.

nescence and the physiological synthesis of acetolactate (synthase reaction). Although corresponding experiments with ketobutyrate as substrate have not been carried out we assume similar results as obtained with ALS II from *S. typhimurium* [11]. Obviously, the oxygenase reaction is inhibited at high concentrations (1 mM) of the physiological substrates of ALS. This observation is consistent with the experiments on bacterial ALS II using the oxygen electrode [11]. The inhibition has been interpreted as the competition between molecular oxygen and pyruvate (or ketobutyrate, respectively) for the TPP-intermediate formed prior to the condensation reaction with the second pyruvate.

Interest in acetolactate synthase has increased by the development of exceptionally potent herbicides which have been shown to specifically inhibit ALS [2]. Table 1 shows that both the synthase and the oxygenase reactions of ALS are inhibited by chlorsulfuron, a sulfonyleurea herbicide. This finding is in agreement with a model suggesting the interference of chlorsulfuron immediately after the formation of the lactyl-TPP [22]. Otherwise a true competition of chlorsulfuron with the decarboxylated intermediate (hydroxyethyl-TPP) would block only the synthase reaction yielding acetolactate, but not the formation of the proposed peroxide-TPP intermediate with the subsequent release of peracetate [11].

The discovery of the oxygenase reaction of ALS obviously accompanied by reactive oxygen species may provide an answer for the molecular mode of action of ALS-specific inhibitors. As reported previously ALS is irreversibly affected by inhibitors [14,23]. Despite the slow dissociation of the tightly-bound inhibitor from the enzyme a permanent inactivation of the enzyme remains. This indicates that neither chlorsulfuron nor imazaquin (an imidazolinone herbicide) match the criteria of irreversible inhibitors or suicide substrates. A comparison of this situation with the radical-induced inactivation of enzymes like fructose-1,6-bisphosphate aldolase by nonphysiological oxidants such as hexacyanoferrate(III) [24] has been made [20,25]. At present, the exact chemistry of this irreversible inactivation

remains to be determined. The physiological concentration of pyruvate within the chloroplast has been estimated as about 0.1 mM [26]. This in turn implicates that there is a substantial and permanent oxygenase activity of ALS in higher plants (see Fig. 2). The presence of the herbicide should increase the likelihood of oxidative inactivation either by trapping the carbanionic intermediate in an oxygen-sensitive state as suggested most recently [20] or by a mechanism whereby the inhibitor is involved directly in the irreversible oxidative damage of ALS (comp. ref. [14]). The sensitive detection of the oxygenase reaction as presented here should allow for further experiments.

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