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EFFECT OF THIOLACTOMYCIN ON FATTY ACID SYNTHESIS IN PEAS

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Abstract—Thiolactomycin inhibited fatty acid synthesis in isolated pea chloroplasts. Using [1-^{14}C]acetate as a precursor, an I_{50} value of *ca* 20 μM was obtained for a (*R*, *S*) mixture of thiolactomycin. The antibiotic inhibited the β-ketoacyl-ACP synthase (condensing enzyme: KAS) and the acetyl-CoA:ACP (ACAT) reactions. An examination of the relative inhibition of the three condensing enzymes revealed that their order of sensitivity to thiolactomycin was KAS II > KAS I > KAS III.

INTRODUCTION

Thiolactomycin (TLM) has been shown to be an inhibitor of type II (dissociable) fatty acid synthases from plants and bacteria [1, 2]. In *Escherichia coli* the partial reactions of β-ketoacyl-acyl carrier protein (ACP) synthases (KAS) and acetyl-CoA:ACP transacylase (ACAT) were shown to be targets for inhibition by TLM [1]. However, a subsequent report [3] found *E. coli* ACAT to be insensitive to TLM.

In *E. coli* and plants, there are three separate condensing enzymes (β-ketoacyl-ACP synthases) involved in *de novo* fatty acid biosynthesis. The short chain condensing enzyme (KAS III) has been shown to catalyse the initial step [4, 5], the condensation of acetyl-CoA with malonyl-ACP, and *in vivo* may carry out more rounds of condensation, although the purified enzyme from spinach was restricted to the initial condensation only [6]. Plant condensing enzyme I (KAS I) catalyses the condensation of acyl-ACPs with malonyl-ACP, from acetoacetyl-ACP (C_4) to myristoyl-ACP (C_{14}). KAS I was originally thought to be responsible for all condensations, from the condensation of acetyl-ACP with malonyl-ACP up to a palmitoyl-ACP product, but acetyl-ACP has recently been shown to be a relatively poor substrate for fatty acid synthesis in spinach leaves [7], thus emphasizing the role of KAS III. The plant condensing enzyme II catalyses the condensation of palmitoyl-ACP with malonyl-ACP and can also use myristoyl-ACP as a substrate [8].

These three condensing enzymes have been reported to have different characteristics with regard to known inhibitors. Cerulenin inhibits KAS I completely and irreversibly at 10 μM. Cerulenin also inhibits animal fatty acid synthases (type I, multifunctional proteins) [10]. This inhibition is irreversible and the mechanism is known to be the covalent binding of the inhibitor to the cysteine in the active site of the condensing subunit of the protein. KAS II is specifically inhibited by arsenite, which inhibits enzymes with vicinal thiol groups. KAS III is insensitive to cerulenin and, indeed, the recently published protein sequences of *E. coli* [11], spinach [12] and red algal (by the translation of an ORF) [13] KAS IIIs, show that the probable active site of these enzymes has only two amino acid residues in common with the active sites of known KAS I enzymes [Siggaard-Andersen, M., personal communication], the (presumed) active site cysteine, plus one adjacent (upstream) alanine. The cysteine residue is known to bind malonyl-ACP in the animal enzyme [10] which is, of course, the common substrate of all condensing enzymes.

It has been demonstrated in both *E. coli* [2] and plants [1] that TLM inhibits fatty acid synthesis and that it inhibits KAS III *in vitro* [14, 15]. We were interested in TLM as part of a study of the characteristics of the KAS III from pea. Because the effects of TLM reported in the literature are very variable even for comparable systems [2, 14–17], our investigation was carried out to see what action TLM had on overall fatty acid synthesis in peas. In addition, we wished to analyse which of the three condensing enzymes involved in *de novo* fatty acid synthesis was most susceptible to this inhibitor.

RESULTS AND DISCUSSION

De novo fatty acid synthesis in plants is concentrated in plastids [8], so we used leaf chloroplast fractions for our initial experiments. TLM does indeed inhibit the synthesis of fatty acids by intact pea chloroplasts (Fig. 1).

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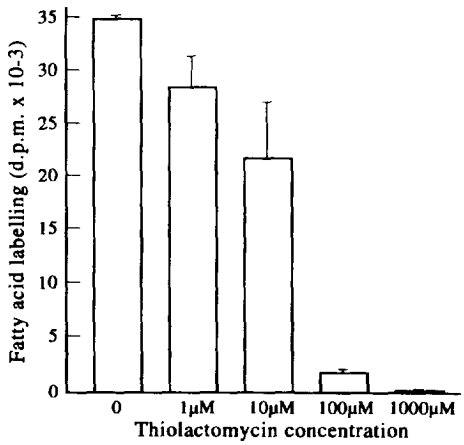

Fig. 1. Effect of concentration of thiolactomycin on fatty acid synthesis from [1-^{14}C]acetate by pea chloroplasts. Data are mean values + SEM ($n = 3$).

The I_{50} value was *ca* 20 μM, which is of the same order as the I_{50} found for spinach fatty acid synthesis *in vitro* and reported by Jaworski *et al.* [14] (i.e. 60% inhibition by 50 μM TLM). However, Yamada [17] found a much lower value (3.8 μM) in spinach chloroplasts. Part, but by no means all of this difference, may be accounted for by the use of natural TLM derived from *Nocardia* spp. by Yamada *et al.* [17], whilst we used a chemically synthesized racemic mixture. A similar situation arises for the inhibitory effect of TLM on fatty acid synthesis in *E. coli*; Jackowski *et al.* [2] reported an I_{50} value of *ca* 75 μM, whereas Hayashi *et al.* [16] reported the I_{50} to be 2 μM. It was considered by Jackow *et al.* [2] that TLM itself was not the active product in inhibiting KAS III *in vitro*, but that some breakdown product, formed after exposure to air, was the active agent in their experiments [14, 15]. However we found that pre-exposure of TLM to air after drying under N_2 made little difference to the inhibition of total fatty acid synthesis observed (Table 1), whereas Jaworski *et al.* [14] found that only air-exposed TLM produced any inhibition of KAS III *in vitro*. Nevertheless, we found that air-exposure of TLM produced a bigger change in fatty acid products (with an increase in shorter chain fatty acids) (Table 1) than for N_2-treated TLM (see later).

The other possible source of variation in the amount of inhibition observed by different workers could be a result of the differing chloroplast concentration used, i.e. the inhibitor/fatty acid synthase ratio. However, when we varied the chloroplast concentration between 50 and 400 μg chlorophyll/ml^{-1} in the incubation system the percentage inhibition by 100 μM TLM remained the same throughout this range (data not shown). Thus, the variations in I_{50} values for TLM reported in the literature do not seem to be explicable simply by the different amounts of target protein being used.

Table 1 shows the results for the effect of thiolactomycin on the pattern of fatty acid synthesis in pea chloroplasts. It is quite clear that the proportion of shorter chain fatty acids is increased, implying that KAS I activity is more susceptible to TLM than KAS III activity. This is consistent with poor inhibition of KAS III activity by TLM *in vitro* (Table 2). However, the most obvious effect of TLM on fatty acid synthesis is the almost complete absence of labelled C_{18} or C_{20} fatty acids (Table 1). Since the formation of such acids is dependent on the activity of KAS II, the results indicate that this condensing enzyme is the most sensitive to TLM (at least 95% inhibited at 100 μM).

Acetyl-CoA:ACP transacylase (ACAT) was originally thought to be an important rate-limiting partial reaction for plant fatty acid synthase [18]. However, with the discovery of KAS III, its role *in vivo* is in some doubt. Indeed, purified KAS III in both *E. coli* [4], spinach [14] and avocado [15] has been shown to have ACAT activity, as a side-reaction. TLM has been reported to inhibit acetyl-CoA:ACP transacylase (ACAT) activity and 100 μM TLM inhibited pea ACAT activity by 40% *in vitro* (Table 2). Nishida *et al.* [1] report a similar finding in *E. coli* but Lowe and Rhodes [3] found no inhibition of this enzyme activity in *E. coli*; in barley, Focke *et al.* [19] also found no inhibition of this enzyme by TLM. One reason for the differences reported for the inhibition of

Table 1. Effect of 100 μM (*R,S*) thiolactomycin on fatty acid synthesis from [1-^{14}C]acetate by pea chloroplasts

^{14}C Fatty acids	Control	TLM (N_2)	TLM (air)
Total labelling	98.9 ± 4.0	16.6 ± 0.8	9.0 ± 2.2
< 14:0	4.0 ± 0.1 (4)	1.9 ± 1.0 (11)	3.2 ± 0.1 (36)
14:0	3.9 ± 0.7 (4)	2.9 ± 0.2 (18)	2.1 ± 0.3 (23)
16:0	24.2 ± 2.6 (24)	7.6 ± 0.9 (46)	2.8 ± 0.2 (31)
18:0	5.8 ± 1.3 (6)	0.7 ± 0.3 (4)	n.d.
18:1	42.1 ± 3.8 (43)	2.5 ± 0.2 (15)	0.9 ± 0.2 (10)
20:0 + 22:0	15.1 ± 4.2 (15)	n.d.	n.d.
Others	3.8 ± 1.9 (4)	1.0 ± 1.9 (6)	n.d.

n.d. = None detected. Data are total dpm (10^{-3}) given as means ± SEM where $n = 3$.

%Total labelling of fatty acids is shown in parentheses.

Table 2. Effect of 100 μM (*R*, *S*) thiolactomycin on activity of β-ketoacyl-ACP synthase III (KAS III) and acetyl CoA:ACP transacylase (ACAT)

	KAS III		ACAT	
	Specific activity	% Inhibition	Specific activity	% Inhibition
Control	22.0 ± 1.1		4.7 ± 0.3	
TLM	16.7 ± 0.8	24	2.7 ± 0.4	43

Specific activity is in pmoles min^{-1} mg^{-1} protein. Data are presented as means ± SD where $n = 4$. Assays were performed using a 40–80% $(NH_4)_2SO_4$ cut from pea leaf soluble proteins. Final concentrations in the ACAT assay: 10 μM [^{14}C]acetyl CoA (55 mCi $mmol^{-1}$), 5 μg ACP (*E. coli*), 0.1 M Tris/HCl pH 7.5. For the KAS III assay, 1 mM NADH, 2 mM NADPH and 10 mM malonyl-CoA were added to the above ACAT assay mixture. Protein (10–20 μg) was used in a final assay volume of 50 μl. Further analysis of the reaction products was as described in ref. [14].

E. coli ACAT by TLM may be due to possible problems with the specificity of the ACAT assay. Thus, because KAS III uses acetyl-CoA and generates an acid-precipitable product, its condensing activity (which is much greater than ACAT) may contribute significantly to the latter's apparent rate, unless appropriate precautions are taken. However, our assay system takes full account of this. We conclude, therefore, that ACAT activity in peas is sensitive to TLM. According to the data in Table 2, ACAT activity is actually slightly more sensitive than KAS III. Because we have detected two separate ACAT activities in pea leaves (one being associated with the KAS III protein) [Jones, A. L., Dancer, J. E. and Harwood, J. L., unpublished results], similar to avocado [15], it is possible that the second ACAT is more sensitive to TLM than the ACAT partial reaction of the KAS III protein.

KAS III is relatively unaffected by TLM but *de novo* synthesis is quite sensitive (Tables 1 and 2). In addition, the change in fatty acids labelled from [1-^{14}C]acetate (Table 1) implies that KAS II was particularly sensitive to TLM. In order, therefore, to by-pass the initial KAS III condensation(s) and look at the relative sensitivity of KAS I and KAS II activities in an independent experiment, we used [^{14}C]laurate. This acid is too large to be used by KAS III but should be accepted by KAS I [see e.g. 20]. Initial experiments showed that exogenous laurate was not readily taken up by isolated chloroplasts under normal assay conditions (data not shown). This problem was overcome by using a leaf disc system to analyse the metabolism of its substrate. Such leaf disc systems have been used previously for many studies of fatty acid metabolism in plants [e.g. 21].

Both KAS I and KAS II are affected by TLM (Table 3). Total metabolism of laurate was reduced by 100 μM TLM to 30% of the controls. Most of the counts in fatty acids were found in palmitate in the TLM-treated tissues. No radioactivity was detected in C_{18} fatty acids, implying complete inhibition of KAS II at 100 μM TLM. These results were supported by experiments using stromal extracts and [^{14}C]malonyl-CoA which gave similar results (data not shown). Yamada *et al.* [17] also found indications in oats and spinach that KAS II was inhibited to a greater extent than KAS I. The lack of metabolism of [1-^{14}C]laurate in these experiments is not due to poor uptake of the precursor, since [1-^{14}C]laurate was detected in high amounts in TLM-treated discs. The results agree well with the data in Table 1 for isolated chloroplasts and [1-^{14}C]acetate substrate, in that KAS II activity is clearly much more sensitive to TLM than KAS I.

Table 3. Effect of 100 μM (*R*, *S*) thiolactomycin on metabolism of [^{14}C]laurate by pea leaf discs

Fatty acid product	Control	+ TLM
	% Total ^{14}C fatty acids	
14:0	2.9 ± 2.5	22.5 ± 2.6
16:0	51.6 ± 5.5	67.2 ± 12.6
16:1	2.8 ± 0.8	10.3 ± 5.3
18:0	8.1 ± 0.7	n.d.
18:1	22.1 ± 5.3	n.d.
18:2	12.5 ± 3.5	n.d.

Total metabolism of [1-^{14}C]laurate in the presence of thiolactomycin was 29.6 ± 3.8% of the control.

It seems clear from our experiments that TLM is an inhibitor of *de novo* fatty acid synthesis in peas as previously reported for other tissues [1, 2, 14–17]. It also inhibits ACAT activity. The different condensing enzymes have different susceptibilities to this inhibitor in pea, KAS II being the most sensitive and KAS III the least sensitive.

EXPERIMENTAL

Pea seeds (*Pisum sativum*, cv. Onward) were obtained from Nutting (Leicester, U.K.) and grown in vermiculite at 20° in a 12 hr light/dark cycle. Leaves were harvested 14 days after sowing. Intact chloroplasts were isolated using the method of ref. [22] as modified in ref. [21]. Once isolated, chloroplasts were kept in the dark on ice until required but were used as soon as possible. Chlorophyll was measured by the method of ref. [23]. Thiolactomycin was a generous gift from AgrEvo U.K. Ltd. (Saffron Walden).

Chloroplast incubations. Chloroplasts were preincubated for 10 min in the light at 25° with TLM that had been added to the tubes in MeOH and dried down under N_2 in amounts as indicated for individual expts. Concs given are the conc of the racemic mixt.; effective concns of active ingredient will be half that indicated. [1-^{14}C]Acetate (Amersham) was added to incubations at 1μCi ml^{-1} and incubation continued for 1 hr. Incubations were stopped by addition of 100 μl of 60% ap. KOH ml^{-1} and heated at 70° for 30 min in Teflon-capped tubes. Fatty acid extraction and analysis was then carried out as described in ref. [21]. Results were analysed using RAMONA Rachel software (LabLogic, Sheffield).

Leaf disc incubations. Leaf discs were cut from 14 day-old pea leaves with a no. 6 cork borer and floated adaxial side uppermost in H_2O until used. Up to 5 discs were added to 5 ml of incubation medium in a 25 ml conical flask. TLM was dried down in the flasks before the water was added (see above), the pea leaf discs added, adaxial side uppermost and then 1 μCi [1-^{14}C]laurate was added to each flask. Flasks were vacuum infiltrated for 10 min before being incubated in the light at room temp. for 6 hr. Discs were then washed with several changes of H_2O, added to 1 ml of *iso*PrOH in a Teflon-capped tube and heated to 70° for 30 min. Lipids were extracted using the method of ref. [24] as modified in ref. [25]. Fatty acid Me esters were produced and analysed as described above.

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