Inhibition of the Plastidic Pyruvate Dehydrogenase Complex in Isolated Plastids of Oat

Andrea Golz and Hartmut K. Lichtenthaler

Botanisches Institut (Pflanzenphysiologie und Pflanzenbiochemie), Universität Karlsruhe, Kaiserstraße 12, D-76128 Karlsruhe, Bundesrepublik Deutschland

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The activity of the plastidic pyruvate dehydrogenase complex (pPDHC) is one source of acetyl-CoA in plastids of higher plants needed for *de novo* fatty acid biosynthesis. This plastidic enzyme reaction is specifically inhibited by acetylmethylphosphinate (AMPI), a compound which had hitherto been known only as an inhibitor of the mitochondrial pyruvate dehydrogenase complex (mPDHC). In the test system of isolated intact oat plastids *(Avena sativa)* [2-^{14}C]pyruvate was used for *de novo* fatty acid biosynthesis. The incorporation of label from [2-^{14}C]pyruvate in fatty acids was inhibited by AMPI in a dose-dependent manner. The inhibition rose with increasing preincubation time of plastids with the inhibitor. I_{50} values for the inhibition of *de novo* fatty acid biosynthesis from [2-^{14}C]pyruvate by AMPI for isolated etioplasts and chloroplasts were 4.5 and 80 μM, respectively. The activity of the pPDHC decreased during greening of oat seedlings, as is seen from the decreasing incorporation of [2-^{14}C]pyruvate into fatty acids during the light-induced transformation of etioplasts into chloroplasts. In contrast to the decreasing pPDHC activity, the activity of the plastidic acetyl-CoA synthetase (ACS), which transfers acetate to acetyl-CoA, rose parallel to the transformation of etioplasts into chloroplasts. During the assay time of 20 min we could not detect an incorporation of radiolabel from pyruvate or acetate into β-carotene or any other carotenoid.

Introduction

In higher plants *de novo* fatty acid biosynthesis is located in the plastid compartment (Ohlrogge *et al.,* 1985) (see Fig. 1). Starting from the C2 unit acetyl-CoA, fatty acids of a chain length of up to C16 and C18 are formed. In plastids acetyl-CoA can be synthesized by the action of the enzyme acetyl-CoA synthetase (ACS), which forms acetyl-CoA from acetate, ATP and CoA by releasing acetyl-CoA, AMP and pyrophosphate. The ACS is exclusively located in the plastids of higher plants (Kuhn *et al.,* 1981). The plant ACS enzyme (EC 6.2.1.1) has been purified from spinach leaves (Zeiher and Randall, 1991) and etiolated radish seedlings (Golz and Lichtenthaler, 1993 a):

$$\text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow{\text{ACS}} \text{acetyl-CoA} + \text{AMP} + \text{PP}_i.$$

Besides the ACS there is another important acetyl-CoA-producing enzyme system present in plastids, the pyruvate dehydrogenase complex (pPDHC), which was first described in 1975 as being present in proplastids of *Ricinus communis* (Reid *et al.,* 1975). Since that time pPDHC activity has been found in various different plant species, for example chloroplasts of pea and maize (Williams and Randall, 1979; Treede and Heise, 1986) and other plants (Golz and Lichtenthaler, 1993 b), as well as in non-green plastids of cauliflower (Journet and Douce, 1985). The pPDHC is a large thiamine pyrophosphate-dependent multienzyme complex, consisting of three enzyme activities (pyruvate dehydrogenase EC 1.2.4.1, lipoate transacetylase EC 2.3.1.12 and dihydrolipoamide dehydrogenase EC 1.6.4.3), which catalyzes the overall reaction:

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \xrightarrow{\text{pPDHC}} \text{acetyl-CoA} + \text{NADH+H}^+ + \text{CO}_2.$$

Today there is still discussion about which enzyme supplies the major part of acetyl-CoA for

Abbreviations: ACC, acetyl-CoA carboxylase; ACS, acetyl-CoA synthetase; AMPI, acetylmethylphosphinate; ethyl-AMP, ethyl-adenylate; FAS, fatty acid synthetase; mPDHC and pPDHC, mitochondrial and plastidic pyruvate dehydrogenase complex; x+c, total carotenoids (xanthophylls and carotenes).

Reprint requests to Prof. Dr. H. K. Lichtenthaler.
Telefax: (0721) 6084784.

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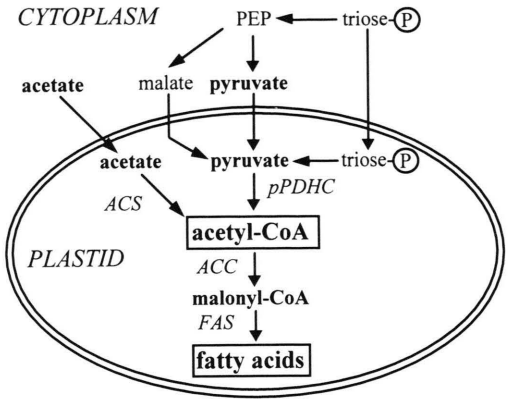
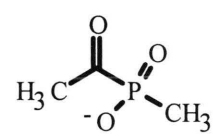


Fig. 1. Acetyl-CoA formation and *de novo* fatty acid biosynthesis in higher plants including the enzymes acetyl-CoA synthetase (ACS), plastidic pyruvate dehydrogenase complex (pPDHC), acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS).

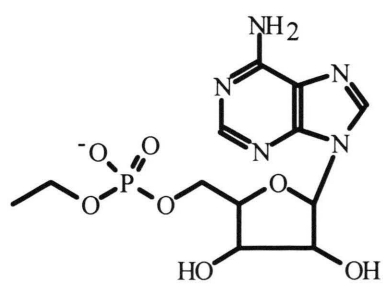
the different acetyl-CoA-consuming pathways within the chloroplasts: the ACS or the pPDHC (Fig. 1). The acetyl-CoA source for *de novo* fatty acid biosynthesis depends on plant species (Liedvogel and Bäuerle, 1986; Springer and Heise, 1989), development of plastids (Heintze *et al.*, 1990; Golz and Lichtenthaler, 1993b) and on plastidic pyruvate and acetate levels (Treede *et al.*, 1986). Acetate enters the plastid by diffusion (Jacobson and Stumpf, 1972). Pyruvate can either be formed in the plastids (Hoppe *et al.*, 1993; Preiss *et al.*, 1993) or comes from glycolysis (Stitt and ap Rees, 1979; Givan, 1983). The mechanism of the pyruvate uptake in plastids seems to be a carrier-mediated process (Flügge *et al.*, 1985).

Isolated chloroplasts and etioplasts are capable of incorporating exogenously applied [^{14}C]acetate as well as [2-^{14}C]pyruvate (Smirnov, 1960; Stumpf and James, 1963; Millerd and Bonner, 1954; Mudd and McManus, 1962) into the total fatty acid fraction and are therefore a suitable test system for the investigation of the total sequence of *de novo* fatty acid biosynthesis (Fig. 1) and of particular inhibitors of this pathway (Kobek *et al.*, 1988; Kobek and Lichtenthaler, 1989). In our studies on the regulation of the plant's *de novo* fatty acid biosynthesis we looked for specific inhibitors of the two main acetyl-CoA-providing enzyme systems in chloroplasts, the ACS and the pPDHC. Knowledge and use of inhibitors of both enzymes will prove to be very helpful in the understanding of the regulation of the plastidic acetyl-CoA formation. Ethyl-adenylate (ethyl-AMP), which mimics the tightly enzyme-bound acetyl-AMP as the intermediate during the ACS reaction (Frenkel and Kitchens, 1977; Golz and Lichtenthaler, 1992), is a specific and potent inhibitor of the plant ACS and of *de novo* fatty acid biosynthesis in isolated plastids starting from [^{14}C]acetate (Golz and Lichtenthaler, 1992) (Fig. 2). In contrast, no specific inhibitor had hitherto been described for the plastidic PDHC.

Acetylphosphinates had been detected in 1987 as inhibitors of the mPDHC by two independent working groups (Baillie *et al.*, 1986; Laber and Amrhein, 1987) (Fig. 2). In one case the aim was to find an inhibitor of mPDHC which would possibly allow the development of new herbicides (Baillie *et al.*, 1986). In the other case the aim was the investigation of 1-aminoethylphosphinate as an inhibitor of the synthesis of anthocyanes; its *in vitro* transamination product acetylphosphinate, however, proved to be a strong inhibitor of the mitochondrial PDHC (Laber and Amrhein, 1987). Acetylphosphinates are inhibitors of the first, thiamine pyrophosphate-dependent step of the PDHC reaction. The mechanism of the inhibition is similar to that of acetylphosphonate, which is a structural analogue to acetylphosphinates (Kluger and Pike, 1977). In contrast to acetylphosphonate,



acetylmethylphosphinate



ethyl-adenylate

Fig. 2. Chemical structure of the mPDHC inhibitor acetylmethylphosphinate (AMPI) and the ACS inhibitor ethyl-adenylate (ethyl-AMP).

which is a reversible non-competitive inhibitor of mPDHC, the inhibition by acetyl-phosphinates is irreversible and time-dependent (Baillie *et al.*, 1986; Laber and Amrhein, 1987; Schönbrunn-Hanebeck *et al.*, 1990). Both classes of inhibitors are analogues of the PDHC substrate pyruvate.

In order to verify whether acetylphosphinates not only block the mitochondrial but also the plastidic PDHC, we tested the effect of acetylmethylphosphinate (AMPI) on the incorporation of [2-^{14}C]pyruvate into the total fatty acid fraction of isolated etioplasts, etio-chloroplasts and chloroplasts from oat seedlings. In addition, we compared the [2-^{14}C]pyruvate incorporation into the total fatty acid fraction with that of [^{14}C]acetate.

Materials and Methods

Oat seedlings (*Avena sativa* L. var. Flämmingsnova) were cultivated and intact plastids (etioplasts, etio-chloroplasts and chloroplasts) were isolated in a sorbitol-containing phosphate buffer pH 8 as previously described (Kobek *et al.*, 1988; Kobek and Lichtenthaler, 1989). The isolated plastids were resuspended in cold isoosmotic isolation buffer pH 8 (according to Hawke *et al.*, 1974) and centrifuged for 90 s at 1500×*g* (isolated chloroplasts) or 3000×*g* (isolated etioplasts) and filtered through two layers of miracloth to remove contaminating cell debris.

The incubation of the chloroplasts with [2-^{14}C]-pyruvate or [1-^{14}C]acetate was carried out for 20 min in an isoosmotic buffer system containing 300 mM sorbitol, 50 mM tricine, 50 mM phosphate, pH 7.9, 5 mM $NaHCO_3$, 2.5 mM DTE, 2 mM ATP, 0.5 mM CoA, 0.5 mM $MgCl_2$, 50 μM [^{14}C]acetate (1 μCi) or 50 μM [^{14}C]pyruvate (1 μCi), 0.2 mM NADH, 0.2 mM NADPH and 0.3 mM thiamine pyrophosphate (cocarboxylase). Chloroplasts with a chlorophyll content of *ca.* 100 μg $(a+b)\cdot ml^{-1}$ were added. Light intensity during the incubation was 1400 $\mu mol\cdot m^{-2}\cdot s^{-1}$. Incubation of etioplasts (total carotenoids *ca.* 1 μg $(x+c)\cdot ml^{-1}$) was performed in the same buffer pH 8 and the same cofactor concentrations as in the case of isolated chloroplasts, with the exception of the addition of 4 mM ATP.

Saponification, acidification and extraction of total fatty acids as well as the measurement of the incorporated radioactivity have been described before (Kobek *et al.*, 1988). During the short assay period of 20 min the radioactivity was only found in the total fatty acid fraction, as was proved by TLC (Silica gel 60 (Merck); solvent: petrol ether/diethylether/acetic acid: 90/20/1) (Malins and Mangold, 1960).

We did not detect a significant incorporation of radiolabel from [^{14}C]acetate or pyruvate into the β-carotene fraction or other carotenoids under these conditions.

The I_{50} values were determined by a double reciprocal plot in which the logarithm of the percentage of inhibition (P) per percentage of control (ln (P/100 – P)) is plotted *versus* the logarithmic inhibitor concentration (log *I*). The regression of this plot is linear. At 50% inhibition, where ln (P/100 – P) = 0, the log I_{50} is obtained.

Results and Discussion

Acetylmethylphosphinate (AMPI) inhibited the incorporation of [^{14}C]pyruvate into the total fatty acid fraction of isolated oat chloroplasts as well as of isolated oat etioplasts. The inhibition was dose-dependent in both cases and increased during preincubation of the plastids with the inhibitor (Fig. 3 and 4). The I_{50} values of the 50% inhibition of [2-^{14}C]pyruvate incorporation into fatty acids by AMPI, as determined with isolated etioplasts, were 10 μM without preincubation and 4.5 μM after a 15 min preincubation time of plastids with the inhibitor (Fig. 3). The I_{50} values determined for oat chloroplasts were, however, much higher: 130 μM without preincubation and 80 μM after 15 min preincubation of the chloroplasts with AMPI (Fig. 3). The I_{50} values obtained for etioplasts are similar to those obtained for acetylmethylphosphinate in the case of the mitochondrial PDHC of pea, where an I_{50} value of 10 μM after a 10 min preincubation had been determined (Baillie *et al.*, 1986). This is similar to the value of 4.5 μM after 15 min of preincubation presented here for the inhibition of fatty acid biosynthesis in isolated etioplasts of oat.

These results described here demonstrate that both the plastidic and the mitochondrial PDHC are sensitive to acetylmethylphosphinate. The inhibition seems to be specific for the mPDHC and pPDHC. The incorporation of [^{14}C]acetate into the total fatty acids by etioplasts or chloroplasts

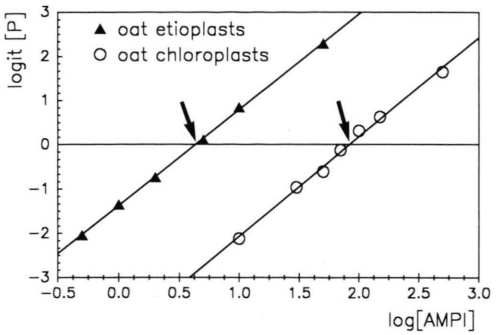


Fig. 3. Double reciprocal plot of the dose-dependent inhibition of the incorporation of [2-^{14}C]pyruvate into the total fatty acid fraction of isolated oat etioplasts and chloroplasts by acetylmethylphosphinate (AMPI). Time of preincubation of plastids with the inhibitor: 15 min. Time of incubation with the substrate: 20 min. The logarithm of the I_{50} values is indicated by arrows. Logit P = ln (% inhibition/% of control). Activity of the control etioplasts is given per mg total carotenoids (x+c): 1054 nmol [^{14}C]pyruvate·h^{-1}·(mg x+c)$^{-1}$ and of control chloroplasts per mg total chlorophyll (a+b): 21.7 nmol [^{14}C]pyruvate·h^{-1}·(mg a+b)$^{-1}$.

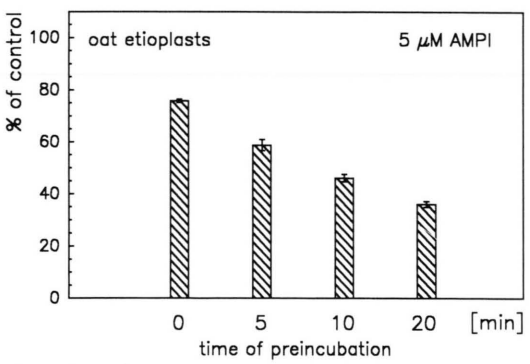


Fig. 4. Influence of the time of preincubation of oat etioplasts with 5 μM acetylmethylphosphinate (AMPI) on the inhibition of the [2-^{14}C]pyruvate incorporation into the total fatty acid fraction. Activity of the control etioplasts per mg total carotenoids (x+c): 968 nmol [^{14}C]pyruvate·h^{-1}·(mg x+c)$^{-1}$.

was not inhibited by AMPI, demonstrating that neither the enzymes ACS or the ACC nor the enzyme activities of the fatty acid synthetase (FAS) were affected by AMPI (*cf.* Fig. 1).

During greening of the oat seedlings and the formation of etio-chloroplasts and chloroplasts the activity of the pPDHC declined, as was seen by a considerable decrease of the incorporation of [^{14}C]pyruvate into the fatty acid fraction of the controls (Fig. 5). The reason for this decline in pPDHC activity is not clear but must be seen in context to the increase in the ACS activity during greening (see below). At the same time the [^{14}C]pyruvate incorporation into fatty acids by a 10 μM AMPI concentration decreased from 75.3% in etioplasts to only 25% in isolated chloroplasts (Fig. 5). One may assume that the lower amounts of pPDHC activity, which still can be detected in chloroplasts after the completion of the thylakoid biogenesis phase, consists of aged and partially degraded pPDHC, which still can bind the inhibitor AMPI, but is no longer physiologically active. One could also assume that in chloroplasts an isoform of the pPDHC complex shows up, which is less sensitive to the inhibitor AMPI. Further experiments with the isolated pPDHC complex to clarify this point are a matter of current research.

A comparison of the incorporation of [^{14}C]-pyruvate relative to that of [^{14}C]acetate into the fatty acid fraction of plastids revealed that isolated etioplasts incorporated a 9.5-fold higher amount of pyruvate than acetate into total fatty acids, whereas in isolated chloroplasts the use of pyruvate for *de novo* fatty acid biosynthesis was much lower than that of acetate (Fig. 6). This indicates that chloroplasts prefer acetate for *de novo* fatty acid biosynthesis, whereas etioplasts make a much better use of pyruvate than acetate. One physiological reason for this change in substrate use for *de novo* fatty acid biosynthesis may lie in the fact that etioplasts, which have no energy or reduction equivalents from photosynthetic reactions, need the NAD(P)H, which is formed besides acetyl-CoA by the activity of the pPDHC, for the

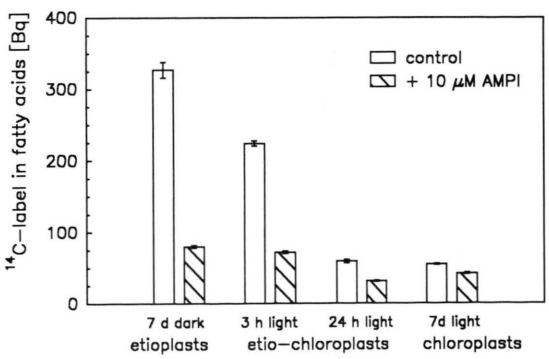


Fig. 5. Influence of the developmental and differentiation stage of oat plastids on the inhibitory potency of 10 μM acetylmethylphosphinate (AMPI) on the [2-^{14}C]-pyruvate incorporation into the total fatty acid fraction of isolated plastids.

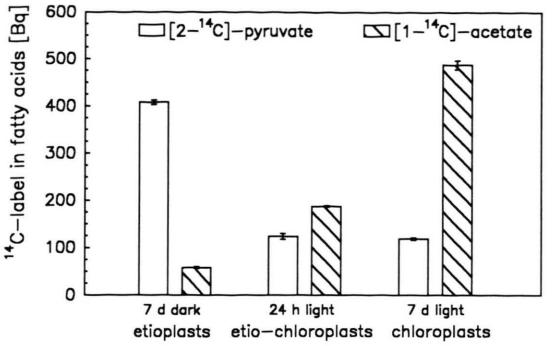


Fig. 6. Influence of the developmental and greening stage of oat plastids (etioplasts, etio-chloroplasts and chloroplasts) on the incorporation of [2-^{14}C]pyruvate and [1-^{14}C]acetate into the total fatty acid fraction of isolated plastids.

de novo fatty acid biosynthesis. In chloroplasts, however, the NADPH and ATP for *de novo* fatty acid biosynthesis are provided by the photosynthetic light reaction. The results also indicate that main pPDHC activity was found before and during the formation of the photosynthetically active thylakoids, which contain high amounts of diacyl lipids such as mono- and digalactosyldiglycerides and certain phospholipids (Lichtenthaler and Park, 1963), the formation of which requires a highly efficient fatty acid biosynthesis.

In solution pyruvate is not completely stable (von Korff, 1969) and the radiolabel of [2-^{14}C]-pyruvate yields small amounts of [2-^{14}C]acetate. These will also be used for *de novo* fatty acid biosynthesis *via* the enzyme ACS. In our [2-^{14}C]pyruvate solution only 2 to 3% of the radiolabel was present in the form of [^{14}C]acetate as determined *via* a yeast ACS test system. These low amounts did not, however, affect our results on the inhibition of [2-^{14}C]pyruvate incorporation into total fatty acids by the pPDHC inhibitor AMPI, as was proved by studying the incorporation of [^{14}C]pyruvate into the fatty acid fraction in the presence of 50 μM ethyl-AMP, a specific inhibitor of plastidic ACS (Golz and Lichtenthaler, 1992). Furthermore, in the case of oat etioplasts the inhibition of ^{14}C-label from pyruvate into fatty acids by AMPI did not increase upon addition of the ACS inhibitor ethyl-AMP.

In intact plant tissue the acetate for *de novo* fatty acid biosynthesis in plastids (chloroplasts) comes from mitochondrial acetyl-CoA which is hydrolyzed by the mitochondrial enzyme acetyl-CoA hydrolase (Murphy and Stumpf, 1981; Liedvogel and Stumpf, 1982). Acetate passes biomembranes very easily, whereas acetyl-CoA cannot enter the plastid (Brooks and Stumpf, 1966; Jacobson and Stumpf, 1972). Theoretically one could consider that a contamination of the isolated plastid fraction by mitochondria and the mitochondrial PDHC could transfer the applied [2-^{14}C]pyruvate to [2-^{14}C]acetyl-CoA, which after hydrolysis to [2-^{14}C]acetate could enter the plastid by diffusion and be used in *de novo* fatty acid biosynthesis. If this would occur the inhibitor ethyl-AMP should inhibit the incorporation of [2-^{14}C]-pyruvate into fatty acids in isolated etioplasts. That ethyl-AMP had no effect demonstrated that the very low I_{50} value found for AMPI (5.5 μM) in isolated oat etioplasts was obtained without a contamination of the plastid preparation with mitochondria.

In the case of isolated oat chloroplasts, the addition of the ACS inhibitor ethyl-adenylate led, however, to a small decrease (*ca.* 10%) in the inhibition by AMPI of the [^{14}C]pyruvate incorporation into the total fatty acid fraction. This may be due to the fact that in isolated oat chloroplasts [^{14}C]acetate is incorporated into fatty acids (*via* ACS) in a 3-fold higher rate than [^{14}C]pyruvate (*via* pPDHC). As a consequence a small contamination by [^{14}C]acetate of the [^{14}C]pyruvate of 2 to 3% should be detectable in the case of chloroplasts. We assume that also the isolated chloroplasts, which had been washed twice with the isolation buffer, were not contaminated by mitochondria or mPDHC.

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