

Fatty acid synthesis by isolated leucoplasts from developing *Brassica* seeds: Role of nucleoside triphosphates and DHAP-shuttle as the source of energy

RANJAN GUPTA and RANDHIR SINGH*

Plant Biochemistry and Molecular Biology Laboratory, Department of Chemistry and Biochemistry, CCS Haryana Agricultural University, Hisar 125004, India

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Abstract. Fatty acid synthesis in leucoplasts isolated from developing seeds of *Brassica campestris* was absolutely dependent on external source of ATP. None of the other nucleoside triphosphates could replace ATP in the reaction mixture. Use of ADP alone also resulted in reduced rates of fatty acid synthesis. However, in combination with inorganic phosphate or inorganic pyrophosphate, it improved the rate of fatty acid synthesis, giving up to 50% of the ATP-control activity. Inorganic phosphate or inorganic pyrophosphate alone again did not serve as an energy source for fatty acid synthesis. AMP, alongwith inorganic pyrophosphate could promote fatty acid synthesis to up to 42% of the activity obtained with ATP. The three components dihydroxy acetone phosphate, oxaloacetic acid, inorganic phosphate of dihydroxy acetone phosphate-shuttle together could restore 50% of the activity obtained with ATP. Omission of any one of the components of this shuttle drastically reduced the rate of fatty acid synthesis to 15–24% of the ATP-control activity. Inclusion of ATP in reaction mixtures containing shuttle components enhanced the rate of synthesis over control. The optimum ratio of shuttle components dihydroxy acetone phosphate, oxaloacetic acid, inorganic phosphate determined was 1:1:2. Maximum rates of fatty acid synthesis were obtained when dihydroxy acetate phosphate was used as the shuttle triose. Glyceraldehyde-3-P, 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate as shuttle trioses were around 35–60% as effective as dihydroxy acetone phosphate in promoting fatty acid synthesis. The results presented here indicate that although the isolated leucoplasts readily utilize exogenously supplied ATP for fatty acid synthesis, intraplastidic ATP could also arise from dihydroxy acetone phosphate shuttle components or other appropriate metabolites.

Keywords. *Brassica*; leucoplasts; dihydroxy acetone phosphate-shuttle; nucleoside-triphosphates; fatty-acid biosynthesis.

1. Introduction

Adenosine triphosphate (ATP) and reduced nucleotides (NADPH and NADH) are essential requirements for fatty acid biosynthesis from acetate in all plastids. While ATP is required for the synthesis of both acetyl-coenzyme A (CoA) and malonyl-CoA by acetyl CoA synthetase and acetyl CoA carboxylase, respectively, reduced nucleotides are needed for the reduction reactions, catalyzed by β -ketoacyl-acyl carrier protein

*Corresponding author (Fax, 0091 01662 73552).

Abbreviations used: CoA, Coenzyme A; ACP, acyl carrier protein; DHAP, dihydroxy acetone Phosphate; PGA, phosphoglycerate; PEP, phosphoenolpyruvate; UTP undine triphosphate; GTP, guanorine triphosphate; Pi, inorganic phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$); PPI, inorganic pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$).

(ACP) reductase and 2-enoyl-ACP reductase, and for the desaturation of stearyl-ACP (Stumpf 1984). In chloroplasts, these cofactors (ATP and reduced nucleotides) are generated during the light reactions of photosynthesis and also produced during glycolytic metabolism, whereas in non-photosynthetic plastids, these are thought to be derived from the reactions of glycolysis and oxidative pentose phosphate pathway (Kleppinger-Sparace *et al* 1992; Emes and Tobin 1993; Qi *et al* 1994). However, a number of studies with non-photosynthetic plastids from different sources have shown that the activities of one or more of the enzymes of glycolytic or pentose phosphate metabolism are either absent or very low (Simcox *et al* 1977; Frehner *et al* 1990; Borchert *et al* 1993; Trimming and Emes 1993), preventing efficient carbon flow completely through these pathways. In view of this, non-photosynthetic plastids must interact with the extraplastidic component to maximize their rates of carbon metabolism by these pathways. One of the possibility as indicated in pea root plastids, could be the operation of dihydroxy acetone phosphate (DHAP)-shuttle in these organelles, supporting fatty acid biosynthesis. In addition to the phosphate translocator, the shuttle requires plastidic glycolytic carbon flow from DHAP to 3-phosphoglycerate (PGA) via NADP-glyceraldehyde 3-P-dehydrogenase and 3-PGA kinase, producing intraplastidic ATP. Besides, the shuttle also needs oxaloacetate, the dicarboxylate translocator and malate dehydrogenase, regenerating NADP for the dehydrogenase reaction. In a recent report, Kleppinger-Sparace *et al* (1992) have shown DHAP shuttle to promote up to 40% of the ATP-control activity for fatty acid biosynthesis which was improved to the level of the ATP control when supplemented with ADP. Similarly, castor bean leucoplasts (Boyle *et al* 1990) and daffodil chromoplasts (Kleinig and Liedvogel 1980) could metabolize various glycolytic intermediates such as phosphoenolpyruvate (PEP) and 2-PGA etc., to produce sufficient intraplastidic ATP. We have recently developed an *in vitro* system with leucoplasts from developing *Brassica* seeds and fully characterized it in terms of various factors influencing the rate of fatty acid synthesis from Na (1 - ^{14}C) acetate (Gupta and Singh 1996). Here, we assess the capacity of internal ATP - generating systems of these leucoplasts to support fatty acid synthesis from Na (1 - ^{14}C) acetate.

2. Materials and methods

2.1 Plant material

The seeds of *Brassica campestris* L., var. toria were procured from the Department of Plant Breeding of the University. The crop was raised in pots filled with farm soil following recommended agronomic practices during rabi season of 1995. Fully opened flowers were tagged on the day of anthesis. The pods (siliqua) formed from such flowers were sampled on day 20 after anthesis and seeds separated for further use.

2.2 Chemicals

All the biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo, USA. Na [1 - ^{14}C] acetate (sp. activity-11.8 mCi/mmol) was obtained from Bhabha Atomic Research Centre, Bombay. All other chemicals used were of analytical grade.

2.3 Isolation of leucoplasts

Leucoplasts were isolated by the method of Smith *et al* (1992). Ten g seeds were hand homogenized in 20 ml of 50 mM Hepes-NaOH (pH 7.5) containing 0.4 M sorbitol, 0.4mM EDTA, 1 mM MgCl₂, 1 mM DTT, 1% BS A and 1% Ficoll. The homogenate was filtered through four layers of cheese cloth and centrifuged at 500 g for 5 min. The resulting supernatant was centrifuged at 6000 g for 10 min. The pellet was resuspended in 5 ml homogenizing buffer and layered onto a discontinuous percoll gradient. The gradient consisting of 5 ml of 80% (w/v), 7.5 ml of 35% , 7.5 ml of 22 % and 7.5 ml of 10% PBF-percoll [containing PEG-4000 (3 % w/v), BSA (1% w/v) and Ficoll (1% w/v)], all in 50mM Hepes-KOH (pH 7.5) containing 0.4 M sorbitol, 2mM EDTA and 1 mM MgCl₂ was centrifuged in a swing-out rotor at 9200 g for 4 min. The band of leucoplasts at 22–35% percoll interface was collected and diluted with 20 volumes of resuspension buffer. The pellet obtained at 10,000 g for 10 min was dissolved in 5 ml of resuspension buffer and used as leucoplast preparation.

2.4 *In vitro* conditions for fatty acid synthesis

Fatty acid synthesis was measured by the method followed by Stahl and Sparace (1991) and Smith *et al* (1992). Leucoplasts equivalent to 80–120 µg protein were incubated in media containing 0.4 M Bis-Tris-propane buffer (pH 8.5), 0.2 M sorbitol, 8 mM MgCl₂, 8mM ATP, 4mM MnCl₂, 0.8mM Na (1 - ¹⁴C) acetate (11-14µCi/µmol), 0.6mM Co A, 20 mM NaHCO₃, 1 mM NADH and 1 mM NADPH in a final reaction volume of 0.2ml. Incubation lasted for 2 h at 25°C. The reaction was then stopped with 0.4 ml of 0.65 N KOH. The radiolabel incorporated into fatty acids was partitioned into chloroform by adding 750 µl of CHCl₃/MeOH (2:1). The CHCl₃ layer was washed three to five times with 750 µl of 0.5 M KCl, mixed with the scintillation fluid and counted using a scintillation counter (Beckman, LS 100C). All experiments were performed twice with duplicates in each experiment. ATP control treatment was carried out for each experiment. The data represents the average of four corresponding observations.

3. Results and discussion

Leucoplasts isolated from developing seeds of *B. campestris* showed absolute requirement of ATP for fatty acid synthesis under *in vitro* conditions (table 1). None of the nucleoside triphosphates tested here could replace ATP in the reaction mixture, giving only 13 and 35 % of the ATP control activity with uridine triphosphate (UTP) and guanosine triphosphate (GTP), respectively. These observations are at variance with those observed with pea root plastids where less than 3 % of the ATP control activity was obtained with other nucleoside triphosphates (Kleppinger-Sparace *et al* 1992). ADP alone or in combination with inorganic phosphate (Pi) or inorganic pyrophosphate (PPi) gave 22 and 50%, respectively of the ATP control activity. Similarly, PPi gave 42 % of the ATP control activity in combination with AMP. Addition of Pi and PPi did not have any effect as the activity obtained in these two cases was 4 and 7%, respectively. These results clearly indicate that though isolated leucoplasts from developing *Brassica* seeds require exogenous supply of ATP for fatty acid synthesis, they do possess some capacity to generate their own ATP when provided with ADP

Table 1. Effect of nucleoside phosphates and inorganic phosphate on fatty acid synthesis by isolated leucoplasts of *Brassica* seeds.

Energy source	Activity (% of control) (nmol 2 h ⁻¹ mg ⁻¹)
ATP (Control)	100 (25.35 ± 1.27)
GTP	34.75 (8.81 ± 0.19)
UTP	13.37 (3.39 ± 0.13)
ADP	21.69 (5.50 ± 0.10)
ADP + Pi	49.78 (12.62 ± 0.62)
ADP + PPi	49.90 (12.65 ± 0.57)
AMP + PPi	41.57 (10.54 ± 0.69)
PPi	6.94 (1.76 ± 0.15)
Pi	4.18 (1.06 ± 0.05)

± SE of mean of four values

Plastids equivalent to 76 µg protein were incubated under standard reaction conditions without ATP and in the presence of 8 mM of each of the nucleoside phosphates and inorganic phosphate.

through the system of adenylate kinase (Murakami and Strotmann 1978). AMP and PPi may also generate ATP through the actions of pyrophosphatase and adenylate kinase. PPi is already known to be involved in a number of energy dependent processes operating in other systems (Stankovic and Walker 1977; Edwards *et al* 1985; Hedrich and Schroeder 1989; Xu *et al* 1989; Botha and Botha 1990).

In absence of ATP, about 50% of the ATP control activity could be achieved by addition of DHAP-shuttle components [DHAP, oxaloacetic acid (OAA) and Pi] in the ratio of 1:1:2 (table 2). These observations are in agreement with the previous studies conducted with pea root plastids (Kleppinger-Sparace *et al* 1992; Qi *et al* 1994) and dark incubated chloroplasts to promote CO₂ fixation (Werdan *et al* 1975), fatty acid biosynthesis (Sauer and Heise 1983) and sulpholipid biosynthesis (Kleppinger-Sparace and Mudd 1987). Omission of any of the component from the mixture lowered the activity to the level of 15–24% of the ATP control indicating operation of the shuttle in leucoplasts of *Brassica* seeds. The DHAP-shuttle promotes ATP synthesis by phosphoglycerate kinase (Werdan *et al* 1975; Kleppinger-Sparace *et al* 1992). To achieve this, a low ratio of NAD(P)H/NAD(P) is maintained, which further favours the oxidation of glyceraldehyde-3-P dehydrogenase, ensuring relatively high levels of substrate for phosphoglycerate kinase. The finding that this enzyme is potentially the

Table 2. Effect of DHAP shuttle components on fatty acid synthesis in absence of ATP by isolated leucoplasts of *Brassica* seeds.

Treatment	Activity (nmol 2 h ⁻¹ mg ⁻¹)
ATP control	33.97 ± 1.20
+ OAA, DHAP, Pi	17.29 ± 1.60
- OAA	7.73 ± 0.07
- DHAP	4.67 ± 0.13
- Pi	6.19 ± 0.12
- OAA, - Pi	4.98 ± 0.17
+ ADP, + OAA, DHAP, Pi	24.69 ± 0.95
+ ATP, + OAA, DHAP, Pi	44.87 ± 1.25

± SE of mean of four values.

Plastids equivalent of 106 µg protein were incubated under standard reaction conditions with all or some of the DHAP shuttle components in absence of ATP. The concentration of OAA, DHAP and Pi used were 1 mM, 1 mM and 2mM, respectively.

second or third most active glycolytic enzyme of non-photosynthetic plastids (Borchert *et al* 1993; Trimming and Emes 1993) supports the opinion that in absence of both externally available ATP and complete plastidic glycolysis, phosphoglycerate kinase might serve as an important source of intraplastidic ATP in plastids. Addition of ADP to shuttle components further enhanced the rate of fatty acid synthesis, giving about 75% of the ATP-control activity. This suggests that ADP rather than the shuttle components (DHAP, OAA and Pi) may limit fatty acid biosynthesis, presumably because of the fact that ADP participates in the shuttle mechanism for generation of ATP. However, as suggested by Kleppinger-Sparace *et al* (1992) the involvement of adenylate kinase as suggested earlier and stimulatory effects of ADP on translocators of the shuttle cannot be ruled out.

On addition of shuttle components to the reaction mixture containing 8 mM ATP, the fatty acid synthesis activity further increased by about 28% compared to that obtained with ATP alone suggesting that the exogenously supplied ATP and the ATP generated through DHAP shuttle have somewhat additive effect. This is contrary to the observations made by Kleppinger-Sparace *et al* (1992) who obtained only 6% greater activity than observed with ATP alone, advocating little synergism between the effects of externally supplied ATP and the ATP generated internally by the shuttle mechanism.

To determine the optimum ratio and concentrations of the shuttle components, a number of experiments were performed where each of the shuttle components was varied individually. The optimum molar ratio of the components (DHAP: OAA: Pi) in the present case was found to be 1:1:2 (table 3) which is in agreement to the ratios obtained earlier by Qi *et al* (1994) and Werdan *et al* (1975). When this ratio was held constant and the absolute concentrations of shuttle components varied from 0.2 to 10 mM, the optimum concentrations of shuttle components were 1 mM DHAP, 1 mM OAA and 2mM Pi (figure 1). Contrary to this, Qi *et al* (1994) reported optimum concentrations of shuttle components to be 2 mM DHAP, 2 mM OAA and 4 mM Pi in

Table 3. Effect of DHAP, OAA and Pi on fatty acid synthesis at different ratios in presence of ATP by isolated leucoplasts of *Brassica* seeds.

Treatment DHAP:OAA:Pi	nmol 2 h ⁻¹ mg ⁻¹
1:1:1	38.64 ± 1.76
1:1:2	44.18 ± 0.72
1:1:3	43.70 ± 1.73
1:1:4	29.94 ± 0.62

± SE of mean of four values.

Plastids equivalent to 120 µg protein were incubated with different ratios of DHAP, OAA and Pi under standard reaction conditions.

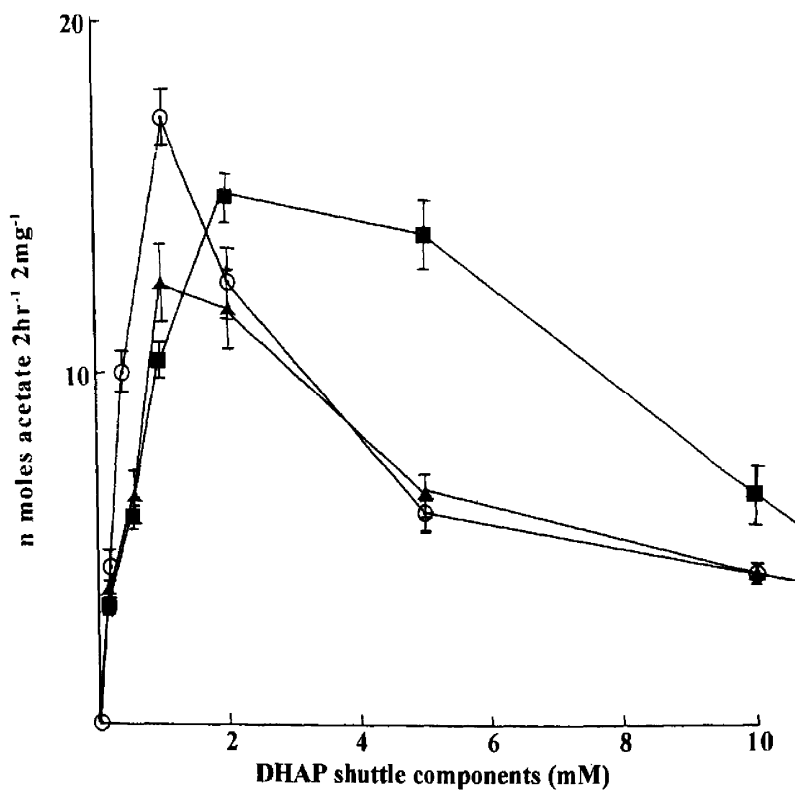


Figure 1. Effect of DHAP shuttle components at different concentrations on fatty acid synthesis in the absence of ATP by isolated leucoplasts of *Brassica* seeds. Plastids equivalent to 81 µg protein were incubated with varying concentrations of DHAP (○) or OAA (▲) or Pi (■) in the presence of the other DHAP shuttle components under standard reaction conditions.

Table 4. Effect of different triose phosphates as shuttle components on fatty acid biosynthesis by isolated leucoplasts of *Brassica* seeds.

Treatment	nmol 2 h ⁻¹ mg ⁻¹
DHAP	17.29 ± 1.60
2-PGA	7.80 ± 0.17
3-PGA	6.53 ± 0.06
PEP	10.15 ± 0.07
G3P	6.19 ± 0.22

± SE of mean of four values.

Plastids equivalent to 106 µg protein were incubated under standard reaction conditions in the presence of various shuttle trioses.

pea root plastids. The inhibitory effect of high shuttle concentrations on fatty acid synthesis may be attributed to decreased uptake of DHAP by excess of extraplastidic Pi (Flugge and Heldt 1984). Similarly, leakage of glyceraldehyde-3-P may also lead to reduced rates of fatty acid biosynthesis. Alternatively, as suggested by Emes and Tobin (1993), the phosphorylation and inhibition of regulatory enzymes of the plastid might also contribute to decreased rates of fatty acid biosynthesis.

Highest rates of fatty acid synthesis were obtained when DHAP was used as the shuttle triose (table 4). Substitution of DHAP by PEP gave only 59% of the DHAP-stimulated activity. Glyceraldehyde-3-P, 3-PGA and 2-PGA as shuttle trioses were even less effective giving only 36–45% of the DHAP-stimulated shuttle activity. The better efficiency of PEP could be explained on the basis of its efficient translocation into the plastid (Borchert *et al* 1993), where only one metabolic reaction catalyzed by pyruvate kinase is required to produce ATP needed for fatty acid biosynthesis. The other metabolites are less efficiently translocated and at the same time require a number of enzymic steps before ATP is synthesized.

The results presented here demonstrate clearly that though *in vitro* fatty acid biosynthesis in *Brassica* leucoplasts is dependent on exogenously supplied ATP, these plastids have the capacity to generate sufficient ATP through adenylate kinase and substrate level phosphorylation of ADP by phosphoglycerate kinase. These findings have gained further support from the observation of Kang and Rawsthorne (1994) who have recently shown the plastids from developing embryos of *Brassica napus* to contain the enzyme complement of complete glycolytic pathway. Thus, for maximum rates of fatty acid synthesis, the leucoplasts besides deriving a portion of their energy requirements from their own metabolisms, also rely on extraplastidic sources of ATP. However, the extent to which the two sources contribute individually remains to be determined.

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