Chloroplast Autonomy in Acetyl-Coenzyme-A-Formation and Terpenoid Biosynthesis

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Isolated intact spinach chloroplasts were supplied with ^{14}C-labeled CO_2, phosphoglycerate, phosphoenolpyruvate, acetate and mevalonate and the incorporation of radioactivity into β-carotene and plastoquinone-9 assayed. All applied substrates were capable of crossing the chloroplast envelope. Except phosphoenolpyruvate all radioactive precursors were incorporated into β-carotene and also into plastoquinone-9. It is concluded that spinach chloroplasts are autonom in acetyl-coenzyme-A-formation and terpenoid biosynthesis. There is a direct carbon flow from intermediates of the Calvin-cycle via acetyl-coenzyme-A and mevalonic acid existing in the chloroplast leading to the carotenoids and isoprenoid side-chains of chlorophylls and quinones.

Introduction

Though Goodwin established already in 1958 an extra- and intrachloroplastic site of the biosynthesis of terpenoids [1], the capability of the chloroplast to synthesize acetyl-coenzyme-A [2] and mevalonic acid is still under investigation. This early assumption was based on the rapid incorporation of ^{14}C-label from $^{14}CO_2$ into chloroplastic terpenoids like β-carotene and plastoquinone-9, while ^{14}C-labeled mevalonate was predominantly incorporated into the extrachloroplastic 3-β-hydroxysterols. Another indication that chloroplasts are autonom in terpenoid biosynthesis was reported by the demonstration of the incorporation of $^{14}CO_2$ into β-carotene by isolated spinach chloroplasts, but the chloroplast preparation contained cytoplasmic contaminations [3]. Only recently it was shown that spinach chloroplasts synthesize fatty acids from photosynthetically fixed CO_2. As potential precursors PGA, PEP and pyruvate were proposed [4, 5].

In this communication isolated intact spinach chloroplasts with a high photosysnthetic activity were supplied with ^{14}C-labeled CO_2, phosphoglycerate, phosphoenolpyruvate, pyruvate, acetate and mevalonate as potential precursors for the biosynthesis of chloroplastic terpenoids. It was investigated, whether isolated intact spinach chloroplasts are capable of synthesizing their own mevalonic acid and terpenoids like β-carotene and plastoquinone-9 from CO_2 via the reductive pentose-phosphate-cycle. Furthermore it was investigated, whether terpenoid biosynthesis proceeds via the same intermediates starting from phosphoglycerate as proposed for the biosynthesis of fatty acids [4, 5].

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Materials and Methods

Spinach, *Spinacia oleracea*, was grown in the botanical gardens. After 3 to 4 weeks leaves were selected, washed and preilluminated for 2 h with white light (20000 Lux; 2.0 KW/m²). Thereafter leaves were homogenized for 5 sec in 0.33 m sorbitol containing 2 mM EDTA; 0.5 mM KH_2PO_4; 1 mM $MgCl_2$; 1.1 mM $MnCl_2$; 20 mM $NaNO_3$; 50 mM MES pH 6.1 and 0.5 g cysteine per liter containing 3.2 ml 0.5 m ascorbate (solution A) [6, 7]. The crude debris was filtered through 15 layers nylon cloth. Chloroplasts were sedimented by a very rapid acceleration to 100 g for 45 sec, the pellet discarded and the supernatant sedimented for 5 min at $660 \times g$. The chloroplast pellet was resuspended in solution A, but in addition 0.04 m HEPES pH 6.7 was added (solution B). The following sedimentation was performed for 5 min at $660 \times g$.

For ^{14}C-incorporation studies chloroplasts with an intactness of 85% and a photosynthetic activity of 280 µmol O_2 per mg chlorophyll per hour were used (Fig. 1). Intactness of the purified chloroplast preparations was determined by phase-contrast microscopy and ferricyanide-dependent oxygen evolution before and after rupturing [7].

At different times from the beginning of the ^{14}C-incorporation aliquots of the chloroplast suspension were removed from the incorporation vessel, extract-

ed with aceton, transferred into light petrol, dried with sodium sulfate and evaporated to a defined volume. Purification of β-carotene and plastoquinone-9 was performed by repeated absorption and partition chromatography until their specific radioactivity remained constant [8, 9]. Light petrol fractions and the purified terpenoids were assayed for radioactivity by scintillation counting. Quench correction was performed by using AES-ratio as well as internal standardisation with ^{14}C-toluene.

Results and Discussion

The isolated intact spinach chloroplasts evolved oxygen at rates of 280 µmol per mg chlorophyll and hour. At 0 °C Hill reaction remained still at 80% after 8 h. After 2 h at 20 °C even 50% of the initial activity could be obtained (Fig. 1).

Incorporation-kinetics of the various ^{14}C-labeled precursors into the light-petrol fraction containing chlorophylls, carotenoids, quinones and minor amounts of acyllipids are shown in Fig. 2. All applied radioactive substrates were incorporated into petrol soluble chloroplast constituents. This indicates that CO_2, PGA, Pyr, PEP, acetate and mevalonate are capable of entering the chloroplast.

Acetate exhibits the highest incorporation rate by far, followed by pyruvate and CO_2. Mevalonate, phosphoglycerate and phosphoenolpyruvate were incorporated to a much lower extent. While the incorporation of acetate, pyruvate and mevalonate approaches saturation within 30 min, the incorporation of phosphoenolpyruvate, phosphoglycerate and CO_2 is maintained even after 120 min. This means, that isolated spinach chloroplasts are still able to fix CO_2 in the Calvin-cycle.

Differences obtained in the incorporation-rate and labeling-kinetic may depend on differences in the permeability of the chloroplast envelope towards the applied radioactive substrates and also on their concentration and metabolic disposability as potential precursors for terpenoid biosynthesis. Predominantly the relative permeability of the chloroplast envelope contributes to the incorporation of the ^{14}C-labeled precursors [10, 11]. It is known that only certain metabolites cross the envelope in a counter exchange process [12–15].

Triose phosphates and aspartate are exported from the chloroplast to the cytoplasm by a specific translocator [10, 12]. Phosphoglycerate enters the chloroplast via a shuttle transfer with dihydroxyacetone phosphate [13]. Pyruvate and phosphoenolpyruvate are also able to cross the chloroplast envelope, the latter predominantly in C_4- and CAM-plants [16]. Particularly mevalonate which is expected to be incorporated exclusively into terpenoids

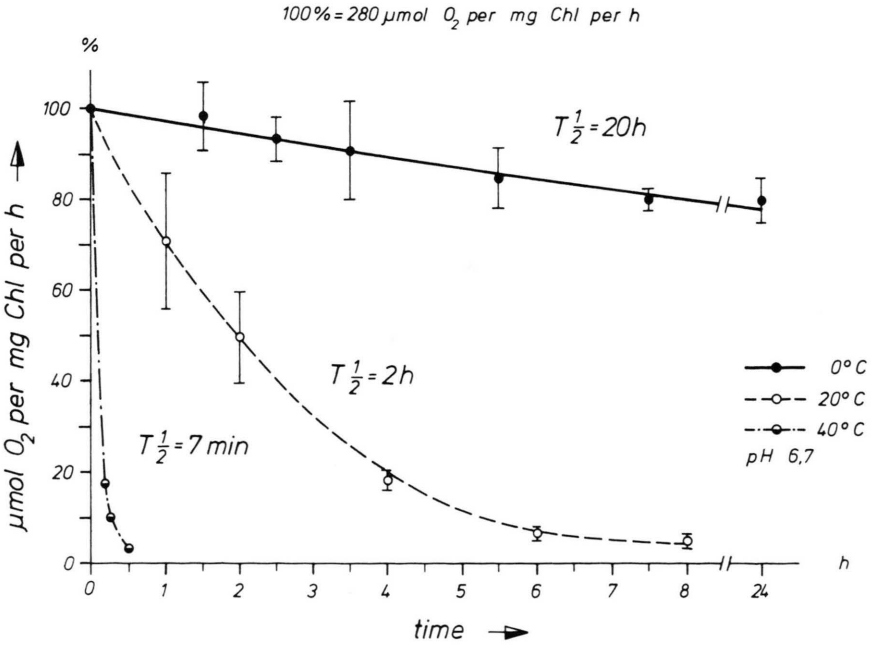

Fig. 1. Life-time of isolated intact spinach chloroplasts in solution B at different temperatures.

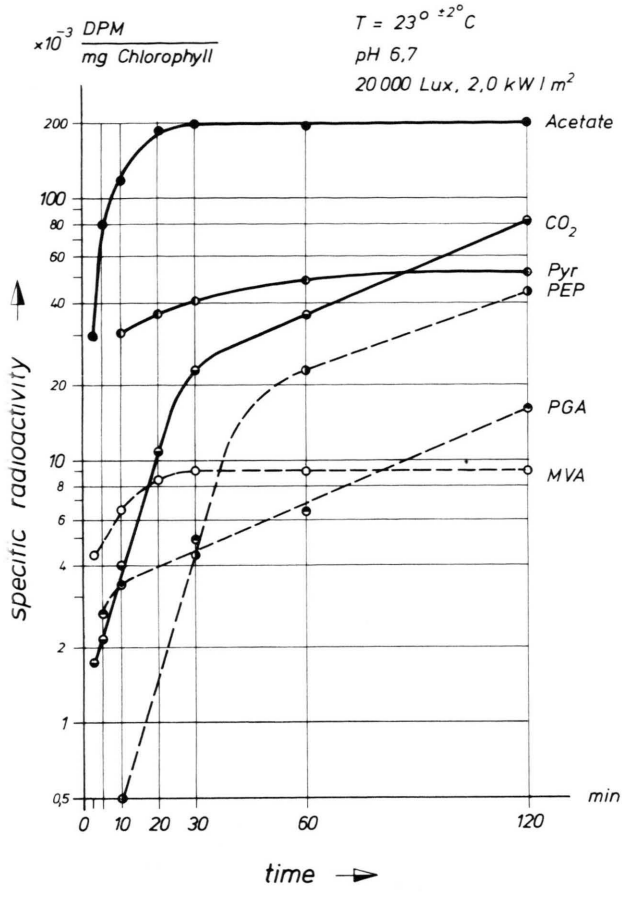

Fig. 2. Incorporation-kinetics of ^{14}C-labeled CO_2, phosphoglycerate (PGA), pyruvate (Pyr), phosphoenolpyruvate (PEP), acetate and mevalonate (MVA) into light-petrol soluble constituents of 4 weeks old spinach chloroplasts.

permeates the envelope very poorly, while acetate as a very specific precursor for the biosynthesis of fatty acids is taken up easily [1, 15]. CO_2 and phosphoglycerate are very unspecific terpenoid and fatty acid precursors and therefore incorporated into nearly all chloroplastic constituents as there are proteins, carbohydrates and also acyllipids and terpenoids.

To prove that all applied ^{14}C-labeled compounds function as precursors for terpenoid biosynthesis in the chloroplast β-carotene and plastoquinone-9 were isolated, purified to constant specific radioactivity and assayed. As shown in Table I β-carotene was labeled by all applied substrates except phosphoenolpyruvate. Plastoquinone-9 was labeled by all substrates.

From this it is concluded that there is a direct carbon flow from intermediates of the Calvin cycle via acetyl-CoA and mevalonic acid existing in the chloroplast, leading to the carotenoids and isoprenoid side-chains of the chlorophylls and quinones [17, 18]. The results further indicate, that acetyl-CoA is synthesized in the chloroplast directly from intermediates of the reductive pentosephosphate-cycle with PGA and pyruvate as precursors. Acetyl-CoA may then be used either for the biosynthesis of chloroplastic terpenoids via mevalonic acid or directly for fatty-acid biosynthesis. The establishment of an autonomy of the chloroplast in terpenoid biosynthesis implies that the enzymes which are involved in the biosynthesis of mevalonic acid like β-ketothiolase, hydroxymethylglutaryl-CoA-synthetase

Table I. Specific radioactivity of purified β-carotene and plastoquinone-9 after 60 min incorporation with ^{14}C-labeled CO_2, PGA, PEP, pyruvate, acetate and mevalonate.

	Applied radioactive precursor						
	CO_2	PGA	PEP	Pyr	Acetate	Mevalonate	
Concentration of the applied precursor	8.98	0.15	1.39	2.58	0.43	1.14	μmol
Total radioactivity of the applied precursor	0.500 18.500	0.025 0.925	0.025 0.925	0.025 0.925	0.025 0.925	0.025 0.925	mCi MBq
mol-specific radioactivity of β-carotene	1712.7	1756.4	0.0	197.1	193.4	677.9	DPM/μmol
mol-specific radioactivity of plastoquinone-9	1632.4	44.1	84.1	237.3	317.4	164.9	DPM/μmol

and hydroxymethylglutaryl-CoA-reductase are also contained in the chloroplast. This is supported by the observation that ^{14}C is incorporated into β-carotene of Chlorella pyrenoidosa very rapidly via $^{14}CO_2$ photosynthesis and also decorporated very fast after replacing $^{14}CO_2$ against $^{12}CO_2$ [17, 18]. Another support is given by the very high incorporation of [^{14}C]acetate and [^{3}H]mevalonate into chloroplastic terpenoids of radish seedlings treated with the bleaching herbicide SAN 6706, which is consistent with a very high specific activity of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme-A-reductase in the chloroplast fraction [19].

Though it was not possible to deduce the exact pathway of the formation of acetyl-coenzyme-A from photosynthetically fixed CO_2, our results give reasonable evidence that spinach chloroplasts are capable of synthesizing acetyl-CoA and mevalonic acid endogenously. Acetyl-CoA may also be synthesized by alternative reactions *e. g.* via glycolate, but from the ^{14}C-label in β-carotene it is evident that phosphoglyceric acid and pyruvate can be metabolized into acetate. Whether the acetate pool in the chloroplast provides the pathway leading to the fatty acids of the acyllipids and alternatively the biosynthesis of carotenoids and isoprenoid side-chains of chlorophylls and quinones is matter of further investigations.

Acknowledgements

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