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THE PLASTIDIC SHIKIMATE PATHWAY AND ITS ROLE IN THE SYNTHESIS OF PLASTOQUINONE-9, α-TOCOPHEROL AND PHYLLOQUINONE IN SPINACH CHLOROPLASTS

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ABSTRACT

The plastidic SkA pathway is operative in the synthesis of aromatic amino acids and of the prenylquinones PQ-9, αT and phylloquinone. Neither exogenous substrates nor coenzymes are needed under photosynthetic conditions. However, addition of PEP - and for Trp formation Gln and Ser - enhances the rates of synthesis. The pathway exhibits a specific feedback: Trp inhibits the pathway at the steps between SkA and chorismate and not at the KDAHP-step as in some microorganisms, whereas Phe and Tyr only inhibit their own synthesis.

The introductory step in PQ-9 and αT-synthesis is the oxidation of p-hydroxyphenylpyruvate to homogentisate /1/. It is prenylated to the methyl-6-prenylquinol by the corresponding prenyl-PP under simultaneous elimination of the carboxylgroup of homogentisate. The only site of αT synthesis is the envelope membrane of the chloroplast, whereas that of PQ-9 synthesis is the envelope and the thylakoid membrane too. The sequence in αT synthesis in spinach is (Fig. 3.):

Homogentisate $\xrightarrow{\text{Phytyl-PP}}$ Me-6-PQH_2 $\xrightarrow{\text{SAM}}$ 2,3-Me_2-PQH_2 $\xrightarrow{\text{Cyclization}}$ γT $\xrightarrow{\text{SAM}}$ αT; for the PQ-9 biosynthesis it is: Homogentisate $\xrightarrow{\text{Solanesyl-PP}}$ Me-6-SQH_2 $\xrightarrow{\text{SAM}}$ PQH_2.

Abbreviations: E4P - erythrose-4-P; GGPP - geranylgeranyl-PP; HPP - p-hydroxyphenylpyruvate; KDAHP - 2-keto-3-deoxyarabinoheptonic acid-7-P; Me-6-$GGQH_2$ - 2-methyl-6-geranylgeranylquinol; Me-6-PQH_2 and isomers - 2-methyl-6-phytylquinol and isomers; 2,3-Me_2-PQH_2 - 2,3-dimethyl-5-phytylquinol; Me_3-PQH_2 - trimethylphytylquinol; Me-6-SQH_2 - 2-methyl-6-solanesylquinol; PGA - 3-D-phosphoglycerate; PQ-9 - plastoquinone-9; PQH_2 - plastoquinol-9; PEP - phosphoenolpyruvate; SkA - shikimate; PRPP - 5-P-ribosyl-1-PP; SAM - S-adenosylmethionine; αT, βT, γT, δT - α-, β-, γ-, δ-tocopherol.

From isotopic studies using CO_2 /2/,SkA /3/ and o-benzoylsuccinate /4/ it has been proved that higher plants are able to synthesize phylloquinone (2-methyl-3-phytylnaphthoquinone vitamin K_1). The envelope membrane is the site of prenylation of 1.4-dihydroxy-2-naphthoate to form 2-methyl-naphthoquinol which is methylated by SAM to yield phylloquinol. Consequently, the sequence in its biosynthesis seems to correspond with that in microorganisms /5, 6, 7/: SkA → (Chorismate $\xrightarrow{\text{Succinylsemialdehyde-TPP}}$) o-Succinylbenzoate $\xrightarrow{\text{Prenyl-PP}}$ 2-Prenylnaphthoquinol $\xrightarrow{\text{SAM}}$ 2-Methyl-3-prenylnaphthoquinol. In plants phytyl-PP is preferred as prenylcompound.

The SkA pathway operates in the synthesis of aromatic amino acids in plants. It is also involved in the synthesis of prenylquinones by synthesizing the aromatic moiety. The prenyl sidechain originates from the plastidic mevalonate pathway /8/. These prenylquinones operate in different ways in the photosynthetic tissue: PQ-9 acts in the photosynthetic electron transport /9/, αT inactivates energetisized oxygen species formed by light (by scavenging radicals and also by quenching $^1O_2$ /10, 11/). αT is also an important membrane constituent. The function of phylloquinone in plants is not yet clear. The first problem was to study the compartmentation of the SkA pathway involved in these syntheses.

Identification of a plastidic SkA pathway

Biosynthesis of aromatic amino acids in spinach chloroplasts under photosynthetic conditions: The findings that isoenzymes of SkA dehydrogenase /12, 13/ and enzymes of Tryp synthesis /14/ are enriched in chloroplast fractions, strongly indicate the occurence of enzymes of SkA pathway in this organelle. To prove the function of this pathway, purified intact chloroplasts were illuminated under photosynthetic conditions with $^{14}CO_2$ and the label in the expected aromatic amino acids and prenylquinones was determined/15, 16, 17/ (see Fig. 1). In all experiments chloroplasts suspensions with at least 1 mg chlorophyll/ml were used; t = 30 min; control expt. disproved microbial contamination /16/. The purity of the chloroplasts isolated acc. to /18/ was tested for marker enzymes /19, 20/.

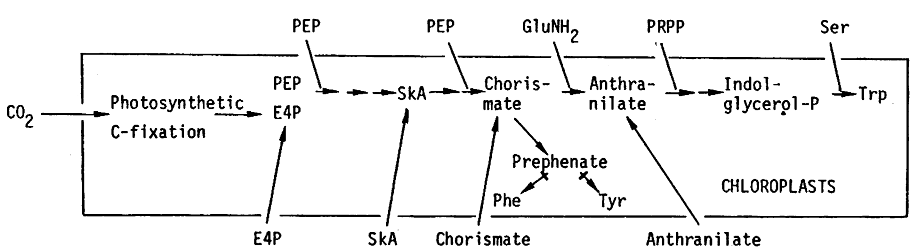

Fig. 1. SkA pathway in spinach chloroplasts and substrates tested /24/

Table 1. ^{14}C-Incorporation from $^{14}CO_2$ into amino acids of illuminated intact spinach chloroplasts of different purity /21/.

	Purified chloroplasts	Non purified chloroplasts
	% of photosynthetically fixed $^{14}CO_2$	
Sum of amino acids	0.34	0.44
	% of label in amino acids	
Asn + Asp + Gln + Glu	16.6	1.8
Ser + Gly	38.9	79.9
Ala	40.3	12.9
Phe + Tyr + Trp	10.2	5.4

Increasing the purity of the chloroplasts, the label of Gly and Ser decreased, whereas that of the aromatic amino acids (and of Ala) increased /21/. Decrease of Gly and Ser synthesis is due to the elimination of the peroxysomes and mitochondria /21/ (Table 1).

As pointed out by /22/ at the onset of illumination the exchange of dihydroxyacetonephosphate from the chloroplasts versus P_i of the suspension medium by the phosphate translocator /23/ of the envelope membranes results in a lag phase of photosynthetic carbon fixation. This is caused by a lack of PGA which is needed by the Calvin-cycle. The same should be valid to metabolic pathways adjacent to the Calvin-cycle, e.g. the SkA pathway investigated here. Experiments with and without P_i in the medium proved this assumption /17/: omission of P_i of the medium effects in a manifold incorporation from $^{14}CO_2$ into aromatic acids (Table 2).

Requirement of exogenous substrates: As shown above, the SkA pathway operates under conditions of photosynthetic carbon fixation by intact chloroplasts at considerable rates. In additional studies the influence of exogenously added substrates on the plastidic SkA pathway was determined /24/. To narrow the study, the metabolic flow of the SkA pathway was directed only to the Trp branch by inhibiting the other two branches by

Table 2. ^{14}C-Incorporation from $^{14}CO_2$ into aromatic amino acids and prenylquinones of illuminated intact spinach chloroplasts adding and omitting P_i to the suspension medium /17/.

	P_i added	P_i omitted
	10^{-3} % of photosynthetically fixed $^{14}CO_2$	
Phe	35	66
Tyr	0.45	4
Trp	2.3	4
PQ-9	0.42	1.3
αT	0.73	1.3

Table 3. ^{14}C-Incorporation from $^{14}CO_2$ or /1,6-^{14}C/-SkA into aromatic amino acids of illuminated intact spinach chloroplasts in the presence of Phe, Tyr and Trp, respectively /25/.

	+Phe	+Tyr	+Trp
	- each 5 mM -		
	% of control[§]		
+ $^{14}CO_2$			
Phe	82	163	18
Tyr	386	25	37
Trp	545	212	12
+ /1,6-^{14}/-SkA			
Phe	5	152	7
Tyr	147	84	10
Trp	120	208	38

§ without addition of aromatic amino acids

adding Phe and Tyr (see below). An addition of PEP, Gln and Ser increased the incorporation from $^{14}CO_2$ into Trp. The optimal concentrations were (without added substrate = 1.0): ca 3.5 fold increase at 5×10^{-4} M PEP; ca 2.5 fold increase at 10^{-6} M Gln and Ser, resp. . Furthermore, /1-^{14}C/-PEP is incorporated into aromatic amino acids of chloroplasts in considerable yields /24/. When E4P, SkA, chorismate and anthranilate, respectively, were applied in $^{14}CO_2$ experiments in increasing concentrations, the label in the aromatic amino acids more or less decreased /24/. This might be caused by isotopic dilution of endogenous intermediates and/or regulatory phenomena.

Feedback control by endproducts: From $^{14}CO_2$-experiments in the presence of Phe, Tyr or Trp (each 5 mM) it could be revealed that the SkA pathway is subject to feedback control by endproducts /25/ (Table 3). Phe and Tyr exert feedback control over their own rates of synthesis, whereas Trp controls the rate of synthesis of all three aromatic amino acids. To determine a point of attack more exactly, /1.6-^{14}C/-SkA was fed as a more direct precursor (Table 3). These results indicate that Trp attacs a step between the synthesis of SkA and chorismate (Fig. 2) and not the KDAHP step as in some microorganisms (for survey see /26/).

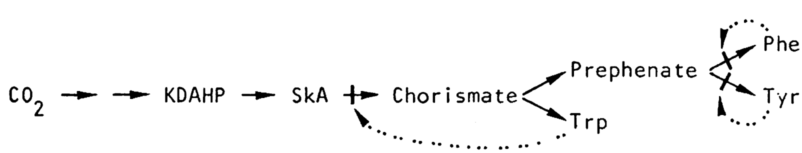

Fig. 2. Feedback control of SkA pathway by Phe and Tyr and Trp in spinach chloroplasts /25/.

Transfer of aromatic amino acids across the envelope membranes: Feeding /1.6-^{14}C/-SkA to spinach chloroplasts, the main portion of label in the aromatic amino acids was found in the suspension medium after a period of 30 min /16/. This finding as well as the considerable incorporation of /ß-^{14}C/-Tyr applied to endosperm, into PQ and flavonoids of leaves of barley seedlings /27/ indicates a relatively rapid transfer of aromatic amino acids across the envelope membranes in vitro and in vivo.

Oxidation of HPP to homogentisate: This step which is a prerequisite for the synthesis of PQ and αT was shown in the thylakoid fraction of Lemna gibba /28/. Furthermore, from the incorporation of ^{14}C labelled CO_2, SkA and Tyr into PQ and αT, it could be conclouded that the HPP oxidation takes place in the chloroplasts /16, 17/.

Biosynthesis of α-tocopherol and plastoquinone-9

The aromatic moiety of both derives from homogentisate /1/ which is formed by an oxydase system from HPP /28/.

α-Tocopherol biosynthesis: The only site of αT biosynthesis in spinach chloroplasts is the envelope membrane /29, 30/. Homogentisate is solely prenylated with phytyl-PP to form Me-6-PQH_2 /30/. There is no stimulation by other chloroplast fractions like thylakoid membranes or stroma /30/. The prenyltransferase in spinach shows a strong specificity for phytyl-PP (26 pmol/h mg envelope protein); GGPP is inactive in this system /30/. From the possible positions isomers only Me-6-PQH_2 is formed; Neither Me-5- nor Me-3-PQH_2 could be found /30/.Consequently,the pathway is strongly directed at this step. A kinase which forms phytyl-PP from phytol plus ATP is localized in the stroma /30/. Phytol and its pyrophosphate arises by reduction from GGPP /31/ which is synthesized by a recombinated system of envelope or thylakoid membranes plus stroma protein /32/.

The following methylation steps with SAM as methyl-group donor to form αT from Me-6-PQH_2 are also performed by enzyme systems localized in the envelope membranes /29/ (see Fig. 3). The quinol is the substrate of the methylation and not the quinone. Comparison of the methylation rates to yield the corresponding dimethyl-compound shows that Me-6-PQH_2 is not only strongly preferred to its isomers Me-5- and Me-3-PQH_2 but also to its chromanol stage δT (ratios are 100 : 10 : 5 : 5) /33/. Thus, the main product is 2,3-Me_2-PQH_2 which undergoes ringclosure to γT and further methylation by SAM to αT. The chromanol stage is the prerequisite for the second methylation, no Me_3-PQH_2 occurs /33, 34/. γT is preferred to ßT to yield αT (100 : 35) /33/. In marked contrast to the prenylation enzyme, the transferase for the first methylation step exhibits a preference for Me-6-$GGQH_2$ (2 nmol/h mg envelope protein) in comparison to Me-6-PQH_2 (0.7 nmol mg envelope protein) /29, 34/.

The ring closure of the dimethylprenylquinol to the corresponding chromanol (in this case 2.3 Me_2-PQH_2 → γT) takes place only in intact chloroplasts /33, 34/ but not in isolated envelope membranes. The concentrations of PQ-9 and αT in the envelope are: PQ 0.53 ug/mg envelope protein (7.1×10^{-4} M), αT 1.03 ug/mg envelope protein (2.4×10^{-3} M) (Soll, Douce unpbl.).

Plastoquinone-9 biosynthesis: PQ-9 biosynthesis, both prenylation and methylation, is not only performed by the envelope membranes (1.2 pmol/h mg protein and 10 pmol/h mg protein, respectively) but also at low rates by the thylakoid membranes (0.013 pmol/h mg protein and 0.35 pmol/h mg protein, respectively) /30/. However, if one takes into account the rate of thylakoid protein to that of envelope protein per mg chlorophyll, the yields in total are not as different as they are calculated on the basis of protein itself. The sequence of reactions involved in PQ-9 biosynthesis is similar to αT. Solanesyl-PP (C_{45}) serves as prenyl compound in the prenylation reaction to form Me-6-SQH_2 with homogentisate. In the following steps Me-6-SQH_2 is methylated with SAM to yield PQ-9.

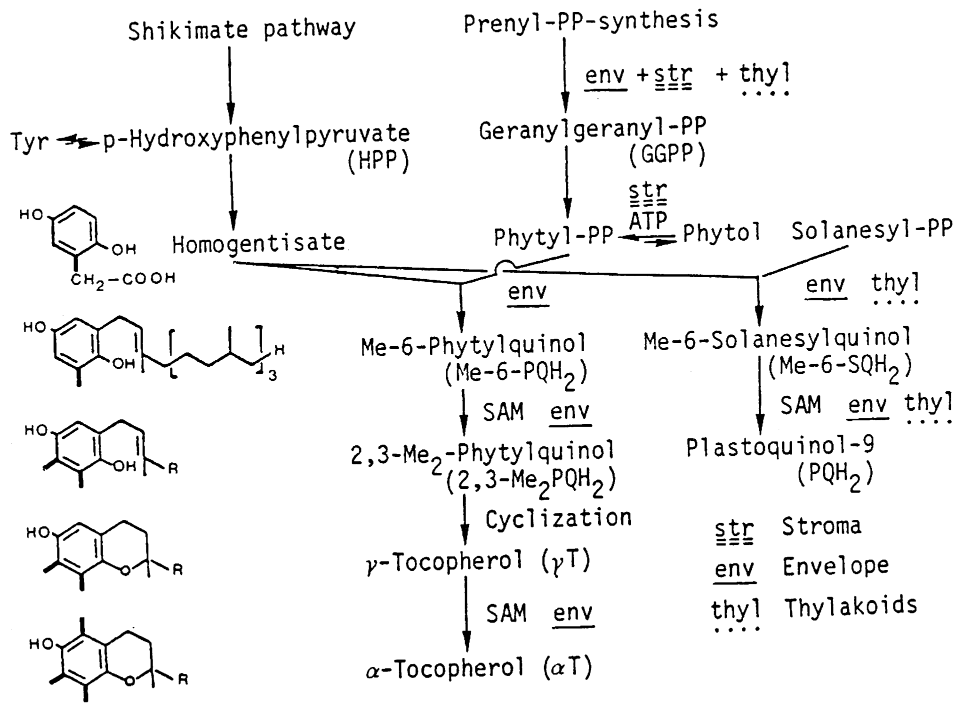

Fig. 3. Biosynthesis of αT and PQ in spinach chloroplasts /29, 30, 33, 34/

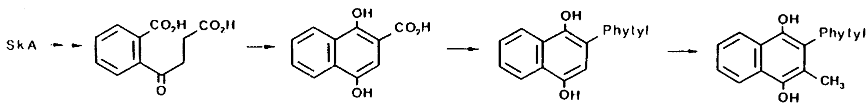

Fig. 4. Proposed scheme for the biosynthesis of phylloquinone in spinach chloroplasts. I -o-succinylbencoic acid; II - 1,4-dihydroxy-2-naphthoic acid; III - 2-phytyl-1,4-naphthoquinol; IV - 2-methyl-3-phytyl-1,4-naphthoquinol

Phylloquinone biosynthesis

As mentioned above, biosynthesis of phylloquinone in plants could be detected by feeding some substrates including SkA by the group of Threlfall /3, 4/. In studies on spinach chloroplast (Schultz and Ellerbrock, unpublished data), 1,4-dihydroxy-2-naphthoate is prenylated by phytol plus ATP to form 2- phytylnaphthoquinol (60 pmol/h mg chlorophyll). (For the biosynthesis of phytyl-PP by chloroplasts see /30/). The quinol is methylated by SAM to yield phylloquinol (6 pmol/h mg chlorophyll). There is a strong specificity of the sequence of both reactions. 2-Phytylnaphthoquinol is preferred to its GG- and farnesyl- homologue. The site of prenylation is the envelope membrane; thylakoid membranes as well as stroma protein seems to be inactive. Both reactions need Mg^{2+} (2.5 mM); light is not required.

CONCLUSIONS

The plastidic SkA pathway is involved in the biosynthesis of aromatic amino acids as well as of prenylquinones of algae and higher plants (for distribution /35/) but how it is combined with the generally occuring secondary metabolism of aromatics in plants is not clear /36/.

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