Glyceraldehyde 3-Phosphate and Pyruvate as Precursors of Isoprenic Units in an Alternative Non-mevalonate Pathway for Terpenoid Biosynthesis

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Abstract: Incorporation of ^{13}C-labeled glycerol or pyruvate into the ubiquinone Q8 of *Escherichia coli* mutants lacking enzymes of the triose phosphate metabolism and of (U-$^{13}C_6$)glucose into the triterpenoids of the hopane series of *Zymomonas mobilis* showed that glyceraldehyde 3-phosphate (or eventually glyceraldehyde) and a C_2 unit derived from pyruvate decarboxylation were the only precursors of the C_5 skeleton of isoprenic units in a novel non-mevalonate pathway for isoprenoid biosynthesis in these bacteria.

Introduction

Isoprenoids are ubiquitous metabolites found in all living organisms. They include for instance essential metabolites such as the sterols acting as membrane stabilizers in eukaryotes or precursors for steroid hormones, the acyclic polyprenols found in the side chain of the prenylquinones, in phytol from chlorophylls or via their phosphodiesters as sugar carriers for polysaccharide biosynthesis, and the carotenoids in photosynthesizing organisms as well as a large variety of compounds (about 22 000 structures known) with a less evident physiological role.[1] The formation of the common isoprene-derived subunit has been extensively studied over the last 50 years, leading to a generally accepted pathway from acetate activated as acetyl-coenzyme A, via acetoacetyl-coenzyme A, 3-hydroxy-3-methylglutaryl-coenzyme A, and mevalonate to isopentenyl diphosphate, the first precursor possessing the branched C_5 isoprenic skeleton.[2−5] A few years ago, however, incorporation of ^{13}C-labeled acetate and glucose into triterpenoids of the hopane series and the prenyl chain of ubiquinone from several bacteria (Figure 1) proved unambiguously that the classical acetate/mevalonate pathway was not operating in all living organisms, and that the isoprenic skeleton can be formed from triose phosphate derivatives via a non-mevalonate pathway.[5−8] A hypothesis was put forward: this C_5 skeleton is formed from the condensation of a C_2 subunit derived from pyruvate decarboxylation and a C_3 subunit derived from a triose phosphate, followed by a transposition step. In this paper, pyruvate and glyceraldehyde 3-phosphate were identified as the precursors in this pathway, acting as donors of the C_2 and C_3 subunits, respectively.

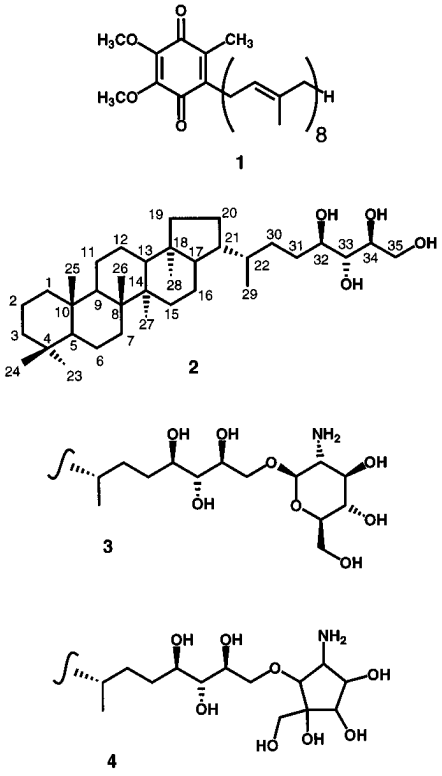

Figure 1. Ubiquinone Q8 (**1**) from *E. coli* and major hopanoids **2**−**4** from *Z. mobilis*.

Experimental Section

Bacterial Cultures. *Escherichia coli* mutants Lin 61, Lin 201, DF 261, and DF 263 were obtained from B. J. Bachmann (*E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven,

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Table 1. Incorporation of ^{13}C-Labeled Pyruvate (Isotopic Abundance 10%) or Glycerol (Isotopic Abundance 10%) into Ubiquinone Q8 of *E. coli* Mutants Lacking Enzymes of the Triose Phosphate Metabolism

E. coli mutant		isotopic abundance (%) of carbon atoms from isopentenyl diphosphate									
		[2-^{13}C]pyruvate + unlabeled glycerol					unlabeled pyruvate + [2-^{13}C]glycerol				
		C-1	C-2	C-3	C-4	C-5	C-1	C-2	C-3	C-4	C-5
DF 261	enolase	1.5	1.6	10.4	1.1	1.4	1.2	7.7	1.5	1.1	1.2
DF 263	phosphoglycerate kinase	1.3	2.5	7.5	1.1	1.0	1.2	7.0	1.1	1.1	1.1
W3CG	glyceraldehyde phosphate dehydrogenase	1.3	1.2	11.0	1.1	1.2	1.1	7.8	1.5	1.1	1.1
DF 502	triose phosphate isomerase	1.2	6.0	6.6	1.1	1.0	1.2	2.4	2.0	1.1	1.0

E. coli mutant		isotopic abundance (%) of carbon atoms from isopentenyl diphosphate									
		[3-^{13}C]pyruvate + unlabeled glycerol					unlabeled pyruvate + [2-^{13}C]glycerol				
		C-1	C-2	C-3	C-4	C-5	C-1	C-2	C-3	C-4	C-5
Lin 201	glycerol phosphate dehydrogenase	6.6	1.3	1.8	1.1	7.2	1.2	1.5	1.4	1.1	1.2
Lin 61	glycerol kinase	7.4	1.2	1.4	1.1	8.1	1.0	1.3	0.8	1.1	1.1

CT), W3CG was obtained from Professor A. Plückthun (Universität Zürich, Switzerland), and DF 502 was obtained from D. Fraenkel (Harvard Medical School). Cultures were grown on a rotatory shaker at 37 °C for 24 h in a synthetic minimal medium (pH 7.0) containing $Na_2HPO_4 \cdot 7H_2O$ (5.3 g·L^{-1}), KH_2PO_4 (12 g·L^{-1}), $(NH_4)_2SO_4$ (2.6 g·L^{-1}), $MgSO_4 \cdot 7H_2O$ (74 mg·L^{-1}), $CaCl_2$ (1.5 mg·L^{-1}), $ZnCl_2$ (0.13 mg·L^{-1}), $FeSO_4 \cdot 7H_2O$ (0.3 mg·L^{-1}), and thiamine (1 mg·L^{-1}). To the medium of DF 502 were added histidine (20 mg·L^{-1}), uracil (20 mg·L^{-1}), and cytosine (20 mg·L^{-1}), and to that of W3CG was added tetracycline (10 mg·L^{-1}). Carbon sources were added at the following concentrations: pyruvate (3g·L^{-1}) and glycerol (0.6 g·L^{-1}) for Lin 61 and Lin 201, pyruvate (5 g·L^{-1}) and glycerol (1 g·L^{-1}) for W3CG and DF 263, and pyruvate (5 g·L^{-1}) and glycerol (1.5 g·L^{-1}) for DF 261 and DF 502. The isotopic abundance of ^{13}C-labeled pyruvate or glycerol was 10%. For each mutant, a 1 L culture afforded about 1 g of freeze-dried cells and ca. 1 mg of ubiquinone. The cells of all mutant cultures were checked after each labeling experiment for their genetic integrity and the absence of revertants. They were plated on Petri dishes containing the same culture medium as that utilized for the incorporation experiments with pyruvate and glycerol together as carbon sources where they grew or pyruvate alone or glycerol alone where they did not grow, with the exception of DF 502. This strain did not grow on pyruvate alone but was able to grow slowly on glycerol as the sole carbon source. This mutant converts glycerol into dihydroxyacetone phosphate from which pyruvate and further glyceraldehyde phosphate are formed via methylglyoxal.

Zymomonas mobilis (strain ZM 6, American Type Culture Collection 29191, 0.75 g, dry weight) obtained from a 1 L culture was grown as previously described on a synthetic medium containing [U-$^{13}C_6$]glucose (isotopic abundance 99%) diluted in a 1:9 ratio with unlabeled glucose as the sole carbon source (final concentration 20 g·L^{-1}).[8]

Isoprenoid Isolation and ^{13}C NMR Specrometry of Labeled Metabolites. Isolation of ubiquinone Q8 (**1**) and acetylated hopanoids **2**, **3**, and **4** and NMR spectroscopy were performed as described earlier.[8] Ubiquinone samples from *E. coli* mutants DF 502 and DF 263 contained significant amounts of ubiquinol (up to 25%) and were therefore oxidized using $FeCl_3$ in ethanol. Isotopic abundances of ubiquinone carbon atoms were evaluated using as internal reference the signal of the carbon atoms of the prenyl chain corresponding to carbon C-4 of isopentenyl diphosphate which was not expected to be labeled. As all isoprenic units presented for all *E. coli* mutants identical labeling, for the sake of clarity the labeling patterns are only indicated for the carbon skeleton of isopentenyl diphosphate whose numbering is given in Figure 3.

In *Z. mobilis* after incorporation of [U-$^{13}C_6$]glucose, all carbon atoms of the hopane skeleton were labeled and most of the signals were multiplets. The isotopic abundances were therefore not evaluated. Only the ^{13}C/^{13}C coupling constants are given. As they were nearly identical for all three examined hopanoids, only those concerning the major compound **3** were reported: (^1J) C-2/C-3, 33.2 Hz; C-5/C-6, 34.6 Hz; C-9/C-11, 34.6 Hz; C-12/C-13, 34.2 Hz; C-16/C-17, 34.6 Hz; C-20/C-21, 33.2 Hz; C-24/C-4, 35.1 Hz; C-10/C-25, 35.4 Hz; C-8/C-26, 36.8 Hz; C-14/C-27, 36.8 Hz; C-18/C-28, 35.1 Hz; C-22/C-30, 34.9 Hz; C-31/C-32, 37 Hz; C-32/C-33, 40 Hz; C-34/C-35, 43 Hz; C-1′/C-2′, 45 Hz; C-2′/C-3′, 40 Hz; C-3′/C-4′, 40 Hz; C-4′/C-5′, 45 Hz; C-5′/C-6′, 45 Hz. Only one ^2J constant was measurable: C-17/C-19, 2.5 Hz. Four ^3J constants could be directly measured: C-2/C-23, 3.5 Hz; C-12/C-15, 1.3 Hz; C-16/C-19, 3.0 Hz; C-20/C-29, 3.5 Hz. The presence of weak ^2J and/or ^3J coupling yielded broad singlets for the signals of C-1 and C-7.

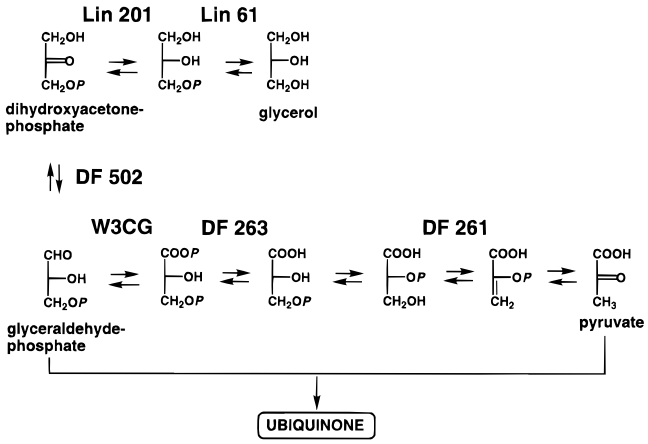

Figure 2. Triose phosphate metabolism mutants of *E. coli*. The reference number of each strain is given beside the enzymatic reaction which is not operative.

Results and Discussion

For identification of the C_3 triose phosphate derivative, six mutants of *E. coli* were employed. They were each defective in only one enzyme of the triose phosphate metabolism and could therefore not interconvert glycerol and pyruvate: Lin 61 lacking glycerol kinase, Lin 201 glycerol 3-phosphate dehydrogenase, DF 502 triose phosphate isomerase, W3CG glyceraldehyde 3-phosphate dehydrogenase, DF 263 3-phosphoglycerate kinase, and DF 261 enolase (Figure 2, Table 1). For each mutant the labeling pattern of the prenyl chain of ubiquinone Q8 (Figure 1; **1**) was determined after two sets of experiments: ^{13}C-labeled pyruvate and unlabeled gycerol on the one hand, unlabeled pyruvate and ^{13}C-labeled glycerol on the other hand (Table 1). In full accordance with the hypothesis, ^{13}C-labeled pyruvate was directly incorporated by all mutants into the C_2 subunit, whereas none of the mutants incorporated ^{13}C-labeled glycerol into these two carbon atoms of the isoprenic skeleton. From pyruvate, incorporation of ^{13}C label into the C_3 subunit occurred only with mutants Lin 61, Lin 201, and DF 502, and from glycerol only with mutants DF 261, DF 263, and W3CG. These results point to the unique position of glyceraldehyde 3-phosphate which remains as the only precursor for the C_3 subunit and exclude any other C_3 compound derived from the triose phosphate pathway as precursor.

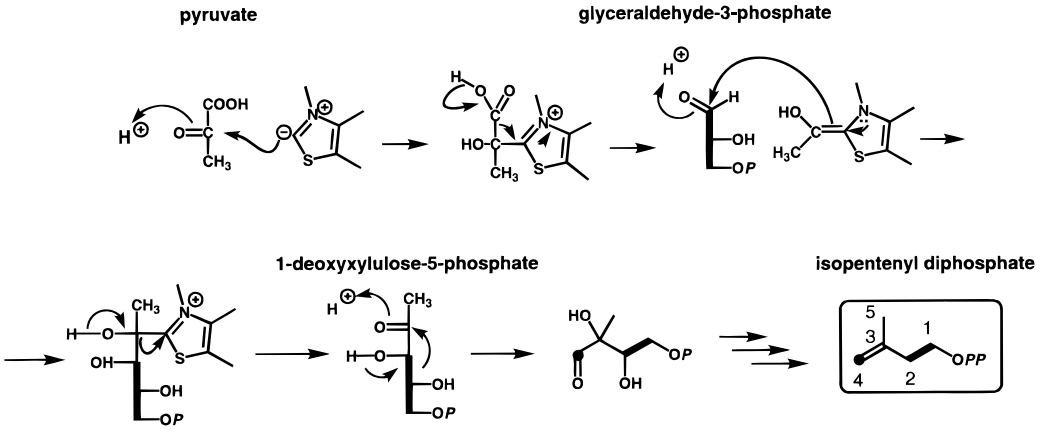

Figure 3. Hypothetical biogenetic non-mevalonate pathway for the early steps of isoprenoid biosynthesis from glyceraldehyde 3-phosphate and pyruvate.

A slight incorporation of [2-^{13}C]glycerol into both C_2 and C_3 subunits was however observed with the triose phosphate defective mutant DF 502. In this mutant, small amounts of [2-^{13}C]glycerol were most probably converted into [2-^{13}C]-dihydroxyacetone 3-phosphate. Two indirect ways of formation of glyceraldehyde phosphate and pyruvate from dihydroxyacetone phosphate are conceivable: one is the chemical equilibration between dihydroxyacetone phosphate and glyceraldehyde phosphate, the latter of which could be incorporated into isoprenoids;[9] the second is the conversion of dihydroxyacetone phosphate via methylglyoxal into pyruvate, and further into glyceraldehyde phosphate.[10] The second alternative is the most likely one since this mutant did not incorporate any label from ^{13}C-labeled pyruvate into the glycerol isolated from its phospholipids after acid hydrolysis and analysis by ^{13}C NMR of its triacetate. This excludes any significant participation of the chemical equilibration between ^{13}C-labeled glyceraldehyde phosphate and dihydroxyacetone phosphate for the formation of the glycerol phosphate pool. [2-^{13}C]Glycerol was likewise slightly incorporated by this mutant into the fatty acids. Their uneven-numbered carbon atoms were weakly labeled with the same isotopic abundance (about 2%) as the labeled carbon atoms of isoprenic units. Pyruvate incorporation into isoprenic units of mutant DF502 was in contrast 3 times higher as compared to that of glycerol.

Final confirmation for the insertion of intact C_2 and C_3 subunits was obtained by incorporation of uniformly labeled [U-$^{13}C_6$]glucose into the hopanoids of *Z. mobilis* (Figure 1; **2**−**4**) and examination of the ^{13}C/^{13}C coupling constants (see the Experimental Section). On the one hand, C-1, C-2, and C-4 from isopentenyl diphosphate were derived from the same C_3 precursor as shown by the ^1J coupling constant between C-1 and C-2 and the ^2J and ^3J constants between C-4 and, respectively, C-2 and C-1. Because of the multiplicity of the signals, most of the ^2J and some of the ^3J could not be measured. Such ^2J coupling constants between C-2 and C-4 from isopentenyl diphosphate could be previously more easily determined after incorporation of [4,5-$^{13}C_2$]glucose.[8] On the other hand, C-3 and C-5 are coupled by a ^1J constant and arise thus from the same C_2 precursor.

Our first hypothetical biosynthetic scheme for the non-mevalonate pathway proposed a condensation step between a C_2 unit derived from pyruvate and a triose phosphate not derived from pyruvate followed by a transposition.[8] This scheme can now be made precise in that the decarboxylation product of pyruvate (most probably as (hydroxyethyl)thiamine diphosphate) and glyceraldehyde phosphate (or eventually glyceraldehyde released by the action of a phosphatase, although the free aldehyde has been rarely reported as a metabolite) are the precursors (Figure 3). This implies that the first C_5 precursor has an unbranched carbon skeleton and should be most probably a 1-deoxypentulose 5-phosphate. Such compounds are already known. 1-Deoxyxylulose is formed from pyruvate and glyceraldehyde by cell-free systems from *Bacillus subtilis* and *E. coli*[11] and is known as a precursor of thiamine diphosphate[12−14] and pyridoxal phosphate.[15] Further, deuterium-labeled 1-deoxyxylulose has been efficiently incorporated into the ubiquinone of *E. coli*, and the labeling pattern was as expected.[16]

The glyceraldehyde phosphate/pyruvate pathway for isoprenoid biosynthesis has been overlooked during the past years. Its presence was clearly identified in several bacteria,[6,8] and more recently for the biosynthesis of diterpenoids in two higher plants, *Gingko biloba* and *Salvia miltiorrhiza*,[17] as well as for the formation of all isoprenoids (*i.e.*, sterols, prenylquinones, phytol, carotenoids) of the unicellular green alga *Scenedesmus obliquus*.[18] Most probably this pathway is more widespread among bacteria and higher plants. This is suggested by the numerous negative or ambiguous results obtained on the biosynthesis of isoprenoids obtained from labeling experiments and which can now be satisfactorily explained by the existence of a different, non-mevalonate pathway.[19]

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