Deuterium-labelled isotopomers of 2-C-methyl-D-erythritol as tools for the elucidation of the 2-C-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis

Lionel CHARON*, Jean-François Hoeffler*, Catherine PALE-GROSDEMANGE*, Luisa-Maria LOIS†, Narciso CAMPOS†, Albert BORONAT‡ and Michel ROHMER†1

* Université Louis Pasteur/CNRS, Institut Le Bel, 4 rue Blaise Pascal, 67070 Strasbourg Cedex, France, and † Departamento de Bioquímica e Biologia Molecular, Facultad de Química, Universitat de Barcelona, Martí i Franquès 1, 08028 Barcelona, Spain

Escherichia coli synthesizes its isoprenoids via the mevalonate-independent 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. The MC4100 dxs−:CAT strain, defective in deoxyxylulose-5-phosphate synthase, which is the first enzyme in this metabolic route, exclusively synthesizes its isoprenoids from exogenous 2-C-methyl-D-erythritol (ME) added to the culture medium. The fate of the hydrogen atoms in the MEP pathway was followed by the incorporation of [1,1-2H]ME and [3,5,5,5-2H]ME. The two C-1 hydrogen atoms of ME were found without any loss in the prenyl chain of menaquinone and/or ubiquinone on the carbon atoms derived from C-4 of isopentenyl diphosphate (IPP) and on the E-methyl group of dimethylallyl diphosphate (DMAPP); the C-5 hydrogen atoms on the methyl groups derived from IPP C-5 methyl group and the Z-methyl group of DMAPP. This showed that no changes in the oxidation state of these carbon atoms occurred in the reaction sequence between MEP and IPP. Furthermore, no deuterium scrambling was observed between the carbon atoms derived from C-4 and C-5 of IPP or DMAPP, suggesting a completely stereoselective IPP isomerase or no significant activity of this enzyme. The C-3 deuterium atom of [3,5,5,5-2H]ME was preserved only in the DMAPP starter unit and was completely missing from all those derived from IPP. This finding, aided by the non-essential role of the IPP isomerase gene, suggests the presence in E. coli of two different routes towards IPP and DMAPP, starting from a common intermediate derived from MEP.

Key words: 1-deoxy-D-xylulose 5-phosphate, dimethylallyl diphosphate, Escherichia coli, isopentenyl diphosphate, ubiquinone.

INTRODUCTION

During the past decades, acetate and mevalonate have been considered to be the unique precursors of isoprenoid compounds in all living organisms [1]. However, a novel metabolic pathway was discovered in the late 1980s for the biosynthesis of isoprenoids in bacteria [2,3], green algae [4] and plants [5–8]. In this alternative biosynthetic route, a condensation step between isoprenoids in bacteria [2,3], green algae [4] and plants [5–8]. In this alternative biosynthetic route, a condensation step between (hydroxyethyl)thiamin diphosphate, resulting from the de-carboxylation of pyruvate (1), and glyceraldehyde 3-phosphate into DXP [16–18], catalysing the condensation of (hydroxyethyl)thiamin diphosphate and thiamin diphosphate into DXP [13–15], ME (4) rather than DXP should be considered to be the first committed precursor of the mevalonate-independent pathway. Only two enzymes of the MEP pathway are known: DXP synthase (DXS), catalysing the condensation of (hydroxyethyl)thiamin diphosphate and glyceraldehyde 3-phosphate into DXP [16–18], and DXP reducto-isomerase, yielding MEP from DXP by a rearrangement followed by a reduction (Scheme 1) [19–21]. The steps between MEP (4) and isopentenyl diphosphate (5) (IPP) and/or its isomer dimethylallyl diphosphate (DMAPP) (6) are still unknown.

The origin and fate of the carbon atoms finally found in the isoprene units derived from the MEP pathway are now well established. In contrast, the origin of the hydrogen atoms is not as clear. Incorporation experiments with deuterium-labelled 1-deoxy-D-xylulose (DX) or 2-C-methyl-D-erythritol (ME) have afforded interesting clues to its elucidation in the bacterium Escherichia coli and in higher plants [12,22–24]. [1,1-2H]ME (4a) was previously incubated with a wild-type strain of E. coli [12]. However, the incorporation yield into the polyprenyl side chain of ubiquinone Q8 was very low. To improve the incorporation yield of ME, an E. coli mutant was constructed in which the gene of DXP synthase was disrupted. Such a mutant had to be complemented with ME, which served as the only isoprenoid precursor. With such a mutant, a quantitative ME incorporation into the prenyl chains of ubiquinone and menaquinone was expected. It therefore represented the best tool for the incorporation of the two deuterium-labelled [1,1-2H]ME (4a) and [3,5,5,5-2H]ME (4b) isotopomers described here (Scheme 1).

EXPERIMENTAL

Insertional disruption of the dxs gene of E. coli by site-directed marker insertion mutagenesis

A 7.9 kb Xhol–PstI fragment containing the E. coli dxs gene was excised from plasmid pLR1 and cloned into plasmid pBluescript

Abbreviations used: DMAPP, dimethylallyl diphosphate; DX, 1-deoxy-D-xylulose; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; FAB, fast atom bombardment; IPP, isopentenyl diphosphate; ME, 2-C-methyl-D-erythritol; MEP, 2-C-methyl-D-erythritol 4-phosphate.

1 To whom correspondence should be addressed (e-mail mirohmer@chimie.u-strasbg.fr).

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to generate plasmid pLR5 (Figure 1A) [17]. The CAT (chloramphenicol acetyltransferase) gene present in plasmid pCAT19 [25] was excised by digestion with XbaI and PstI, treated with T4 DNA polymerase and cloned between the two HindIII sites present in the dxs gene by blunt-end ligation (HindIII cohesive ends were previously converted to blunt ends by treatment with T4 DNA polymerase) resulting in plasmid pLR6 (Figure 1A). Restriction enzyme mapping was used to identify those clones in which the CAT gene was in the same orientation as the dxs gene. Plasmid pLR7 was constructed by subcloning the NheI–SphI fragment excised from pLR6 into the corresponding sites of plasmid pBR322 (Figure 1A). Plasmid pLR7 was linearized by digestion with PstI, incubated with calf intestine alkaline phosphatase and purified by agarose gel electrophoresis. The purified linear plasmid (250 ng) was used to transform strain JC7623 [26]. Transformed cells were plated on Luria–Bertani plates containing tryptone (10 g/l), yeast extract (5 g/l), NaCl (5 g/l), DX (2 mM) and chloramphenicol (17 μg/ml). Colonies showing both chloramphenicol resistance and DX auxotrophy were selected for further studies. The presence of the CAT insertion into the dxs gene was checked by PCR with primers Pr1 (5'-CCGGTTTTATCGCCCCACTG-3') and Pr2 (5'-GGAGTCCAGTGCCCTGAG-3') (Figure 1B). Bacteriophage P1 lysates obtained after infection of one of the selected strains was used to transduce the CAT insertion into strain MC4100 [27]. The resulting strain was designated MC4100dxs::CAT. The presence of the CAT insertion into the dxs gene was checked by PCR with the above-mentioned primers. Although strain MC4100dxs::CAT showed good growth rates when the medium was supplemented with DX, its growth in the presence of ME (2 mM) was significantly lower. Derived strains with an improved capacity for ME utilization were selected by repeated plating on Luria-Bertani medium supplemented with ME.

**Cell culture, labelling experiments and isolation of ubiquinone Q8 and menaquinone MK8**

*Escherichia coli* MC4100dxs::CAT mutant was grown for 24 h in a medium containing tryptone (16 g/l), yeast extract (10 g/l), NaCl (5 g/l), chloramphenicol (17 mg/l), thiamin (1.5 μM), pyridoxol (1.5 μM) and ME (0.1 mM). The pH was adjusted to 7. Media were sterilized by heating at 120 °C for 30 min. Thiamin, pyridoxol and ME were sterilized separately by filtration (Millipore 0.22 μm filters). Incubation of [1,1-2H2]ME (4a) was performed with a 1-litre culture yielding ubiquinone Q8 (7a).
Extracted with chloroform and washed with hexane. After evaporation of the hexane, the non-polar lipids were separated by TLC on silica gel (dichloromethane), yielding ubiquinone Q8 (7, Rf 0.60) and menaquinone MK8 (8, Rf 0.90), which were further purified by TLC [dichloromethane/cyclohexane (60:40, v/v), Rf 0.60].

Analytical methods

1H-NMR spectra were measured either on a Bruker AV400 or on a Bruker ARX500 spectrometer, and 13C-NMR spectra on a Bruker ARX500 spectrometer equipped with a Silicon Graphics station under the following conditions: 30° pulse, 4 s repetition time, 20.03 p.p.m. spectral width, 0.15 Hz fid resolution, digital acquisition mode, 1H decoupling by WALTZ 16 during acquisition and relaxation. All NMR spectra were recorded at 300 K. Electron-impact MS was performed by direct inlet on a Finnigan-MAT TSQ 700 spectrometer with a 70 eV ionization energy; positive fast atom bombardment (FAB) MS was performed on a ZAB-HF spectrometer with an acceleration potential of 8 keV by using m-nitrobenzyl alcohol as matrix and xenon as ionization gas.

NMR data of deuterium-labelled ubiquinone and menaquinone

Ubiquinone Q8 (7a) resulting from the incubation of [1,1-2H2]ME. 1H-NMR (C6D6, 400 MHz): δ (p.p.m.) = 1.57 (3H, broad s, 33'-CH3), 1.59 (18H, broad s, 34'-, 35'-, 36'-, 37'-, 38' and 39'-CH3), 1.64 (1H, broad s, 32'-C2H5H), 1.74 (3H, broad s, 40'-CH2), 2.01 (3H, s, 6-CH3), 2.05 (14H, m, 5'-, 9'-, 13'-, 17'-, 21'-, 25'- and 29'-CH2), 3.18 (2H, d, J = 7.0 Hz, 1'-CH2), 3.98 (3H, s, -OCH3), 3.99 (3H, s, -OCH3), 4.93 (1H, t, J = 1.5 Hz, J = 7.0 Hz, 2'-H), 5.02 (1H, t, J = 1.5 Hz, J = 7 Hz, 6'-H), 5.11 (5H, t, J = 1.5 Hz, J = 7.0 Hz, 10'-, 14'-, 18'-, 22'-, 26'- and 30'-H).

Ubiquinone Q8 (7b) resulting from the incubation of [3,5,5,5-2H4]ME. 1H-NMR (C6D6, 500 MHz): δ (p.p.m.) = 1.67 (3H, s, 32'-CH3), 1.97 (14H, m, 4'-, 8'-, 12'-, 16'-, 20'-, 24'- and 28'-CH2), 2.01 (3H, s, 6-CH3), 2.05 (14H, m, 5'-, 9'-, 13'-, 17'-, 21'-, 25'- and 29'-CH2), 3.18 (2H, d, J = 7 Hz, 1'-CH2), 3.98 (3H, s, -OCH3), 3.99 (3H, s, -OCH3), 4.93 (1H, t, J = 7.1 Hz, 2'-H), 5.06 (1H, t, J = 6.9 Hz, 6'-H), 5.09 (1H, t, J = 6.9 Hz, 10'-H), 5.11 (4H, t, J = 6.9 Hz, 14'-, 18'-, 22' and 26'-H).

H-NMR (C6D6, 61 MHz): δ (p.p.m.) = 1.57 (2H, 33', 34', 35', 36', 37', 38' and 39'-CH3), 1.71 (3H, 40'-CH3), 5.14 (1H, 30'-H).

Ubiquinone Q8 (natural abundance). 13C-NMR (C6D6, 125 MHz): δ (p.p.m.) = 1.96 (methyl group at C-5), 16.04 (C-3', C-5', C-35', C-36', C-37', C-38' and C-39'), 16.38 (C-33'), 17.70 (C-40'), 25.33 (C-1'), 25.71 (C-32'), 26.55, 26.71, 26.74 and 26.80 (C-5', C-9', C-13', C-17', C-21', C-25' and C-29), 39.75 and 39.77 (C-4', C-8', C-12', C-16', C-20', C-24' and C-28), 61.17 (-OCH3), 118.88 (C-2'), 123.87 (C-6'), 124.18 (C-10'), 124.30 (C-14', C-18', C-22' and C-26'), 124.44 (C-30'), 131.27 (C-31'), 134.91 and 134.96 (C-15', C-19', C-23' and C-27), 135.03 (C-11'), 135.28 (C-7'), 137.66 (C-3'), 138.89 and 141.73 (C-5 and C-6), 144.27 and 144.41 (C-2 and C-3), 183.94 and 184.80 (C-1 and C-4).

H-labelled ubiquinone Q8. 13C-NMR (C6D6, 125 MHz): δ (p.p.m.)/1H-induced shift (p.p.b.) = 11.95 (methyl group at C-5), 15.22 (-C2H5, 300 MHz: δ (p.p.m.)/1H-induced shift (p.p.b.) = -820 p.p.m., heptet, JCH/CH2 = 2000 Biochemical Society
RESULTS

Incorporation of [1,1-\textsuperscript{2}H\textsubscript{5}]ME

[1,1-\textsuperscript{2}H\textsubscript{5}]ME (4a) was synthesized from 3-methylfuran-2(5H)-furanone as described previously [12]. Ubiquinone Q8 (7a) was verified by \textsuperscript{13}C-NMR spectroscopy and MS. All analytical data showed that the quinone synthesized de novo was synthesized solely from deuterium-labelled precursor added to the culture medium. Indeed, only one ubiquinone isotopomer with 16 deuterium atoms synthesized from [1,1-\textsuperscript{2}H\textsubscript{5}]ME (4a) (representing 92\% of the total ubiquinone content) was detected next to small amounts of unlabelled ubiquinone (8\%) derived from the cells of the preculture on ME of natural isotopic abundance. The \textsuperscript{1}H-NMR spectrum displayed a broad signal at 1.64 p.p.m.; it corresponded to the C-32'-methyl group and integrated for one hydrogen atom in the place of the three hydrogen atoms found in the spectrum of the unlabelled ubiquinone. No signal corresponding to C-4', C-8', C-12', C-16', C-20', C-24' and C-28' protons was detected at 1.98 p.p.m. The \textsuperscript{4}H-NMR spectrum was recorded with H-\textsuperscript{2}H-coupling as well as in H-\textsuperscript{2}H-decoupling conditions, the former allowing quantitative integration of the signals. It displayed only two signals: a doublet at 1.69 p.p.m. (\textit{J}_{\text{H-H}} = 2.2 Hz), integrating for two deuterium atoms and corresponding to the deuterated C-32'-methyl group, and a second signal at 1.96 p.p.m., integrating for 14 deuterium atoms, representing the signature of the C-4', C-8', C-12', C-16', C-20', C-24' and C-28' deuterium atoms of deuterated methylene groups. Strong evidence for an isoprenoid biosynthesis solely from the deuterated ME was obtained by FAB-MS. With this ionization method, ubiquinone is efficiently reduced into ubiquinol in the source of the mass spectrometer [12,29]. The mass spectrum displayed an \textit{m/z} 744 molecular ion in place of the \textit{m/z} 728 ion for ubiquinone Q8 (7a) of natural isotopic abundance. This corresponded to the presence of 16 deuterium atoms in the eight isoprene units of the prenyl side chain. No other ubiquinone isotopomer with a lower number of deuterium atoms was detected.

Incorporation of [3,5,5,5-\textsuperscript{2}H\textsubscript{4}]ME

[3,5,5,5-\textsuperscript{2}H\textsubscript{4}]ME (4b) was synthesized from butyne-1,4-diol [30]. The steps allowing the introduction of deuterium were a palladium(II)-catalysed hydrostannation of the triple bond and a coupling reaction between a vinyl iodide and a methyl-cycanoacrylate. Deuterated ubiquinone Q8 (7b) and menaquinone MK8 (8b) resulting from the incubation of [3,5,5,5-\textsuperscript{2}H\textsubscript{4}]ME (4b) with the \textit{E. coli} DXS-defective strain were analysed by MS and \textsuperscript{13}C-NMR spectroscopy. In the \textsuperscript{13}C-NMR spectrum of ubiquinone Q8 (7b) displayed three signals at 1.57 p.p.m. (corresponding to the C-33', C-34', C-35', C-36', C-37', C-38' and C-39'), 15.70 (C-40'), 25.72 (C-32'), 26.03 (C-1'), 26.54, 26.69, 26.70 and 26.74 and 26.80 (C-5', C-9', C-13', C-17', C-21', C-24' and C-29'), 93.71, 93.75 and 93.76 (C-1', C-8', C-12', C-16', C-20', C-24' and C-28'), 119.04 (C-2'), 123.87 (C-12'), 124.17 (C-14', C-18', C-22' and C-26'), 124.44 (C-30'), 126.22 (C-8'), 126.34 (C-5'), 131.27 (C-31'), 131.19 and 132.22 (C-4a and C-8a), 133.29 and 133.35 (C-6 and C-7'), 134.92, 134.94, 134.96, 134.97, 134.99 and 135.26 (C-7', C-11', C-15', C-19', C-23', C-27'), 136.70 (C-33'), 143.38 and 146.20 (C-2 and C-3), 184.55 and 185.50 (C-1 and C-4).


\textsuperscript{13}C-NMR spectra displayed an \textit{z}-shifted heptet at 15.22 p.p.m.
Incubation of [1,1-^3H]ME (4a) into the ubiquinone of the E. coli DXS-defective strain shed light on the quantitative incorporation of the labelled substrate into the prenyl chain from ubiquinone and menaquinone. This was in striking contrast with the modest yields observed with a wild-type strain [12]. This experiment confirmed our previous results with this wild-type strain: the two C-1 protons of MEP were integrally preserved on the corresponding carbon atoms of IPP and DMAPP, without any loss. No change occurred in the oxidation state of this carbon atom when MEP was converted into IPP and DMAPP. This suggested a key role for the carbon atom corresponding to C-3 of IPP in the reaction sequence leading from MEP to IPP.

Such a hypothesis has been corroborated by the incorporation of [4-^3H]DX into ubiquinone in a wild-type E. coli [22] and of [2-^14C, 4-^3H]DX into phytol and lutein in Catharanthus roseus cell cultures [24].

[3,5,5,5-^3H]ME (4b) was therefore incubated with the E. coli DXS-defective strain. This experiment confirmed former results obtained with [5,5,5-^3H]DX [23]. In E. coli, the methyl group of DXP or MEP is not modified and retains its three protons throughout the whole reaction sequence leading to IPP and DMAPP. Accordingly, the deuterated methyl group could serve as an internal reference for ^3H-NMR for following the fate of the C-3 deuterium when [3,5,5,5-^3H]ME (4b) was incorporated into the prenyl chains of ubiquinone and menaquinone. This C-3 deuterium was retained only in the terminal ω-isoprene unit derived from DMAPP, which served as starter for the prenyltransferase, and was completely absent from all other units derived from IPP. This result was in accord with a former incorporation of [4-^3H]DX into the ubiquinone of a wild-type E. coli that showed deuterium retention in the DMAPP-derived unit only [22].

The C-4 and C-5 carbon atoms of IPP and DMAPP were to some extent found equivalent in many fungi and plants, when label from C-2 of mevalonate was not stereospecifically incorporated into the E-methyl group of the DMAPP-derived isoprene units [33]. Extensive studies on the yeast IPP isomerase shed light on a significant lack of selectivity of this enzyme, at least in vitro [33]. No deuterium scrambling between C-4 and C-5 of IPP or DMAPP was detected by NMR spectroscopy in the E. coli isoprenoids after feeding of deuterium-labelled ME isoprenomers labelled with deuterium at C-1 or C-5, i.e. on the two carbon atoms corresponding to the methyl groups of DMAPP. Deuterium labelling from [1,1-^3H]ME (4a) was found only on carbon atoms corresponding to the C-4 E-methyl group of the DMAPP starter unit (corresponding to the C-32’ methyl group of the ω-isoprene unit of ubiquinone) and on those corresponding to C-4 of IPP. No deuterium was found on the carbon atoms derived either from the Z-methyl group of DMAPP or from the C-5 methyl group of IPP. Reciprocally, after the incubation of [3,5,5,5-^3H]ME (4b), deuterium was found only on the carbon atoms corresponding to the Z-methyl group of DMAPP (i.e. the methyl group at C-31’) and to the C-5 methyl group of IPP. Furthermore, no deuterium loss was observed on the methyl groups, either by NMR spectroscopy or by the more sensitive MS after incubation of [1,1-^3H]ME or [3,5,5,5-^3H]ME. No deuterium/proton exchange occurred on any of the carbon atoms derived from C-4 of IPP or DMAPP, indicating that a reversible activity of the IPP isomerase was not significant in the cells under the growth conditions used. The absence of methyl scrambling in the isoprene units from E. coli implies either that the reaction catalysed by the IPP isomerase is fully stereoselective in this bacterium, at least in vivo or, in the hypothesis of an incompletely selective reaction, that the isomerase activity was not significant in the labelling conditions and that IPP and
DMAPP were independently synthesized via two different reaction sequences starting from a common intermediate derived from MEP. The latter hypothesis is supported by the non-essential role in isoprenoid biosynthesis of the ido gene encoding the IPP isomerase in *Escherichia coli* [34].

The retention of the 3-H proton of MEP (i.e. of the 4-H proton of DXP) only in the DMAPP-derived isoprene units from *E. coli* and its complete absence from those formed from IPP is in accord with such a branching in the MEP pathway in *E. coli*. Even the knowledge of the enantioselectivity of an *E. coli* IPP isomerase [35] and of a farnesyl diphosphate synthase [36] (i.e. both eliminating the pro-R proton at C-2 of IPP) did not permit the decipherment of this enigma [37]. In contrast with *E. coli*, the C-4 proton of DX (corresponding to the C-3 proton of ME) was not found in all isoprene units of phytol and lutein from *Catharanthus roseus* cell cultures [24], whether they were derived from DMAPP or from IPP. In the bacterium *Zymomonas mobilis*, a similar loss of the hydrogen corresponding to the C-4 hydrogen of DX was observed: it was replaced by a hydrogen atom derived from NAD(P)H on all carbon atoms derived from C-2 of IPP and DMAPP in the triterpenes of the hopane series [28]. The 4-H of DX (i.e. the 3-H of ME) is accordingly not eliminated in the isomerization of IPP into DMAPP or in the elongation process catalysed by the prenyltransferase but it is lost in *Z. mobilis* before the formation of IPP. This indicates that DMAPP might be obtained by two different routes in the MEP pathway, depending on the organism.

The incorporation of 13C-labelled precursors allowed us to determine the formation of the carbon skeleton of isoprene units via the MEP pathway, including the transposition step, and permitted the identification of DX and ME as IPP precursors. The incorporation of deuterium-labelled glucose into the phytol of the cyanobacterium *Synechocystis* [37] or into the linalol of *Mentha citrata* [38] were characterized either by low yields and/or significant deuterium loss and scrambling resulting from glucose metabolism, yielding no clearcut data on the localization of labelling. In contrast, deuterium-labelled isomoterpomers of DX and ME proved to be useful tools for detailed investigations of the tentative identification of further intermediates and has thrown light on at least two different routes from MEP to IPP and DMAPP.

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