C-METHYLATION OF DESMETHYLMENAQUINONES: SPECIFICITY OF THE ENZYMATIC SYSTEM OF MYCOBACTERIUM PHLEI

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1. Introduction

It was previously shown that a cell-free extract from *M. phlei* was able to methylate DMK-9 * to MK-9 in the presence of S-adenosyl methionine, then to reduce it to MK-9(IIH_2) when NADH or NADPH was added [1]. We have now studied the specificity of this C-methylation system towards possible substrates differing from DMK-9 either by their chain or their ring structure. The results we report here show that only molecules having a naphthoquinonic ring are good substrates; modifications of chain length or chain structure have less important effects on methylation rate, provided they leave untouched the first isoprenoid unit.

2. Methods

Synthesis of DMK species and analogous compounds will be described elsewhere [2]. Analyses by T.L.C. showed that the *trans* form was largely predominant.

Soybean lecithins were used as micellar solutions to solubilize all DMK species; these micellar solutions were prepared either by the Fleischer-MacKlouwen technique [1,3], or by the following method: Sigma soybean lecithins (400 mg) were sonicated at 25°C during 10 min with 20 ml of a tris 0.1 M buffer, pH 7.8 containing 1.15% KCl. The resulting suspension was centrifuged at 75,000 *g* for 1½ hr. The DMK compounds (0.1 ml of a 30 mM solution in dioxane) were solubilized in 1 ml of micelles by sonicating 15 min at 25°C, then centrifuging for 25 min at 20,000 *g*. DMK concentrations in the supernatant were estimated spectrophotometrically.

Dialysed cell-free extracts were prepared as described previously [1,4].

Incubations were performed at 30°C [1] with the following modifications: incubation periods were shortened to 1 or 2 hr and total additions of ATP reduced to 1.5–2.5 μmoles/ml of final volume (12–35 ml). All incubation mixtures contained 0.5 μC/ml of L-methionine-$^{14}CH_3$ (specific activity: 10.8 mC/mMole). DMK were present at a 0.05 mM final concentration. In each experiment the amount of micellar phospholipids was adjusted to a constant value (generally 0.66 mg/ml). Incubation mixtures contained 20–25 mg of proteins/ml of final volume. From some experiments with increased amounts of DM phylloquinone, it was assumed that measured methylation rates were maximal under the above conditions.

After incubation, the quinones were extracted and purified as described previously [1] except that the hexane extract was chromatographed on silicagel G with benzene. The quinone bands were localized by comparison with test samples and eluted with ether; an aliquot was chromatographed on vaseline-impregnated paper with a vaseline-saturated acetone–water mixture (95:5 or 9:1). Chromatograms were cut in 1 cm portions which were immersed in liquid scintillation vials and subsequently counted. The total radioactivity incorporated into a methylquinone was

* Abbreviations: menaquinone-9: MK-9; II-dihydromenaquinone-9: MK-9(IIH_2); 2-desmethylmenaquinone-9: DMK-9; as recommended by the I.U.P.A.C., I.U.B. Committee (Biochim. Biophys. Acta 107 (1965) 5).

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obtained by addition of the total dpm found at the same Rf as an authentic cochromatographed specimen.

3. Results and discussion

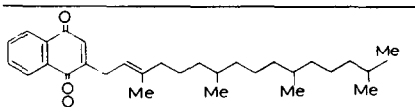
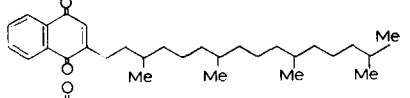
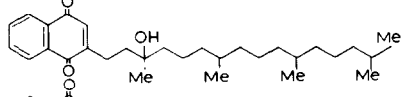
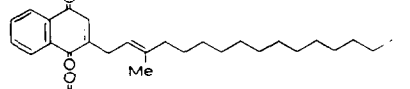
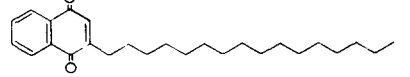
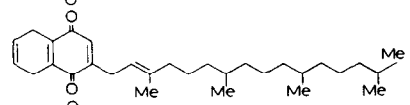
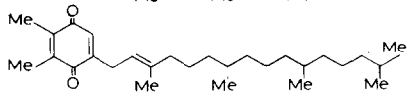
C-methylation rates of some DMK homologues are reported in table 1. In each case, it was found by chromatographic comparison with authentic MK species that the labeled methylquinones obtained were the C-methylation products of the parent DMK.

Table 1
C-methylation of desmethylmenaquinones

	Incorporation (%) *	
DMK-1	0.5 ± 0.1 **	(2 ***)
DMK-2	32 ± 7	(4)
DMK-3	100	
DMK-3(IIH_2)	104 ± 24	(3)
DMK-4	96 ± 9	(3)
DMK-6	15 ± 5	(4)
DMK-7	1.5 ± 0.8	(3)
DMK-9	3.2 ± 1.2	(4)
DMphylloquinone	60 ± 14	(3)

* Expressed for each experiment as % of the incorporation observed in MK-3.
** Mean ± standard error.
*** Number of experiments.

Table 2
C-methylation of DM phylloquinone and DM phylloquinone analogs

	Incorporation (%) *
	100
	7.5 **
	0
	26 ± 8 (5)
	< 1 (2)
	0
	0

* Expressed for each experiment as % of the incorporation observed in phylloquinone.
** Remaining activity obtained with 2',3'-dihydro-desmethyl-phylloquinone is probably due to a residue of non-hydrogenated quinone.

Several striking facts emerge from an examination of these results. First, methylation is only possible with DMK molecules having a side chain of two or more prenyl units. DMK-1 is not a substrate for the enzymatic system. Methylation of 1,4-naphthoquinone itself could not be tested in this system because it was impossible to recover added menadione from the incubation mixtures; this is probably due to its high affinity for sulphydryl groups of proteins [5,6]. However, the dependence of C-methylation activity on the presence of a polyprenyl side chain (and the lack of activity with DMK-1) indicates that 1,4-naphthoquinone would possibly remain unmethylated in this system. Consequently we can conclude that one of the biosynthetic ways to MK is the methylation of a previously formed DMK. The alternative pathway, necessitating menadione as an intermediate, cannot however be excluded by our experiments.

Another fact to be noted from the results in table 1 is the relative non-specificity of the methylation system. All DMK species tested from DMK-2 to DMK-9 are methylated, but activity reaches a maximum for DMK-3 and DMK-4; it decreases rapidly for longer chains. It is noticeable that saturation of one or more isoprenoid units, other than the first one, has little effect on the methylation rate (see DMK-3(IIH_2) or DM phylloquinone).

The results of table 2 afford complementary data about the influence of side chain structure upon methylation rate. Any modification in the *first prenyl unit* (saturation, hydroxylation of the double bond)

prevents enzymatic activity. On the other hand, replacement of the remaining part of the side chain, for example by an unbranched chain, leads only to a lowered but nevertheless appreciable activity. Any structural change introduced in the naphthoquinone part, either by partial saturation (5,8-dihydro-2-phytyl-1,4-naphthoquinone) or by replacement by a 2,3-dimethyl benzoquinone ring, induces total loss of activity.

It is somewhat surprising that the maximal rate of methylation is obtained for a side chain considerably shorter than that of the natural dihydromenaquinone. (For the structure of MK-9(IIH_2) see ref. [7].) Two interpretations can be suggested: this phenomenon may only reflect the *in vivo* relative availability of long chain polyprenyl pyrophosphates [8]; or this apparent relative specificity is an artefact derived from the DMK-solubilization technique. Anyway, it appears that most of the known enzymes of polyprenoid biosynthesis similarly exhibit a low specificity: simultaneous formation of homologous polyprenyl pyrophosphates [8,9], and ubiquinone [10] or menaquinone [11] isoprenologs, has been recently described *in vivo* and *in vitro*.

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