ENZYMATIC FORMATION OF A PHOSPHORYLATED DERIVATIVE OF VITAMIN K₁*

BY TETSUO WATANABE AND ARNOLD F. BRODIE

DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF SOUTHERN CALIFORNIA

SCHOOL OF MEDICINE, LOS ANGELES

Communicated by Martin D. Kamen, July 8, 1966

A role for naphthoquinones in oxidative phosphorylation has been demonstrated with a cell-free system from Mycobacterium phlei.¹⁻² The natural naphthoquinone of this microorganism, vitamin K₉H, functions between the flavoprotein and cytochrome b on the NAD⁺-linked and malate-vitamin K reductase pathways.³, ⁴ Evidence has been presented for the existence of a phosphorylative site between the natural quinone and cytochrome b.², ⁵, ⁶ Restoration of the quinone function has been shown to occur on supplementation with vitamin K₁ (I) to the quinone-depleted system.¹ ² ⁵

Evidence has been obtained which suggests that certain naphthoquinones may take part directly in the phosphorylative events.⁵⁻⁸ Although oxidation by the quinone-depleted system can be restored by numerous naphthoquinones, the requirements for restoration of phosphorylation are more specific. For example, certain quinones such as dihydrophytyl vitamin K₁ restore oxidation by the same pathway as vitamin K₁, but fail to restore phosphorylation at this site. The recent finding of a dependence on inorganic phosphate for tritium incorporation into the naphthoquinone under conditions of oxidative phosphorylation further suggests a direct role for phosphate during the reductive process.⁷

A number of mechanisms have been proposed for the participation of quinones in oxidative phosphorylation.⁹⁻¹³ Chemical and biological experiments with model compounds such as menadiol phosphate¹⁰, ¹⁴ and the 6-chromanyl phosphate derivative of vitamin K₁,¹⁵ have suggested a means by which ATP can be formed from the oxidation of a hydroquinone. In an earlier report,⁸ organically bound phosphate was found in association with the enzymatically formed naphthoquinol fraction. Nevertheless, the formation of a biologically active quinol phosphate has not been demonstrated. Evidence for the enzymatic formation of a naphthoquinol phosphate derivative of vitamin K₁ has now been obtained with the system from M. phlei.

Materials and Methods.—Mycobacterium phlei, ATCC 354, was grown, and cell-free homogenates were prepared by methods previously described.¹⁶ The system
for preparation of $^{32}$P- and C$^{14}$-labeled quinol phosphates consisted of 10 ml of cell-free homogenate (260 mg of protein), 10 $\mu$moles of vitamin K$_1$ prepared in Asolectin by the method of Asano et al., 60 $\mu$moles of malate, 0.25 $\mu$mole of FAD, 30 $\mu$moles of inorganic phosphate, 30 $\mu$moles of MgCl$_2$, and 200 $\mu$moles of Tris-HCl buffer, pH 7.6. The reaction mixture was incubated anaerobically for 20 min at 30°. In addition, the system contained either Pi$^{32}$ (10$^7$ cpm) or C$^{14}$-labeled vitamin K$_1$ (120, 180 cpm/$\mu$mole). The reaction was terminated by chilling the mixture to 0° and the mixture lyophilized. The lyophilized material was extracted with diethyl ether: methanol (2/1, v/v), taken to dryness, re-extracted with pure diethyl ether, and again taken to dryness. The residue was chromatographed on liquid paraffin-impregnated paper (Whatman #3 MM) with a solvent system consisting of n-butanol, ethanol, distilled water, and glacial acetic acid (100/40/60/4, v/v) in an atmosphere of nitrogen and argon. The quinones were detected by their absorption or fluorescence under UV light. The radioactivity was detected on a Nuclear-Chicago strip counter.

Results and Discussion.—A phosphorylated derivative of vitamin K$_1$ was formed by the quinone-depleted system supplemented with substrate amounts of vitamin K$_1$. The reaction mixture was incubated anaerobically in order to permit the naphthoquinone to act as an "electron sink" and thus favor the accumulation of reduced quinone derivatives. Incubation of the quinone-depleted extract, with vitamin K$_1$, inorganic phosphate containing Pi$^{32}$, and an electron donor resulted in the formation of a lipid-extractable quinone which was distinct from vitamin K$_1$ and dihydrovitamin K$_1$ (Fig. 1A). This material had an $Rf$ of 0.85 and was found to comigrate with the synthetic chromanyl phosphate derivative of vitamin K$_1$ (II).

Examination of the distribution of $^{32}$P-labeled material in the ether-extractable fraction revealed that only one main area contained $^{32}$P label and that this area corresponded to the new quinol derivative of vitamin K$_1$ ($Rf$ 0.85). A spot corresponding to dihydrovitamin K$_1$ ($Rf$ 0.60) was occasionally observed; however, phosphate was not associated with this material. The inability to find dihydrovitamin K$_1$ consistently as a product was due to the rapid autoxidation which occurs with this compound.

The conditions for maximal formation of this compound were investigated. Incubation of the system for 30 min was required for accumulation of the derivative; however, further incubation failed to yield larger amounts. The phosphorylated derivative of vitamin K$_1$ was not formed in the absence of substrate, or under
aerobic conditions. In the absence of inorganic phosphate, the compound was not formed (as determined by the lack of fluorescent material at Rf 0.85); however, material corresponding to dihydrovitamin K$_1$ (Rf 0.60) was detected. The extent of conversion of vitamin K$_1$ to the new compound was estimated by the amount of P$^{32}$ associated with the purified material and ranged between 0.3 and 0.7 per cent. Formation of the phosphorylated derivative of vitamin K$_1$ was completely inhibited by Dicumarol (10$^{-4}$ M), and partially inhibited by pentachlorophenol (10$^{-4}$ M). Both KCN (10$^{-3}$ M) and 2-nonylhydroxyquinoline N-oxide (15 µg), compounds which inhibit electron transport after the quinone site, failed to inhibit the formation of the quinol phosphate derivative.

The fractionated components of the system from M. phlei obtained by centrifugation at 140,000 × g were tested for their ability to form the new compound. Formation of the phosphorylated derivative of vitamin K$_1$ was found to occur with the particulate fraction alone or in combination with the supernatant fraction, but failed to occur with the supernatant fraction alone. This is of particular interest since the site of the endogenous naphthoquinone interaction with the respiratory chain is in the particles.

Evidence that the enzymatically formed quinol phosphate was derived from vitamin K$_1$ was obtained following incubation of C$^{14}$-labeled vitamin K$_1$ with the system from M. phlei. Material corresponding to the phosphate derivative was formed and detected by its fluorescence. Two areas containing C$^{14}$ label were

---

**Fig. 1.**—The formation of P$^{32}$- and C$^{14}$-labeled quinol phosphate. The system in A contained P$^{32}$; the system in B contained C$^{14}$-labeled vitamin K$_1$. The chromatographic behavior of some synthetic derivatives of vitamin K$_1$ is shown below (C). K refers to pure vitamin K$_1$; DHK refers to dihydrovitamin K$_1$; and CP refers to the 6-chromanyl phosphate derivative of vitamin K$_1$.

**Fig. 2.**—Formation of P$^{32}$-labeled quinol derivative. The conditions were similar to those described in Fig. 1, except that 10 µmoles of dihydrophytyl vitamin K$_1$ or desmethyl vitamin K$_1$ were substituted for vitamin K$_1$ in experiments 2 and 3.
found; one spot corresponded to vitamin K₁ (the starting material), while the other corresponded to the new quinol phosphate derivative, *R*₁ 0.85 (Fig. 1B). The C¹⁴ label was found in the same area as the P³² label in Figure 1A. The dependence on inorganic phosphate was tested with C¹⁴-labeled vitamin K₁. In the absence of inorganic phosphate, the C¹⁴ label was only detected in the spot corresponding to vitamin K₁. The dependence on inorganic phosphate for formation of this compound may explain the requirement of inorganic phosphate observed for the incorporation of tritium into vitamin K₁.¹⁷

A number of naphthoquinone analogues have been shown to restore oxidation by the same pathway as vitamin K₁, but fail to restore phosphorylation.², ⁵ Substitution of dihydrophytyl vitamin K₁ (III) or desmethyl vitamin K₁ (IV) for vitamin K₁ failed to result in the formation of the quinol phosphate of either analogue (Fig. 2). These results further indicate the specificity of the reaction with respect to the β position of the side chain and the methyl group in the 2 position of the naphthoquinone nucleus. Similar results have been obtained in studies of the specific quinone structures which are required for restoration of oxidative phosphorylation², ⁴ and for tritium incorporation into vitamin K₁.⁷

The absorption spectrum of the enzymatically formed phosphorylated derivative of vitamin K₁ was obtained following purification by two-dimensional paper chromatography (Fig. 3). This material had two major absorption peaks at 230 and 248 μm and two minor peaks at 324 and 338 μm. This spectrum of this compound was found to lack the four peaks that are characteristic of the conjugated naphtho-
quinone ring, but was similar to that exhibited by quinol derivatives. Although the absorption at 230 μ was first thought to be due to contamination with another compound, attempts to remove it by further purification have been unsuccessful. The absorption spectrum of the enzymatically formed compound differed from that exhibited by the mono- or diphosphate derivative of vitamin K₁ and from that exhibited by dihydrovitamin K₁. In many respects the enzymatically formed compound exhibited a spectrum that was similar, but not identical, to the 6-hydroxychromanol and the 6-chromanyl phosphate derivatives of vitamin K₁, differing in the additional peak at 230 μ, which is associated with the new compound. The IR spectrum of the enzymatically formed derivative of vitamin K₁ was compatible with that of a phosphorylated quinol structure.

Although the enzymatically formed material was similar to the synthetic 6-chromanyl phosphate derivative of vitamin K₁ in UV, IR, and chromatographic behavior, other properties of the enzymatically formed compound were found to differ considerably from the synthetic compound. Unlike synthetic chromanyl phosphate, the enzymatically formed compound was unstable and decomposed in 4–7 days when stored in the dry state at −14°. Some stabilization was achieved by storage under nitrogen. In addition, the compound, unlike the synthetic material, was decomposed on silica gel plates. Thus, although the new phosphorylated derivative resembles the 6-chromanyl phosphate derivative of vitamin K₁, it probably differs in structure. Further analysis is in progress to elaborate this point.

The enzymatic formation of a phosphorylated derivative of vitamin K₁ is of particular interest, since vitamin K₁ is necessary for restoration of oxidative phosphorylation by the quinone-depleted system. The mechanism and site of phosphate addition are unknown; however, studies of the site of deuterium incorporation into vitamin K₁ suggest that it does not occur following quinone methine formation.

Although the role of the phosphorylated derivative of vitamin K in biological oxidation is unknown, this bacterial system may shed some light on both the mechanism of phosphate addition and utilization at the quinone site in the respiratory chain.

Summary.—A phosphorylated derivative of vitamin K₁ was formed enzymatically by extracts of Mycobacterium phlei. Formation of the new derivative was dependent on the presence of an electron donor, inorganic phosphate, extract, and vitamin K₁ or the natural naphthoquinone (vitamin K₉H). The ultraviolet absorption spectrum of the derivative was similar to that of synthetic chromanol derivatives of vitamin K₁ and provided evidence that the enzymatically formed compound is a quinol. Although the enzymatically formed quinol phosphate co-migrated with the synthetic chromanyl phosphate derivative of vitamin K₁, the two compounds exhibited marked differences in stability. Phosphorylated derivatives were not formed with vitamin K₉ in which the β-γ position of the side chain was saturated or in which the methyl group (2 position) was lacking.

* We would like to acknowledge the technical assistance of Mrs. Hiroko Sakamoto and Mrs. Lillian Lin. This work was supported by grant AI-05637 from the National Institutes of Health, USPHS, by a grant from the National Science Foundation (GB-3583), and by the Hastings Foundation of the University of Southern California School of Medicine. This is the 21st paper in a series dealing with oxidative phosphorylation in fractionated bacterial systems.


C¹⁴-labeled vitamin K₃ was prepared by Mr. Joel Adelson by condensing phytol alcohol with C¹⁴-labeled menadione by the procedure described by L. F. Fieser, in Experiments in Organic Chemistry (Boston: D. C. Heath and Co., 1955). The labeled material was repeatedly purified by chromatography on Permutit. The purified material chromatographed as a single spot on paraffin-impregnated paper and comigrated with pure vitamin K₃.

Gutnick, D., and A. F. Brodie, unpublished observations.