Stereospecificity of Vitamin K-epoxide Reductase*

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Vitamin K epoxide can occur as a pair of optical isomers due to the asymmetry of the oxirane ring substituents. The stereoselectivity of vitamin K-epoxide reductase for the oxirane ring configuration was determined by recovery of the partially resolved unreacted substrate following incubations of racemic vitamin K epoxide with rat liver microsomes. The substrate was enriched for the (−)-enantiomer, but selectivity for the biologically relevant (+)-enantiomer was low. This result was confirmed by direct comparison of the rates of reaction for the racemic substrate and (+)-vitamin K epoxide. The selectivity of vitamin K-epoxide reductase for the cis- or trans-phytyl configuration of the vitamin K side chain was also low. These results suggest an enzyme-active site which is open toward the 2,3-orientations with respect to the positions of the methyl and phytyl side chain substituents.

A microsomal vitamin K-epoxide reductase catalyzes the reductive elimination of the oxirane ring oxygen of vitamin K-2,3-epoxide to form vitamin K quinone (1). A related microsomal vitamin K reductase activity, possibly catalyzed by the same enzyme, reduces vitamin K quinone to vitamin K hydroquinone (2–3). Both activities are sensitive to inhibition by coumarin anticoagulants, and this sensitivity is decreased in microsomal preparations from warfarin-resistant rat liver (4–6). Both activities use disulfhydryl compounds, usually dithiothreitol, and vitamin K epoxide as reductant is not known. Vitamin K-epoxide reductase appears necessary to reduce vitamin K epoxide formed by an epoxide activity reaction that is probably identical to the vitamin K-dependent carboxylation reaction (7). The disulfhydryl-dependent vitamin K reductase as well as at least two NAD(P)H:quinone dehydrogenases complete this metabolic cycle by reducing vitamin K to the hydroquinone substrate of the vitamin K-dependent carboxylase. This recycling is necessary for the continued synthesis of vitamin K-dependent proteins at physiological vitamin concentrations. (For reviews of vitamin K function, see Refs. 8–9.)

The absolute configuration of the biologically generated vitamin K epoxide has recently been determined (10). Previous studies of vitamin K-epoxide reductase have used racemic vitamin K epoxide prepared from commercially available vitamin K which contains a mixture of cis- and trans-phytyl isomers using the classical chemical synthesis (11). We now report the stereoselectivity of vitamin K-epoxide reductase for the configurations of the phytyl side chain and the oxirane ring.

MATERIALS AND METHODS

Postmitochondrial supernatants and resuspended whole microsomes equivalent to 0.5 g of liver/ml were prepared from livers of vitamin K-deficient male Holtzman rats (250 g, body weight) in 0.25 M sucrose, 0.025 M imidazole HCl, pH 7.2, as previously described (12). Incubations were at 27 °C with 0.5 ml of microsomes or postmitochondrial supernatant, 5 mM dithiothreitol, and vitamin K epoxide added in 10 μl of ethanol for a total volume of 0.52 ml. The reactions were stopped and vitamin K and vitamin K epoxide extracted by addition of two volumes of isopropanol/hexane (3:2). Vitamin K hydroquinone formed during the incubation (2) was recovered as vitamin K quinone. A 0.5-ml aliquot of extract was evaporated under nitrogen, redissolved in 200 μl of methanol, and 50 μl was analyzed by HPLC using a Waters Associates μBondapak C18 10-μm analytical column run at 2 ml/min 95% methanol, 5% water (retention times: vitamin K epoxide = 9.32 min, vitamin K = 13.17 min). External standard quantitation was based on the total integrated absorbance at 254 nm using extinction coefficients of 30,800 M⁻¹cm⁻¹ at 225 nm for vitamin K epoxide (11) and 18,900 M⁻¹cm⁻¹ at 248 nm for vitamin K (13) in hexane.

For large scale preparations, approximately 50 ml extracts were washed with saturated sodium chloride, dried over anhydrous sodium sulfate, filtered, then evaporated in vacuo at 37 °C. A trace of [3H]vitamin K epoxide was added, and the extract was chromatographed on Silica CC-4 Special (Mallinkrodt) as described by Sadowski et al. (12). The radioactive fractions were pooled, dried under nitrogen, then chromatographed using a Waters Associates μBondapak C18 10-μm semi-preparative column run at 4 ml/min 100% methanol (retention times: vitamin K epoxide = 6.87 min, vitamin K = 8.73 min). The collected fractions were concentrated in vacuo and rechromatographed using 95% methanol, 5% water (retention times: vitamin K epoxide = 15.33 min, vitamin K = 21.17 min). Purity and concentrations of the isolated materials were determined by analytical HPLC and comparison of their absorbance spectra with those of synthetic standards. Absorption spectra were measured with a Varian model 635 spectrophotometer and CD spectra with a Jasco J41-C spectropolarimeter in HPLC grade hexane using 1-cm path length cells. The isolated vitamin K was analyzed for cis- and trans-phytyl isomers by HPLC on a μPorasil 10-μm analytical column (Waters) run with 1 ml/min 7.5% chloroform in hexane (retention times: cis = 3.38 min, trans = 4.94 min).

(+) Vitamin K epoxide was isolated as above from a large scale epoxidase reaction. Liver microsomes from five rats given 5 mg/kg of warfarin intraperitoneally 18 h before being killed were resuspended in sucrose/imidazole buffer to a concentration equivalent to 0.5 g of liver/ml. The 130-ml reaction was incubated at 27 °C with constant stirring and contained 100 ml of microsomes, 20 ml of an ATP-generating mix, plus cycloheximide (12), 50 μg/ml of sodium warfarin, 10 mM sodium cyanide, 1.4 mM NADH, and 6.5 μg/ml of reduced vitamin K (12) added last dropwise in 2 ml of ethanol. After 2 h, the epoxide was extracted and purified.

Vitamin K₁, obtained from Sigma and containing 24% cis- and 76% trans-phytyl isomers, was separated by Silica Gel G column chromatography using 7.5% isobutyl ether in hexane (14). Racemic vitamin K epoxides were prepared as described by Tischler et al. (11) and purified by preparative HPLC before use. [3H]-vitamin K (148 mCi/
Vitamin K-epoxide Reductase

RESULTS AND DISCUSSION

The stereoselectivity of enzymatic vitamin K-epoxide reductase was investigated by recovering the vitamin K quinone product and the partially resolved unreacted substrate from large scale incubations using racemic vitamin K epoxide. Selective reduction of the (+)-enantiomer resulted in enrichment of the unreacted substrate for the (+)-enantiomer (Table I). The recovered epoxide exhibited a CD spectrum (negative maximum at 342 nm, positive maximum at 386 nm, \( [\theta]_{342}/[\theta]_{386} = -2.7 \)) of the same form as (+)-vitamin K epoxide isolated from rat liver (10), but of opposite sign. Collection of the unreacted substrate for the (-)-enantiomer and the similarity observed for both (+)- and (-)-vitamin K epoxide reduction was calculated. Though vitamin K epoxide reductase acted preferentially on the physiologically relevant (+)-enantiomer, the stereoselectivity of the reaction was low. The vitamin K product was also found to have about the same cis-isomer content as the vitamin K epoxide substrate. The apparent stereoselectivity for the phytanyl configuration was also low. The stereoselectivity of the reaction was essentially constant over a range of substrate concentrations and resulting percent conversions. This finding indicates the general validity of the method and suggests that the two enantiomers compete for the same enzyme, though the occurrence of two different enantiomERICALLY specific vitamin K-epoxide reductases with similar kinetic properties cannot be excluded.

Microsomal recycling of vitamin K epoxide may be presumed to occur, as vitamin K epoxide will support vitamin K-dependent carboxylations (4, 6) in intact microsomes. The coupled formation of vitamin K epoxide by vitamin K-dependent carboxylase is highly stereospecific (10), and extensive recycling of the epoxide through reduction of the racemic epoxide and subsequent stereospecific epoxidation would lead to enrichment of the recovered epoxide for the (+)-enantiomer. The apparent stereoselectivity of epoxide reductase would be decreased in proportion to the fraction of initially formed quinone which was re-epoxidized. The relative constancy of the stereoselectivity when the per cent epoxide conversion was varied, and the similarity observed for both per cent conversion and stereoselectivity for incubations conducted under air or nitrogen atmospheres, indicate that little recycling occurred in these experiments. Further, any cytosolic factors present in postmitochondrial supernatants, such as endogenous reductants or epoxide reductase stimulatory activity (15), that might be present in the postmitochondrial supernatant but not in washed microsomes, did not influence the degree of stereoselectivity of the reaction.

These results were confirmed by directly assaying vitamin K-epoxide reductase with optically-active or racemic substrate. (+)-Vitamin K epoxide was isolated from large-scale incubations of vitamin K hydroquinone with rat liver microsomes. This material had essentially the same substrate activity as racemic vitamin K epoxide (Table II). Both substrates gave approximately the same apparent \( K_m \) value of 8 \( \mu \)M. Similarly, the activity of racemic vitamin K epoxides prepared

![Fig. 1. Activity of cis- and trans-phytanyl racemic vitamin K epoxides. Double reciprocal plot of nmol of K formed versus concentration of vitamin K epoxide. The data for the two substrates were from separate microsomal preparations, and the curves have been normalized to the same maximum velocity and replotted. Samples containing 1 ml of microsomes equivalent to 0.1 g of liver/ml, 5 mM dithiothreitol, and the indicated concentrations of racemic vitamin K epoxide isolated from large scale microsomal epoxidation reactions added in 15 \( \mu \)l of ethanol. Incubations were at 27 °C for 30 min.](image-url)

### Table I

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Gas phase</th>
<th>[Vitamin K epoxide]</th>
<th>Conversion</th>
<th>[( \theta )] of recovered epoxide</th>
<th>Stereoselectivity</th>
<th>cistrans recovered product</th>
</tr>
</thead>
</table>
| PMS Open    | 52        | 12                  | -42        | 1.17 (14)                         | PMS Open (+)
| PMS Open    | 42        | 4.4                 | -21        | 1.27 (21)                         | PMS Open (+)
| Micro Open  | 21        | 22                  | -81        | 1.16 (14)                         | Micro Open (+)
| Micro Open  | 21        | 11                  | -40        | 1.19 (16)                         | Micro Open (+)
| Micro Air   | 42        | 4.7                 | -10        | 1.13 (19)                         | Micro Air (+)
| N2           | 42        | 4.8                 | -13        | 1.15 (13)                         | N2 (+) |

*Incubations of postmitochondrial supernatant (PMS) or intact microsomes (micro) were conducted as described under "Materials and Methods" for 1 h at 27 °C using 5 mM dithiothreitol and the indicated final concentrations of racemic vitamin K epoxide prepared from vitamin K containing 24:76 cistrans phytanyl isomers and were open to the air or sealed after gassing with air or N2.

* Stereoselectivity of reduction = \( SS = \frac{\text{conversion (+)}}{1 - \left(\frac{1 - \text{conversion (+)}}{1 - \text{conversion (-)}}\right)} \)

* Where ee is the fractional enantiomeric excess present in the recovered epoxide calculated as {(+)-total - [-]-total)}/[(+)-total - [-]-total]. Values in parentheses are per cent stereoselectivity = 100 for completely stereospecific reaction = 0 for completely nonselective reaction = 1 - (1/ee).

* Phytanyl isomer composition of the recovered vitamin K product.

### Table II

<table>
<thead>
<tr>
<th>[Vitamin K epoxide]</th>
<th>Activity (nmol vitamin K/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>From (+)-epoxide</td>
<td>From (-)-epoxide</td>
</tr>
<tr>
<td>[( \mu )M]</td>
<td>2.13</td>
</tr>
<tr>
<td></td>
<td>5.32</td>
</tr>
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<td></td>
<td>21.3</td>
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<td></td>
<td>42.6</td>
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![Graph](image-url)
from pure cis- and trans-vitamin K were compared. In a number of experiments, observed $K_m$ values ranged from 9 to 20 $\mu M$ for cis-phytyl vitamin K epoxide and from 3 to 12 $\mu M$ for trans-phytyl vitamin K epoxide (example in Fig. 1). These values differ only slightly and are in agreement with the low stereoselectivity observed when both isomers were present.

The low stereoselectivity of vitamin K-epoxide reductase suggests that the enzyme has a binding site which is open toward the 2,3-position and is able to accommodate the significantly different steric requirements imposed by a cis-versus trans-phytyl side chain. These observations are consistent with the inhibition of vitamin K-epoxide reductase in vivo and in vitro by a wide range of 4-hydroxycoumarin anticoagulants having varied and quite large 3-substituents (3, 5, 16-18). Most of the specificity for anticoagulant activity resides in the aromatic head groups with only slight changes in activity resulting from differences in the side chain beyond the first methylene. The approximately equal activity of both enantiomers of vitamin K epoxide suggests that substrate binding to the enzyme may occur with both possible orientations of the 2-methyl and 3-phytyl groups. Rotation of the naphthoquinone plane would then place the oxirane ring in the same position at the active site (Fig. 2). Alternately, if binding occurs with a specific side chain orientation, the catalytic residues must interact mainly with the planar diketobenzenoid portion of the molecule. This latter possibility would place significant limitations on the possible mechanism of vitamin K-epoxide reductase action.

REFERENCES