JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 481–486

Received July 24, 2000
Accepted November 28, 2000
ISSN 0022-3573

Characterization and purification of the vitamin K_1 2,3 epoxide reductase system from rat liver

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Abstract

The enzyme vitamin K_1 2,3 epoxide reductase is responsible for converting vitamin K_1 2,3 epoxide to vitamin K_1 quinone thus completing the vitamin K cycle. The enzyme is also the target of inhibition by the oral anticoagulant, *R,S*-warfarin. Purification of this protein would enable the interaction of the inhibitor with its target to be elucidated. To date a single protein possessing vitamin K_1 2,3 epoxide reductase activity and binding *R,S*-warfarin has yet to be purified to homogeneity, but recent studies have indicated that the enzyme is in fact at least two interacting proteins. We report on the attempted purification of the vitamin K_1 2,3 epoxide reductase complex from rat liver microsomes by ion exchange and size exclusion chromatography techniques. The intact system consisted of a warfarin-binding factor, which possessed no vitamin K_1 2,3 epoxide reductase activity and a catalytic protein. This catalytic protein was purified 327-fold and was insensitive to *R,S*-warfarin inhibition at concentrations up to 5 mM. The addition of the S-200 size exclusion chromatography fraction containing the inhibitor-binding factor resulted in the return of *R,S*-warfarin inhibition. Thus, to function normally, the rat liver endoplasmic reticulum vitamin K_1 2,3 epoxide reductase system requires the association of two components, one with catalytic activity for the conversion of the epoxide to the quinone and the second, the inhibitor binding factor. This latter enzyme forms the thiol-disulphide redox centre that in the oxidized form binds *R,S*-warfarin.

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Acknowledgement and funding: G. B. Steventon would like to thank RP Scherer, UK for the donation of the HPLC instrumentation. L. A. Begent was supported by a Biotechnology and Biological Sciences Research Council quota studentship and A. P. Hill by a University of the West of England Development Research studentship.

Introduction

Vitamin K_1 is an essential co-factor in hepatocytes for the endoplasmic reticulum enzyme, vitamin K_1-dependent carboxylase (Sadowski & Suttie 1977), which converts inactive precursors of vitamin K_1-dependent blood-clotting factors into coagulation zymogens (Suttie 1987). This is achieved by the carboxylation of glutamic acid side chain groups in the precursor proteins resulting in the formation of γ-carboxyglutamic residues in the proteins (Suttie 1987). This post-translational modification via γ-carboxylation of glutamic acid residues, first discovered for prothrombin (Nelsestuen et al 1974), is also seen in other coagulation factors, namely factor VII, IX, X (Stenflo et al 1974) and protein Z (Di Scipio et al 1977) and the coagulation factors, protein C (Stenflo 1976) and S (Prowse & Esnouf 1977).

The hepatic endoplasmic reticulum enzyme, vitamin K_1 2,3 epoxide reductase, is responsible for the conversion of the vitamin K_1 2,3 epoxide into the quinone form, vitamin K_1 (Gardill & Suttie 1990). Whether vitamin K_1 2,3 epoxide reductase converts the metabolite to the hydroquinone form or whether a second enzyme is responsible is a matter of debate. The available evidence suggests that these are

identical proteins in terms of their cellular location, catalytic activities, substrate specificities and inhibitor sensitivity (Gardill & Suttie 1990). Vitamin K_1 2,3 epoxide reductase and the postulated vitamin K_1 quinone reductase are highly sensitive to inhibition by coumarin drugs (Suttie 1987; Shearer 1990), of which *R,S*-warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin) is the most commonly prescribed oral anticoagulant in the UK. Inhibition of vitamin K_1 2,3 epoxide reductase in-vivo by *R,S*-warfarin results in the accumulation of vitamin K_1 2,3 epoxide in the plasma of patients (Shearer et al 1977; Choonara et al 1988).

To date there are two alternative hypotheses concerning the nature of vitamin K_1 2,3 epoxide reductase. Mukharji & Silverman (1985), using bovine microsomes as the source of enzyme, suggested that at least two enzymes were responsible for the metabolism of vitamin K_1 2,3 epoxide. One enzyme was found to be insensitive to warfarin but converted the substrate into the apparent "dead end metabolites", 2- and 3-hydroxy-2-methyl-3-phytyl-2,3-dihydronaphthoquinone. This non-warfarin sensitive enzyme was purified to homogeneity and was found to be a dimer of molecular weight 25 000 Da (Mukharji & Silverman 1985). A second protein from bovine liver was also found to mediate the conversion of vitamin K_1 2,3 epoxide to vitamin K_1 quinone and this second enzyme was found to be sensitive to *R,S*-warfarin inhibition. This second protein was, however, not purified to homogeneity (Mukharji & Silverman 1985).

A second investigation using rat liver microsomes yielded a more complex picture (Cain et al 1997; Wallin & Guenthner 1997). Here the vitamin K_1 2,3 epoxide reductase was found to be a complex formed from the association of a dimeric cytosolic α-class glutathione S-transferase enzyme and the endoplasmic reticulum enzyme, epoxide hydrolase (microsomal epoxide hydrolase). The glutathione S-transferase enzyme possesses two glutathione-binding sites, one on each sub-unit. One of the glutathione-binding sites on the glutathione S-transferase protein interacts with one of the two vitamin K_1 2,3 epoxide-binding sites on the microsomal epoxide hydrolase. This association of proteins results in the formation of the warfarin sensitive thiol-disulphide redox centre, which is known to exist in the enzyme (Lee & Fasco 1984). This thiol-disulphide redox centre is now inaccessible to glutathione and vitamin K_1 2,3 epoxide. The second vitamin K_1 2,3 epoxide-binding site on the microsomal epoxide hydrolase binds the substrate, vitamin K_1 2,3 epoxide (Cain et al 1997). The cytosolic 66–74000 Da glutathione S-transferase has been purified to homogeneity (Cain et al 1997) but the catalytic protein, microsomal epoxide hydrolase was only purified 118-fold (Wallin & Guenthner 1997). Thus from the available evidence it appears that either vitamin K_1 2,3 epoxide reductase exists as more than one catalytic protein or that the enzyme is a complex between cytosolic and endoplasmic reticulum proteins.

We report on the purification and characterization of rat hepatic vitamin K_1 2,3 epoxide reductase to investigate these hypotheses further.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma-Aldrich Chemical Co. Ltd (Poole, Dorset, UK) with the exception of the high performance liquid chromatography (HPLC) grade methanol which was obtained from BDH Laboratory Supplies (Poole, Dorset, UK). A Spherisorb 10 μ ODS2 (250 × 4.6 mm i.d.) HPLC column was obtained from Phenomenex (Macclesfield, UK). The mono S ion exchange column (8.8 × 1.2 cm i.d.), hydroxyapatite macro-prep ceramic type I ion exchange column (11.5 × 2.8 cm i.d.) and a DEAE (Diethylaminoethyl)-10-bioscale ion exchange column (15.0 × 2.0 cm i.d.) were obtained from Bio-Rad Labs Ltd (Hemel Hempstead, Hertfordshire UK). The Sephacryl S-200 HiPrep 26/60 size exclusion column (72.0 × 2.8 cm i.d.) was obtained from Pharmacia Biotech Ltd (St Albans, Hertfordshire, UK).

Apparatus

The HPLC system consisted of a Dionex GMP2 quaternary pump in line with a Marathon variable injection autosampler and Dionex UVW UV/vis detector. Quantification was carried out via a Hewlett Packard (HP 3394) integrator.

The fast protein liquid chromatography system consisted of a P-500 pump/injector in line with a single path monitor UV-1 detector and a Frac 100 fraction collector. The system was controlled by a LCC-501 plus controller.

Synthesis of vitamin K_1 2,3 epoxide

Vitamin K_1 2,3 epoxide was synthesized by the method of Tishler et al (1940).

Purification of vitamin K_1 2,3 epoxide reductase and microsomal enzyme assay

Rat liver microsomes were prepared and solubilized according to the method of Mukharji & Silverman (1985) with the exception that the livers were from

female Wistar rats, 200–250 g. Microsomal vitamin K_1 2,3 epoxide reductase activity and enzyme purification were carried out as described by Mukharji & Silverman (1985).

HPLC assay of reaction products from in-vitro enzyme assay

HPLC analysis was based on the method of Mukharji & Silverman (1985). Using methanol (100 %) as the eluant at a flow rate of 2.0 mL min^{-1}, the vitamin K_1 and vitamin K_1 2,3 epoxide were separated on a Spherisorb 10 μ ODS2 column with UV detection at 247 nm. Retention times were 5.20 min for vitamin K_1 2,3 epoxide and 8.25 min for vitamin K_1.

Protein assay

The dye-binding method using Coomassie Brilliant Blue G-250 (Bradford 1976) was utilized to determine protein concentration.

Enzyme kinetics

Enzyme activity was analysed by the "Leonora" steady-state enzyme kinetics program version 1 for both Michaelis–Menten and Michaelis–Menten with substrate inhibition kinetics (Cornish-Bowden 1994). Statistical analysis of the data was carried out by the Student's *t*-test.

Results

The kinetic parameters, K_m (Michalis–Menten Constant) and V_{max} (maximal velocity), for the substrate and cofactor of vitamin K_1 2,3 epoxide reductase determined by using rat liver microsomal preparations are presented in Table 1. The substrate, vitamin K_1 2,3 epoxide had a K_m of 1.3 ± 0.14 μM and a V_{max} of 0.17 ± 0.015 nmol min^{-1} (mg protein)$^{-1}$. The cofactor, DTT (D,L-dithiothreitol), had a K_m of 0.42 μM and a V_{max} of 0.80 ± 0.25 nmol min^{-1} (mg protein)$^{-1}$. The effect of 1.7 μM *R,S*-warfarin (concentration cause 50 % inhibition, IC50 concentration, see Table 1) on the K_m and V_{max} of substrate and cofactor showed that for the substrate, the K_m (1.60 ± 0.20 μM, $P > 0.05$) was unaffected by the inhibitor but the V_{max} (0.07 ± 0.008 nmol min^{-1} (mg protein)$^{-1}$, $P < 0.01$) showed a 2.4-fold decrease (Table 1). In contrast, the cofactor showed a fivefold increase in the K_m (2.12 ± 0.95 μM, $P < 0.05$). The V_{max}, however, was not significantly affected by the inhibitor (0.99 ± 0.25 nmol min^{-1} (mg protein)$^{-1}$,

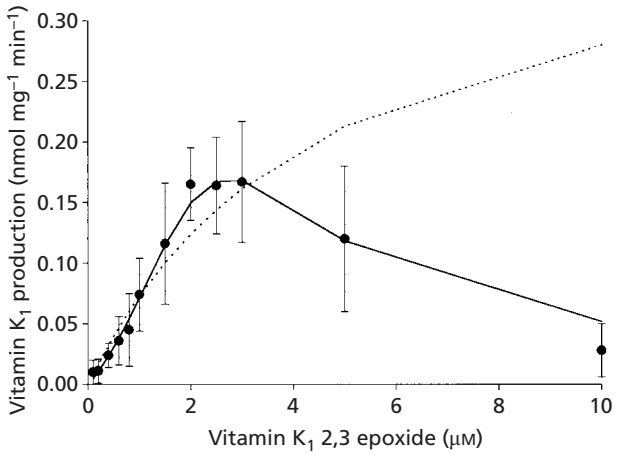

Figure 1 V against S plot for vitamin K_1 2,3 epoxide reductase using vitamin K_1 2,3 epoxide as the substrate. ●, Experimental data; – – –, Leonora generated best fit to experimental data using Michaelis–Menten equation; –, Leonora generated best fit to experimental data using Michaelis–Menten with substrate inhibition equation. Data expressed as mean ± s.d., n = 6.

Table 1 Kinetic data for microsomal vitamin K_1 2,3 epoxide reductase in the presence and absence of *R,S*-warfarin.

Substrate/cofactor	K_m (μM)	V_{max} (nmol min^{-1} (mg protein)$^{-1}$)	IC50 *R,S*-warfarin (μM)
No *R,S*-warfarin present			
Vitamin K_1 2,3 epoxide	1.30 ± 0.140	0.170 ± 0.015	1.7 ± 0.16
DTT	0.42 ± 0.120	0.800 ± 0.250	1.6 ± 0.14
1.7 μM *R,S*-warfarin present			
Vitamin K_1 2,3 epoxide	1.60 ± 0.200	0.070 ± 0.008**	
DTT	2.12 ± 0.950*	0.990 ± 0.250	

The results are the mean ± s.d. of six determinations. *$P < 0.01$, **$P < 0.05$, Student's *t*-test.

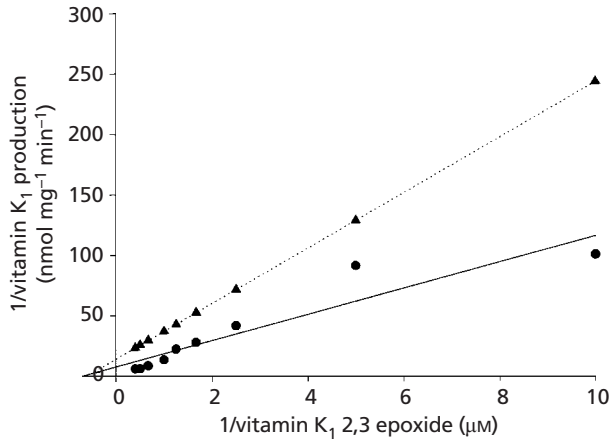

Figure 2 Lineweaver-Burk plots of vitamin K_1 2,3 epoxide reductase activity in the presence and absence of *R,S*-warfarin. –, No *R,S*-warfarin; – – –, 1.7 μM *R,S*-warfarin.

$P > 0.05$). The kinetic data for the substrate showed considerable deviation from Michaelis–Menten kinetics (Figure 1) and was therefore modelled by the Michaelis–Menten equation with substrate inhibition, since above a concentration of 3 μM substrate inhibition was a factor (Figure 1). Analysis of the K_m and V_{max} data from Table 1 and the linear plots in Figure 2 indicated that *R,S*-warfarin was a mixed non-competitive inhibitor vs the substrate, vitamin K_1 2,3 epoxide (K_m unchanged and V_{max} altered, see Table 1) and a competitive inhibitor against the cofactor, DTT (K_m altered and V_{max} unchanged, see Table 1).

The solubilization of the microsomal enzyme from the membrane was carried out using homogenizing buffer containing 1% (w/v) 3-[(3-cholamidopropyl) dimethylaminonio]-1-propane sulphonate and 500 mM NaCl. (Table 2). This solubilized fraction was then subjected to a series of ion exchange chromatography steps that resulted in a 100-fold purification of the rat hepatic enzyme. A similar purification process carried out on bovine hepatic vitamin K_1 2,3 epoxide reductase resulted in a 336-fold purification (Mukharji & Silverman 1985). Since a warfarin sensitive and insensitive vitamin K_1 2,3 epoxide reductase has been reported in the literature, the following procedure was carried out after each step in the purification procedure. All fractions containing vitamin K_1 2,3 epoxide reductase activity were assayed for the ability of the IC50 *R,S*-warfarin concentration to produce inhibition of the enzyme activity. This was observed in all the fractions with the exception of the Sephacryl S-200 size-exclusion column chromatography stage. The size exclusion column produced two fractions when monitored at 280 nm. The first fraction, peak 1 exhibited no reductase activity (see Table 3) but the peak 2 fraction did exhibit vitamin K_1 2,3 epoxide reductase activity. However, the partially purified enzyme (327-fold, see Table 2) was no longer susceptible to inhibition by *R,S*-warfarin. Increasing the *R,S*-warfarin from 1.7 μM to 5 mM, a concentration that normally resulted in > 99% inhibition of enzyme activity, resulted in no inhibition of enzyme activity. A series of experiments were then undertaken to establish if the partially purified fraction could regain its warfarin sensitivity (Table 3). Combining both fractions from the size exclusion column (peak 1 + 2 fractions) resulted in a combined fraction with enzyme activity but which was refractory to warfarin inhibition. Combining the void

Table 2 Purification factors and specific activities of vitamin K_1 2,3 epoxide reductase following various column chromatography procedures.

Procedure	Total volume (mL)	Total protein (mg)	Total activity (nmol min^{-1})	Protein (mg mL^{-1})	Activity (nmol min^{-1} mL^{-1})	Specific activity (nmol min^{-1} mg^{-1})	Purification factor
Microsomes*	200	8600	3.6	43.0	0.018	4.186×10^{-4}	
Solubilized microsomes*	200	8000	16.0	40.0	0.080	2×10^{-3}	5
MonoS eluant*	100	800	12.0	8.0	0.120	0.015	36
Hydroxyapatite eluant*	98	708	11.3	7.08	0.113	0.016	38
DEAE eluant*	80	258	11.0	3.23	0.138	0.042	100
S-200 eluant†	50	62	8.5	1.24	0.170	0.137	327

*Fifty percent inhibition of vitamin K 2,3 epoxide reductase activity in presence of 1.7 μM *R,S*-warfarin. †Zero percent inhibition of vitamin K 2,3 epoxide reductase activity in presence of 1.7 μM *R,S*-warfarin.

Table 3 Warfarin inhibition studies on vitamin K_1 2,3 epoxide reductase activity following size exclusion chromatography.

Sephacryl S-200 column fraction	Vitamin K_1 2,3 epoxide reductase activity (nmol mg^{-1} min^{-1})	Vitamin K_1 2,3 epoxide reductase activity (nmol mg^{-1} min^{-1}) in presence of 1.7 μM *R,S*-warfarin
Peak 1 fraction	< 0.005	< 0.005
Peak 2 fraction	0.139 ± 0.009	0.137 ± 0.008
Peak 1 + 2 combined fractions	0.140 ± 0.008	0.135 ± 0.010
Void volume fraction	< 0.005	< 0.005
Peak 1 + void volume fraction	< 0.005	< 0.005
Peak 2 + void volume fraction	0.138 ± 0.007	0.070 ± 0.005
Peak 2 + heat-denatured void volume fraction	0.140 ± 0.008	0.136 ± 0.009

volume fraction and peak 1 fraction produced no enzyme activity. However, combining the void volume fraction with peak 2 fraction produced a combined fraction that showed enzyme activity and was also warfarin sensitive. The final experiment was to heat-denature the void volume fraction and combine this with the peak 2 fraction. This combined fraction possessed enzyme activity that was now warfarin insensitive. Thus the void volume fraction from the size exclusion column contained one or more components that appeared to be the target for warfarin binding in the rat microsomal membranes.

Discussion

Examination of rat hepatic microsomal preparations for the metabolism of vitamin K_1 2,3 epoxide indicated that at substrate concentrations > 3.0 μM substrate inhibition occurred (Figure 1). A similar observation has been reported previously where vitamin K_1 2,3 epoxide concentrations > 10 μM were shown to cause substrate inhibition (Hazelett & Preusch 1988). The substrate inhibition seen with vitamin K_1 2,3 epoxide concentrations above 3 μM were probably not physiological since intra-hepatic concentration of vitamin K_1 2,3 epoxide are probably below 1.5 μM (Haroon & Hauschka 1983; Thijssen & Drittij-Reijnders 1993). In man, hepatic vitamin K_1 concentrations ranged from 1.3 to 6.9 ng (g tissue)$^{-1}$ and from 17.5 to 70 pg (mg microsomal protein)$^{-1}$ (Thijssen & Drittij-Reijnders 1993). Those reported in the rat ranged from 4.9 to 12.2 ng (g tissue)$^{-1}$ in male rats and from 3.0 to 11.1 ng (g tissue)$^{-1}$ in female rats (Haroon & Hauschka 1983). The K_m and V_{max} values for the substrate and cofactor were in agreement with literature values (Lee & Fasco 1984; Gardill & Suttie 1990).

The results of the enzyme kinetic study in the presence of the IC50 concentration of *R,S*-warfarin can be seen in Table 1 and Figure 2. The V_{max} for vitamin K_1 2,3 epoxide ($P < 0.05$) and K_m values for DTT ($P < 0.05$) were significantly different from the values reported in the absence of the inhibitor (Table 1). Analysis of the Lineweaver-Burk plot (Figure 2) indicated that *R,S*-warfarin was a mixed non-competitive inhibitor against the substrate, vitamin K_1 2,3 epoxide. Thus, the inhibitor and substrate were not binding to the same site on the enzyme but the binding of the inhibitor to the enzyme was either influencing the binding of the substrate or the flow of reducing equivalents to the enzyme's active site. The results for the cofactor, DTT, indicated that *R,S*-warfarin was a competitive inhibitor vs DTT (see Table 1), which indicated that the cofactor and inhibitor bind to the same site on the enzyme, presumably the thiol-disulphide redox centre (Lee & Fasco 1984; Cain et al 1997). This data is in agreement with previous reports (Soute et al 1992; Preusch 1992). There was no significant difference between IC50 values for the *R,S*-warfarin against either DTT or vitamin K_1 2,3 epoxide, indicating that the effect of the inhibitor at the cofactor binding site similarly affected the substrate binding site.

The protein involved in the conversion of vitamin K_1 2,3 epoxide to vitamin K_1 quinone was not purified to homogeneity in this investigation (Table 2). However, significant advances were made in this study. Following the procedures of Mukharji & Silverman (1985), a 327-fold purification was achieved which should be compared with that reported by Wallin & Guenthner (1997) for the rat of a 118-fold purification. During the purification procedure (Table 2), the fractions containing vitamin K_1 2,3 epoxide reductase activity were investi-

gated for *R*,*S*-warfarin inhibition by the IC50 concentration of the inhibitor. All the procedures up to and including the DEAE-cation exchange chromatography resulted in fractions of eluant containing vitamin K_1 2,3 epoxide reductase activity that were *R*,*S*-warfarin sensitive (Table 2). However, the size exclusion chromatography on the Sephacryl S-200 column produced a fraction containing vitamin K_1 2,3 epoxide reductase activity that was *R*,*S*-warfarin insensitive (Table 2 and 3). An unknown factor, called here the "warfarin binding factors" was found to be present in the void volume fraction, the addition of which to the vitamin K_1 2,3 epoxide reductase active fraction resulted in the restoration of *R*,*S*-warfarin inhibition of vitamin K_1 2,3 epoxide to vitamin K_1 quinone conversion.

The results of this investigation support the hypothesis of Cain et al (1997) that the vitamin K_1 2,3 epoxide reductase is a complex of at least two components, one possessing catalytic activity for the conversion of vitamin K_1 2,3 epoxide to vitamin K_1 quinone. The second component contains the binding site for the exogenous (DTT) and endogenous disulphide reductant. This thiol-disulphide redox centre was also the site of *R*,*S*-warfarin binding. Once bound to this element (warfarin binding factors), the inhibitor prevents the flow of reducing equivalents to the vitamin K_1 2,3 epoxide reductase. The identification of the warfarin binding factors and the identification of vitamin K_1 2,3 epoxide reductase as microsomal epoxide hydrolase were not possible in this study. However, the possibility that the warfarin binding factors and the cytosolic α-class glutathione S-transferase are the same component is difficult to rationalize due to the fact that the size exclusion limits for the Sephacryl S-200 column are < 5000 and $> 250\,000$ Da and the molecular mass of the glutathione S-transferase is 66–74 000 Da. Thus the glutathione S-transferase should not appear in the void volume. The possibility that there may be more than one enzyme involved in the metabolism of vitamin K_1 2,3 epoxide cannot, however, be discounted.

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