Vol. 104, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1982 Pages 187-192

VITAMIN K_1 HYDROQUINONE FORMATION CATALYZED BY DT-DIAPHORASE

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Received November 19, 1981

SUMMARY: Vitamin K_1 hydroquinone has been identified as a metabolite of vitamin K_1 biotransformation catalyzed by highly purified DT-diaphorase (NAD(P)H dehydrogenase, EC 1.6.99.2) isolated from livers of 3-methylcholanthrene induced rats. The hydroquinone was sufficiently stable to permit enzymatic reactions to be conducted under an atmosphere of air and quantitation of hydroquinone by high performance liquid chromatography. Based on kinetic data reported here, warfarin and probably dicoumarol at therapeutic levels do not appreciably affect DT-diaphorase catalyzed vitamin K hydroquinone formation.

INTRODUCTION

DT-diaphorase (NAD(P)H dehydrogenase, EC 1.6.99.2) is a flavoenzyme which catalyzes the reduction of a variety of redox-dyes such as DCIP[1](1). The enzyme occurs primarily in the cytoplasm of the liver although traces of it have been detected in mitochondria and microsomes(2). DT-diaphorase is induced 3 to 4 fold in rats by 3-methylcholanthrene and the induced enzyme is electrophoretically and immunologically indistinguishable from the native form(3).

4-Hydroxycoumarin and indanedione anticoagulants, especially dicoumarol, are effective inhibitors of DT-diaphorase(1). This coupled with the ability of the diaphorase to rapidly reduce menadione (vitamin K_3) led to the hypothesis that a physiological role of this enzyme is to reduce vitamin K to vitamin K hydroquinone(4). Vitamin K_1, however, was initially shown to be an extremely poor substrate for the diaphorase in a number of in vitro

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††To be submitted as part of a thesis by L.M.P. in partial fulfillment of the Ph.D. requirement, Albany Medical College of Union University, Albany, N.Y.

[1]The abbreviations used are: DCIP, 2,6-dichloroindophenol; and HPLC, high performance liquid chromatography.

0006-291X/82/010187-06$01.00/0

assays(2). Hydroquinone formation was finally achieved by incorporating the vitamin into liposomes and linking the reaction to potassium ferricyanide reduction(5). Recently a number of investigators(6,7) have demonstrated that purified DT-diaphorase in the presence of vitamin K_1 and NADH can produce vitamin K_1 hydroquinone dependent-γ-carboxyglutamate formation in hepatic microsomal precursor protein.

Assays previously utilized to detect and/or quantify DT-diaphorase catalyzed-vitamin K_1 hydroquinone formation have employed indirect methods such as secondary chromogens or enzymes which accept reducing equivalents from the hydroquinone. We recently reported a technique using HPLC which directly quantifies vitamin K_1 hydroquinone formed by hepatic microsomes in the presence of dithiothreitol(8). We report here our investigations of highly purified DT-diaphorase catalyzed vitamin K_1 hydroquinone formation using a direct quantitation method. The effects of dicoumarol and warfarin on vitamin K_1 hydroquinone formation were determined with the object of probing the role of the diaphorase in vitamin K metabolism and its function in coagulation.

METHODS

DT-diaphorase Preparation: Male Wistar rats (220±10g) from a colony maintained in this Division were administered 3-methylcholanthrene (5 mg/ml corn oil) ip at 25 mg/kg once daily for three days. Forty-eight hours after the last injection the rats were rendered unconscious with N_2 and their livers perfused *in situ* with physiological saline. The livers were removed, homogenized with two volumes by weight of 0.25 M sucrose and the homogenate centrifuged at 10,000 g for 30 min. Microsomes were separated from the supernatant by centrifugation at 105,000 g for 90 min and the protein content of the cytosol was adjusted to 6 to 7 mg/ml by dilution with 0.02 M Tris-HCl, 0.25 M sucrose buffer, pH 7.4. DT-diaphorase was purified from the cytosol by azodicoumarol and Sephracryl S-200 chromatography essentially as described by Höjeberg et al.(9), except that the Sephracryl column was 2 x 70 cm and the flow rate was 10 ml/h. Protein contents were determined by the method of Bradford(10) using Bio-Rad reagents.

Assays: Assays of DT-diaphorase employing DCIP as substrate were performed essentially as described by Ernster(1), except that 0.01% (v/v) Emulgen 911 (Kao Atlas, Tokyo, Japan) was used as the detergent and the buffer was 0.2 M Tris-HCl, 0.15 M KCl, pH 7.4. Reactions were initiated by the addition of 0.29 µg of DT-diaphorase to 3 ml of buffered NADH-DCIP solution equilibrated at 25°C. Rates of reactions were calculated using an extinction coefficient for reduced DCIP of 21 $mM^{-1}cm^{-1}$ at 600 nm.

Stock solutions of vitamin K_1 were prepared at 20 mg/ml in 10% (v/v) Emulgen 911 as described previously(6) and were diluted with water for use in metabolism studies. Reaction mixtures in 0.5 ml of 0.2 M Tris-HCl, 0.15 M KCl buffer, pH 7.4, contained 2.6 µg DT-diaphorase, and NADH. Following incubation at 25°C for 1 min, reactions were initiated by the addition of 20 µl of vitamin

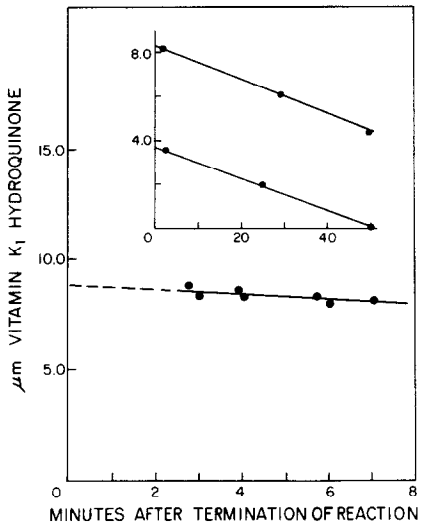
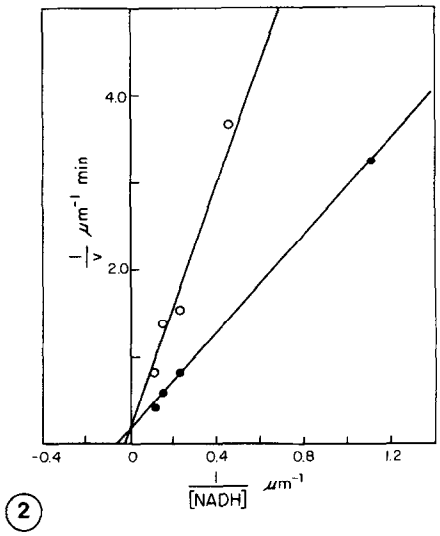


Fig. 1 Effect of time on vitamin K_1 hydroquinone oxidation by air. The NADH and vitamin K_1 concentrations were 0.9 mM and 40 μM respectively. Incubations were for 3 min at 25°C. Vitamin K_1 hydroquinone concentrations were determined as described in METHODS at various times after termination of reaction by addition of dicoumarol. Insert: Effect of hydroquinone concentration and time on rates of oxidation by air. Values were determined by repetitive injections of the same sample.

K_1 solution containing sufficient Emulgen 911 to yield a final detergent concentration of 0.01%. Reactions were terminated by the addition of 20 μl of dicoumarol solution (2 mg disodium salt/ml). A 0.1 fraction of the mixture was assayed for vitamin K_1 hydroquinone concentration by HPLC essentially as described previously(8) except that the column was a 5 mm ID Radial Pak C_{18} (Waters Associates, Milford, MA) and solvent B was acetonitrile/isopropanol (4/1) and solvent A was water/solvent B (1/1).

RESULTS AND DISCUSSION

The product of vitamin K_1 transformation catalyzed by DT-diaphorase cochromatographed with synthetically prepared vitamin K_1 hydroquinone(8) under the HPLC conditions used. Upon prolonged exposure to air the product oxidized back to vitamin K_1 thus establishing it as vitamin K_1 hydroquinone. This provides the first direct evidence that DT-diaphorase catalyzes vitamin K_1 hydroquinone formation and establishes that DT-diaphorase has the potential to function in the vitamin K-vitamin K 2,3 epoxide metabolic cycle by providing vitamin K hydroquinone cofactor for the carboxylase which catalyzes γ-carboxyglutamate formation in postribosomal precursor protein. Such a role for DT-diaphorase was previously proposed by Wallin et al.(6) and Wallin and Suttie (7) based on results obtained with purified DT-diaphorase and Triton X-100 treated microsomes.



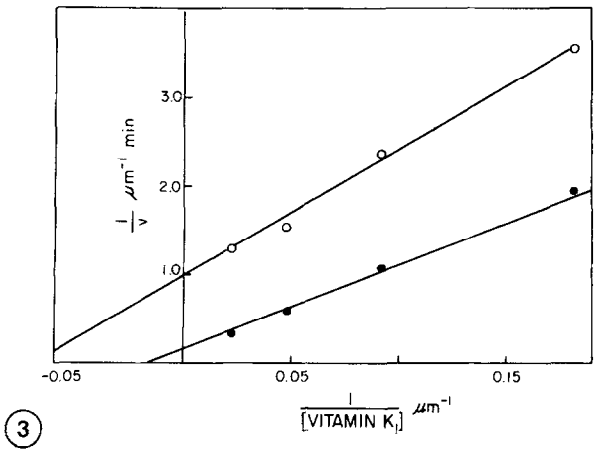


Fig. 2 DT-diaphorase catalyzed-vitamin K_1 hydroquinone formation at varying NADH concentrations in the absence (•) and presence of dicoumarol (o). The vitamin K_1 concentration was 40 μM and the dicoumarol concentration was 26 μM. Incubations were for 3 min at 25°C. Other conditions were as described in METHODS. Slopes were calculated by linear regression analysis.

Fig. 3 DT-diaphorase catalyzed-vitamin K_1 hydroquinone formation at varying vitamin K_1 concentrations in the absence (•) and presence of dicoumarol (o). The NADH concentration was 0.9 mM and the dicoumarol concentration was 26 μM. Incubations were for 3 min at 25°C. Other conditions were as described in METHODS. Slopes were calculated by linear regression analysis.

Since vitamin K_1 hydroquinone oxidizes to vitamin K_1 in air, experiments were undertaken to determine the extent to which oxidation affected quantitation of the hydroquinone. The hydroquinone contents obtained from three min incubations of vitamin K_1, NADH and DT-diaphorase and analyzed at various times thereafter are provided in Fig. 1. Extrapolation of these values to the time of termination of reaction demonstrated that hydroquinone concentrations determined within five min were within 95 percent of the actual concentration and that oxidation was linear with time. Repetitive HPLC analysis of samples containing two different initial hydroquinone concentrations (Fig. 1, insert) demonstrated that over the range of these concentrations oxidation rates were virtually independent of hydroquinone concentration. Based on these data hydroquinone concentrations in incubation mixtures were determined within four min after termination of reaction and were not corrected for loss due to oxidation.

Rates of vitamin K_1 hydroquinone formation catalyzed by DT-diaphorase were linear for three min at 25°C and were dependent upon the NADH concentration over the range of 1 to 10 μM (Fig. 2). Dicoumarol was a competitive inhibitor with respect to NADH (Fig. 2) and at 26 μM increased the Km for NADH from 17.5 to an apparent value of 30.3 μM. The Vmax was 5.6 μM/min. In contrast, dicoumarol was an uncompetitive inhibitor of vitamin K_1 hydroquinone formation with respect to vitamin K_1 (Fig. 3): dicoumarol decreased the Km from 64.1 to 14.4 μM and the Vmax from 7.2 to 1.0 μM/min. At a concentration of 520 μM, warfarin did not inhibit vitamin K_1 hydroquinone formation at any of the NADH or vitamin K_1 concentrations used in these investigations.

Using DCIP as substrate for partially purified DT-diaphorase, Hollander and Ernster(11) previously demonstrated that dicoumarol was a competitive inhibitor with respect to NADH and was an uncompetitive inhibitor with respect to DCIP. We obtained data for DCIP similar to their data with the highly purified DT-diaphorase under experimental conditions similar to those used for investigations of hydroquinone formation from vitamin K_1. The modes of inhibition of DT-diaphorase by dicoumarol are therefore the same for the substrates vitamin K_1 or DCIP. Major kinetic differences are observed, however, when the two substrates are compared: i) the maximum rate of hydroquinone formation occurs at 10 μM NADH whereas that of DCIP reduction occurs at 900 μM; ii) the Km for vitamin K_1 is 64.1 μM and for DCIP is 23.3 μM; iii) at optimum NADH concentration the Vmax for hydroquinone formation is 7.2 μM/min and that for DCIP reduction is 483 μM/min; and iv) with respect to NADH dicoumarol was approximately 40 fold less effective as a competitive inhibitor of vitamin K_1 reduction than of DCIP reduction. In reference to the latter it is apparent that dicoumarol and particularly warfarin are very much less effective as inhibitors of DT-diaphorase with a "physiological" rather than an "artificial" substrate.

In conclusion vitamin K_1 hydroquinone has been identified as a metabolite of the DT-diaphorase catalyzed-reduction of vitamin K_1 and a quanti-

tative HPLC assay of its formation has been developed. Dicoumarol and particularly warfarin inhibit vitamin K_1 reduction much less effectively than DCIP reduction. Based on the in vitro data presented here, DT-diaphorase could function physiologically in the production of vitamin K hydroquinone, but inhibition of the diaphorase is not a primary mode of action of the 4-hydroxycoumarin anticoagulants.

ACKNOWLEDGEMENT

This work was supported in part by Grant HL-19772 (MJF) from the National Institutes of Health.

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