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A survey of rat tissues for phylloquinone epoxidase activity

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The vitamin K-dependent post-translational modification of prothrombin is the γ-carboxylation of a number of glutamic acid residues in the polypeptide to convert them into γ-carboxyglutamic acid (GLA) residues [1]. GLA has also been identified in the vitamin K-dependent coagulation factor X [2] as well as in protein C [3], a plasma protein of unknown function, and in a protein isolated from bone, osteocalcin [4], the formation of which also appears to be vitamin K dependent [5]. Low levels of [^{14}C]GLA have been found after incubation of $NaH^{14}CO_3$ with chick calvaria [4], thus suggesting that tissues other than liver may also synthesize GLA-containing proteins.

Epoxidation of phylloquinone (K_1), in which K_1 is converted to its stable 2,3-epoxide, occurs in liver microsomes in the presence of oxygen and reduced pyridine nucleotides [6]. Epoxidase activity, which is not inhibited by carbon monoxide and does not appear to be P-450-mediated, is proportional to the amount of prothrombin precursor present in liver [6]; inhibitors which block carboxylation also block epoxidation [7]. All evidence to date suggests that locations where epoxidation occurs are sites at which carboxylation will also occur. Epoxidation is easily measured in crude liver homogenates, while vitamin K-dependent carboxylation is not. In the latter, significant non-vitamin K-dependent fixing of $^{14}CO_2$ into molecules other than γ-carboxyglutamic acid occurs, which obscures vitamin K-dependent carboxylation.

Because of the prior association of the carboxylase and epoxidase reactions, we have conducted a survey for K_1 epoxidase activity in homogenates from the tissues of the rat. Such information might reveal likely locations of the vitamin K-dependent carboxylation reaction.

Materials and methods

Rats were purchased from Charles River Farm. Since it has been shown that liver microsomes from K-deficient rats give higher levels of epoxidation than those from normal rats [6], in most experiments 250-g male CD rats fed K-deficient chow (Teklad Industries) for 8 days in coprophagy minimizing wire-bottom cages were used. After 8 days, prothrombin levels were reduced to 20 per cent of normal. Placentas and fetal liver were obtained from an 18-day pregnant rat. In one experiment, bone and liver were obtained from 4 hr newborns; calvaria, vertebrae and long bones were scraped clean of soft tissue in a cold room at 4°. Animals were killed by a single blow to the head, and after exsanguination through the neck vessels, tissues were rapidly removed in the cold, rinsed in cold 0.05 M potassium phosphate buffer, pH 7.5, containing 0.25 M sucrose (PS buffer), blotted, weighed, finely minced, and then homogenized in 3 vol. of PS buffer with several passes on a motor driven Potter Elvehjem grinder with a Teflon pestle rotating at 2000 rev/min. The homogenates were used directly. When bones were used, they were minced as fine as possible; then 1 ml of the PS buffer was added for each 500 mg of bone, and the minced bones were homogenized at top speed for 1 min in the microcontainer of a Virtis homogenizer set in ice. The bony debris was centrifuged at 1000 *g* for 5 min and the supernatant assayed.

Tritiated phylloquinone (K_1) was synthesized from tritiated menadiol sodium diphosphate (Amersham-Searle; sp. act. 96 ci/mole) by a published procedure [8]. Phylloquinone epoxide was prepared chemically from K_1 [9]. Epoxide formation in homogenates was measured by reversed phase thin-layer chromatography, using paraffin-impregnated silica gel G plates and a solvent system of acetone–water (92:8) as has been described [8]. Tetrachloro-4-pyridinol (4-TCP) was a gift of the Dow Chemical Co., Indianapolis, IN.

Reactions were run in air in a total volume of 1.0 ml and contained: potassium phosphate, pH 7.5, 50 μmoles; sucrose, 250 μmoles; homogenate or extract, 800 μl; NADH, 4 μmoles; warfarin, 20 μg; tritiated K_1, 400 ng (10,000 cpm); and 4-TCP 50 μg, when present. All reactants except K_1 were mixed and, after a 30-sec incubation at 37°, K_1 was added and a further 20-min incubation ensued. After the second incubation, 1 ml water was added and the K_1 and its epoxide were extracted into 6 ml hexane–isopropanol (3:2). It could be shown that 85–90 per cent of the radioactivity present after incubation could be extracted from the tissues studied by this method. Aliquots (2 ml) of the organic phase were evaporated to dryness under N_2; the residue was dissolved in 0.1 ml ethanol, containing carrier K_1 and epoxide; 0.05 ml samples were spotted on paraffin-impregnated silica gel G plates which were developed as described above [8]. After drying, K_1 and epoxide spots were detected with an ultraviolet lamp; they were scraped into scintillation vials to which 1 ml methanol and 10 ml Instagel were added, and the samples were counted for radioactivity. Unincubated complete reaction mixtures served as controls.

Results and discussion

The epoxidase activities of the tissues studied are summarized in Table 1. Epoxidase activity is present in an 18-day rat fetal liver, and there is a progressive increase in the epoxidase activity in fetal, newborn and adult liver, expressed as activity per unit volume. 4-TCP, an inhibitor of K-dependent carboxylation and K_1 epoxidation [7, 10], effectively inhibited epoxidation in fetal, newborn and adult liver. Substantial epoxidase activity, which was inhibited by 4-TCP, was also found in placenta and in kidney. Lesser but easily measurable amounts of epoxidation were found in splenic homogenates as well as in the bone extract. The activity in the bone extract was low (the radioactivity found in the epoxide spot of the thin-layer chromatograph is only 50 per cent above the background level). However, since it is consistently reproducible, it is likely that we are measuring a low rate of epoxidation in bone, a finding consistent with the observation that *in situ* GLA synthesis occurs in bone. Low levels of epoxidation were also found in incubations of minced bones with tritiated K_1. In this preparation, however, 4-TCP did not inhibit the reaction, perhaps reflecting its inability to penetrate the bony matrix. The following tissue homogenates had no detectable activity: lung, heart, skeletal muscle, pancreas, brain, skin and small intestine. It should be emphasized that a negative result does not exclude the existence

Table 1. Phylloquinone epoxidase activity in rat tissues*

Tissue homogenate	Per cent K_1 converted to epoxide	Per cent K_1 converted to epoxide in presence of 4-TCP
Fetal liver	19.4	5.6
Newborn liver	44.5	8
Adult liver	70	14
Kidney	8	0.9
Placenta	22	10
Spleen	4.8	2.5
Bone extract	1.2	<0.3
Heart	<0.3	<0.3
Peripheral muscle	<0.3	<0.3
Forebrain	<0.3	<0.3
Hindbrain	<0.3	<0.3
Pancreas	<0.3	<0.3
Small Intestine	<0.3	<0.3
Lung	<0.3	<0.3
Skin	<0.3	<0.3

* For reaction conditions, see Materials and Methods. Conversion is expressed as the percentage of the radioactivity converted to the epoxide (ratio cpm epoxide spot/cpm epoxide plus K_1 spots minus the same ratio from a zero time control). All numbers are the average of duplicate determinations that differed by less than 5 per cent.

of epoxidase activity in these tissues but may only reflect the inability of our assay to detect activity.

Apart from identifying tissues capable of converting K_1 to its epoxide, it was hoped that this study might reveal tissues that synthesize GLA-containing proteins. In preliminary experiments, GLA-containing protein has been identified in kidney cortex, while vitamin K-dependent formation of radioactive GLA from $^{14}CO_2$ has been detected in kidney microsomes prepared from K-deficient rats and dicoumarol-treated chicks [11]. The nature of the GLA-containing protein(s) is as yet unknown. Vitamin K-dependent carboxylation may also occur in spleen and placenta.

Finally, it is interesting to attempt to relate tissue epoxidase activity to the distribution of K_1 in rat tissues. It has been shown that radioactive K_1 fed to male rats is widely distributed in the animals [12]; the highest concentrations (expressed as dis./min/mg of tissue) were observed in liver and spleen; bone and cartilage, as well as lung, each had about two-thirds the concentration of liver while kidney had about one-third. Other tissues had lesser concentrations. Although the levels of epoxidase activity do not correlate with the concentrations of K_1 found in the various tissues, with the exception of lung, those tissues with the highest concentrations of K_1 are those with measurable epoxidase activity. Since there is no reason to predict a correlation between tissue epoxidase activity and K_1 concentration, neither the lack of such a correlation nor the absence of measurable epoxidase activity in lung is unexpected. Experiments in which lung and liver homogenates were mixed did not show evidence for an inhibitor of epoxidation in lung homogenate.

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