

Vitamin K-dependent carboxylases from non-hepatic tissues

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Received 27 September 1982

The presence of vitamin K-dependent carboxylase was investigated in the microsomal fraction of 20 different types of bovine tissue. Except for muscle, veins, lymphocytes and bone membrane, carboxylase was found in all these preparations, albeit in varying amounts. No differences could be detected between these carboxylating systems with respect to their affinity for vitamin K and warfarin. Most of the endogenous substrates had some affinity towards antiprotease or antifactor X.

<i>Carboxylase</i>	<i>Vitamin K</i>	<i>Warfarin</i>	<i>Liver γ-carboxyglutamic acid</i>	<i>Anticoagulant therapy</i>
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1. INTRODUCTION

Vitamin K-dependent carboxylase has been detected in the liver of a wide variety of species, including man [1–3]. In the first years after its discovery, this carboxylase was thought to be uniquely involved in the synthesis of blood clotting factors, and hence it was supposed to be located in the liver exclusively. During the last few years, however, a number of investigators has reported the presence of vitamin K-dependent carboxylase in non-hepatic tissues, such as kidney, spleen, lung and bone [4–6]. The importance of these non-hepatic carboxylases remained obscure until now, because in most cases: (a) the reaction products could not be identified; and (b) the relative amounts of these carboxylating systems were not compared with the amounts of enzyme present in hepatic tissue. Here, we describe experiments by which we try to make an inventory of the carboxylating systems in different types of bovine tissue.

2. MATERIALS AND METHODS

2.1. Chemicals

Vitamin K was obtained from Hoffmann-La Roche and vitamin K hydroquinone was prepared as in [2]. Warfarin and dithiothreitol were obtained from Sigma and the pentapeptide Phe–Leu–Glu–Glu–Leu (FLEEL) from Vega Fox. $\text{NaH}^{14}\text{CO}_3$ (40 Ci/mol) was purchased from New

England Nuclear. All other chemicals were obtained from Merck.

2.2. Preparation of washed microsomes

Soft tissues were homogenized and used for the preparation of crude microsomes as in [2]. The microsomes were washed 3 times with a buffer containing 30% ethylene glycol, 0.8 M KCl and 0.02 M Tris–HCl (pH 7.4). Arteries, veins, bone membrane and tendon were frozen in liquid nitrogen, crushed to powder and subsequently homogenized in the same buffer. Bone and cartilage were powdered in a similar way, extracted with a buffer containing 0.1 M EDTA, 0.8 M KCl and 0.02 M Tris–HCl (pH 7.8) and the extracts were homogenized as described for the soft tissues.

2.3. Measurement of carboxylase activity

The vitamin K-dependent incorporation of $^{14}\text{CO}_2$ was measured in the absence of detergent in a similar way to [2]. Unless stated otherwise, vitamin K was used in its reduced form.

3. RESULTS

3.1. Detection of vitamin K-dependent systems in various tissues

A 3-week-old calf was anticoagulated by oral administration of warfarin (1.5 g daily) for 1 week. After this period the animal was slaughtered and the various organs were excised and homogenized

as in section 2. The amounts of vitamin K-dependent carboxylase present in the respective microsomal fractions were measured in the presence of an excess of exogenous substrate and are expressed as a percentage of the amount of carboxylase in liver. The results are summarized in table 1.

Table 1

Amounts of microsomal carboxylase and endogenous substrate in various tissues

Tissue	Amount of carboxylase (%)	Amount of endogenous substrate (%)
Liver	100	100
Testis	210	130
Kidney	73	12
Spleen	70	41
Lung	63	6
Thyroid	31	7
Pancreas	11	6
Thymus	11	3
Heart muscle	0	0
Skeletal muscle	0	0
Diaphragm	0	0
Arteries	62	10
Veins	0	0
Lymphocytes	0	0
Bone marrow	10	30
Cartilage	40	16
Bone	25	8
Bone membrane	0	0
Tendon	18	35
(Uterus)	(32)	(6)

The amounts of carboxylase were measured as the maximal rate of carboxylation of endogenous + exogenous (FLEEL, 10 mM) substrate and are expressed per mg microsomal protein (cf. [2]). In liver this value was 0.95 pmol CO₂ incorporated · min⁻¹ · mg protein⁻¹. The amounts of endogenous substrate were measured as the total amount of CO₂ that could be incorporated into trichloroacetic acid-precipitable material. In liver this value was 35 pmol CO₂/mg microsomal protein. In both cases the amounts in liver were arbitrarily considered to be 100%. The data for uterine tissue were obtained from a female animal with comparable characteristics of carboxylase from liver, spleen and kidney. Because they are obtained from a different animal, these data are given in parentheses

3.2. Characterization of the various vitamin K-dependent systems

To compare the various carboxylating enzyme systems, we measured the K_M of vitamin K hydroquinone in these systems and their susceptibility to warfarin. The latter was accomplished by establishing the warfarin concentration required for 50% inhibition of the vitamin K-driven reaction. The results of these experiments are summarized in table 2.

3.3. Characterization of the various endogenous substrates

Finally, we tried to identify the various endogenous substrates by immunospecific adsorption of the carboxylated reaction products onto Sepharose-bound antibodies against prothrombin and clotting factor X. About 40% of the total amount of endogenous substrate in the liver could be bound to one of these antibodies (table 3). Most of the other tissues, however, also contained some material (10–20%) with antigenic determinants that are recognized by either antiprothrombin or antifactor X.

Table 2

Characteristics of carboxylase from various tissues

Tissue	K_M (μ M) of vitamin KH ₂	[Warfarin] (μ M) required for 50% inhibition
Liver	14	18
Testis	11	18
Kidney	7	25
Spleen	8	13
Lung	8	15
Thyroid	7	16
Thymus	8	16
Arteries	16	10
Cartilage	12	12
Bone	11	9
(Uterus)	(8)	(14)

The K_M of vitamin K hydroquinone (KH₂) was measured as in [2]. [Warfarin] required for 50% inhibition was measured in the presence of 25 μ M vitamin K quinone

Table 3

Immunochemical characterization of endogenous substrates from various tissues

Tissue	Carboxylated substrate (%) bound to:	
	Antiprothrombin	Antifactor X
Liver	17.2	21.5
Testis	10.4	6.2
Kidney	7.0	13.1
Spleen	5.3	8.1
Lung	6.4	3.9
Thyroid	9.5	1.0
Pancreas	0	0
Thymus	6.9	2.6
Arteries	8.8	13.6
(Uterus)	8.7	3.0

Reaction mixtures (1 ml) were incubated for 1 h at 25°C in the presence of 50 μ Ci NaH¹⁴CO₃. Purified antibodies against either prothrombin or factor X were immobilized and used for the extraction of these mixtures [7]

4. DISCUSSION

When we screened a great number of tissues for the presence of carboxylase, it became clear that this vitamin K-dependent enzyme is present in all tissues except lymphocytes, veins, muscle and bone membrane. The other tissues that were checked all contained carboxylase, though in variable amounts. It should be kept in mind, that in all cases carboxylase was tested with the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu. This peptide has been derived from prothrombin and it might be a poor substrate for some non-hepatic carboxylases, especially because we have observed some difference in substrate specificity between carboxylase obtained from liver and that from placenta (unpublished). It is quite possible therefore, that the data in table 1 underestimate the real amounts of carboxylase. We were unable to detect substantial differences between the various carboxylase preparations with respect to the K_M for vitamin K and the ability of the vitamin K-antagonist warfarin to inhibit these systems. It seems likely therefore, that in patients receiving vitamin K-antagonists, the vitamin K-dependent carboxylases in all tissues will probably be inhibited to the same extent. In an attempt to characterize the carboxyl-

ated substrates, present in a number of tissues, we extracted carboxylating reaction mixtures with Sepharose-bound antibodies against prothrombin and factor X. As might be expected, the endogenous substrate in the liver had more antigenic determinants in common with these two clotting factors than had the substrates from other tissues. Nevertheless, except for the pancreas they all had some affinity towards the Sepharose-bound antibodies, although to a variable extent. Since it seems unlikely that coagulation factors are produced by all these tissues, we think that in most cases the affinity of the various substrates towards the antibodies is caused by a certain amino acid sequence which they have in common with the clotting factors and which might serve as a recognition site for carboxylase.

It is tempting to speculate about the function of the non-hepatic vitamin K-dependent proteins, but conclusions have to be drawn with great care. However, we are inclined to make some critical remarks. In the first place, it is surprising that only few side-effects are known in patients who undergo long-term oral anticoagulant therapy. Of course this may be an indication that most vitamin K-dependent processes (except for their role in the maturation of coagulation factors) are rather unimportant reactions, which can be blocked without further damage for the organism. On the other hand, it remains possible that side-effects of anticoagulant therapy are obscured by secondary reasons. The high amount of carboxylase in testis, for instance, suggests that vitamin K is involved in the synthesis of a sperm protein (for instance acrosin, a trypsin-like serine protease which has many similarities with prothrombin and which is required for the penetration of the sperm cell into the ovum [8]). During long-term anticoagulation, the synthesis of such a vitamin K-dependent protein would be inhibited, possibly resulting in an impaired production of active sperm cells. Most patients treated with vitamin K-antagonists are over 50 years old, and maybe for this reason their fertility has never been checked (at least as far as we know).

A second striking observation was the difference in the carboxylase content between arteries and veins. The protein(s) produced by arterial carboxylase might be involved in the calcification of the vessel wall (which occurs almost exclusively in the

arteries) or in the formation of hardened atherosclerotic plaque (in which a Gla-containing protein has been detected [9]). A possible dominant role of this protein in thrombus formation would be obscured for the following reasons. High-risk patients (for instance those who suffered from myocardial infarction) are generally kept on oral anti-coagulant therapy, because it has been demonstrated that the therapy reduced the risk of reinfarction [10]. Is it believed that the reduced risk is related to the reduced plasma concentration of the vitamin K-dependent clotting factors. However, during antivitamin K treatment also the production of the arterial vitamin K-dependent protein(s) is reduced and this reduction might be of much greater importance than the reduced concentration of coagulation factors.

Since at present we can only guess at the function of the various non-hepatic vitamin K-dependent proteins it seems important that they are purified and further characterized. Possibly this will lead to the detection of their role in a number of biological processes.

ACKNOWLEDGEMENTS

This research was supported in part by grant MD 82145 from the Thrombosesstichting Nederland. The authors wish to thank Drs H.C. Hemker and R. van Dam-Mieras for their suggestions and

stimulating discussions and Dr A.E.J.M. van den Bogaard for providing us with the various bovine organs.

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