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Isolation and partial characterization of a vitamin K-dependent carboxylase from bovine aortae

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Vitamin K-dependent carboxylase activity has been demonstrated in the crude microsomal fraction of the intima of bovine aortae. The procedure for the isolation of vessel wall carboxylase is a slight modification of the general preparation procedure for tissue microsomes. The highest activity of the non-hepatic enzyme was observed at 25 °C and hardly any NADH-dependent vitamin K reductase could be demonstrated. The optimal reaction conditions for both vessel wall as well as liver carboxylase were similar: 0.1 M-NaCl/0.05 M-Tris/HCl, pH 7.4, containing 8 mM-dithiothreitol, 0.4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid (CHAPS), 0.4 mM-vitamin K hydroquinone and 2 M-$(NH_4)_2SO_4$. Warfarin inhibits the hepatic and non-hepatic carboxylase/reductase enzyme complex more or less to a similar degree. We have measured the apparent K_m values for the following substrates: Phe-Leu-Glu-Glu-Leu ('FLEEL'), decarboxylated osteocalcin, decarboxylated fragment 13–29 from descarboxyprothrombin and decarboxylated sperm 4-carboxyglutamic acid-containing (Gla-)protein. The results obtained demonstrate that liver and vessel wall carboxylase may be regarded as isoenzymes with different substrate specificities. The newly discovered enzyme is the first vitamin K-dependent carboxylase which shows an absolute substrate specificity: FLEEL and decarboxylated osteocalcin were good substrates for vessel wall carboxylase, but decarboxylated fragment 13–29 and decarboxylated sperm Gla-protein were not carboxylated at all.

INTRODUCTION

Vitamin K-dependent carboxylase is a microsomal enzyme system, discovered in rat liver more than 10 years ago (Esmon *et al.*, 1975). Since then, carboxylase activity has been demonstrated in the livers of a wide variety of species, including man (Suttie *et al.*, 1979; Vermeer *et al.*, 1982*a*; Soute *et al.*, 1982). The enzyme is involved in the post-translational carboxylation of distinct glutamic acid (Glu) residues into 4-carboxyglutamic acid (Gla) residues (Suttie, 1980; Vermeer, 1984). For many years it has been thought that the carboxylating enzyme system was only restricted to the liver, but recently carboxylase has also been detected in other tissues such as lung, kidney, spleen, testis and bone (Buchtal & Bell, 1983; Vermeer *et al.*, 1982*b*). Whereas it is known that hepatic carboxylase is involved in the production of a number of proteins involved in blood coagulation, the importance of the non-hepatic systems is far less understood.

In the liver three metabolites of vitamin K have been identified: vitamin K quinone (K), vitamin K hydroquinone (KH_2) and vitamin K epoxide (KO). Of these three metabolites KH_2 is the coenzyme for carboxylase and its oxidation to KO probably provides the energy required for the carboxylation reaction. KO is then reduced to K and KH_2 by two or more reductases. In this way there is a cyclic interconversion of vitamin K metabolites resulting in a constant supply of KH_2, so that the coenzyme can be re-used many times. DTT may serve *in vitro* as a reducing cofactor for these reductases, but the physiological cofactor has not yet been identified (Wallin *et al.*, 1978). Like the carboxylase, also the reductases are found in the microsomal fraction of tissue homogenates, where they seem to occur closely associated with carboxylase.

In this paper we describe the isolation and partial characterization of a non-hepatic vitamin K-dependent carboxylase present in the microsomal fractions of the intima of bovine aortae. Unfortunately the reaction product of this vessel wall carboxylase has not yet been identified. It might be related to the Gla-containing protein found in hardened atherosclerotic plaque (Levy *et al.*, 1979, 1986). Since a number of non-hepatic Gla-containing proteins are found in calcified tissues (Hauschka *et al.*, 1976; Lian *et al.*, 1977), it might be possible that the product of vessel wall carboxylase plays a role in the deposition of calcium salts in the vessel wall during atherosclerosis.

MATERIALS AND METHODS

Chemicals

Vitamin K_1 (Konakion) was obtained from Hoffmann–La Roche (Basel, Switzerland) and chemically reduced into the hydroquinone form (Vermeer *et al.*, 1982*a*). Warfarin, dithiothreitol, CHAPS and Triton X-100 were purchased from Sigma, and the synthetic substrate Phe-Leu-Glu-Glu-Leu (FLEEL) was from Vega Biochemicals (Tucson, AZ, U.S.A.). Sucrose was obtained from BDH. $NaH^{14}CO_3$ (40–60 Ci/mol) was from Amersham International and Atomlight from New England Nuclear. CNBr-activated Sepharose, QAE-

Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid; K, KH_2, KO, vitamin K and its hydroquinone and epoxide; DTT, dithiothreitol; the prefix d- refers to a decarboxylated protein; Gla, 4-carboxyglutamic acid.

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Sephadex and Sephadex G-25 were purchased from Pharmacia. The proteinase subtilisin and NADH were obtained from Boehringer-Mannheim. All other chemicals were from Merck.

Preparation of liver microsomal carboxylase

Crude microsomes were prepared from the liver of normal cows as described earlier (Vermeer *et al.*, 1982*a*). The microsomes obtained were suspended in buffer A [0.5 M-NaCl/0.05 M-Tris/HCl (pH 7.4)/1 mM-EDTA/ 20% (v/v) ethylene glycol] to a final protein concentration of 10 mg/ml. The preparations were kept at −80 °C until use.

Preparation of vessel wall carboxylase

Bovine aortae were obtained from normal cows within 1 h after slaughter and were quickly cooled in ice-cold buffer B [0.25 M-sucrose/0.1 M-NaCl/0.05 M-Tris/HCl (pH 7.4)/20% (v/v) ethylene glycol]. The aortae were prepared free from media and adventitia and subsequently homogenized in buffer B using a high-speed homogenizer (Breda Scientific). A 20% homogenate (w/v) was used for the preparation of the crude microsomal fraction (Vermeer *et al.*, 1982*a*). It was observed that during the centrifugation at 10000 *g* of the homogenate at 4 °C, the carboxylating enzyme was coprecipitated with collagen, resulting in a hardly detectable carboxylase activity in the microsomal fraction. Elevation of the temperature to 20 °C during the centrifugation at 10000 *g* could solve this problem. After one wash the obtained microsomal fraction was suspended in buffer A to a concentration of 10 mg of protein/ml, frozen in liquid N_2 and stored at −80 °C until use.

Measurement of carboxylase activity

Unless stated otherwise, the vitamin K-dependent incorporation of $^{14}CO_2$ was measured by incubating reaction mixtures (0.25 ml) in buffer A containing: 1.0 mg of microsomal protein and 0.01 mCi of $NaH^{14}CO_3$. The concentrations of exogenous substrates were as indicated. The optimal reaction conditions for the two enzymes were similar: 0.1 M-NaCl/0.05 M-Tris/ HCl, pH 7.4, containing 8 mM-DTT, 0.4% CHAPS, 2 M-$(NH_4)_2SO_4$ and 0.4 mM-vitamin K hydroquinone, which was the last compound added to the reaction mixture. After sealing the tubes the mixtures were incubated at 25 °C and at the indicated times the reaction was stopped by adding 2 ml of 5% (w/v) trichloroacetic acid. Subsequently traces of non-bound label were removed by degassing the samples at elevated temperatures for 3 min, before 10 ml of Atomlight was added. The samples were counted in a Beckman LS 1801 scintillation counter.

Preparation of substrates

Osteocalcin was prepared from bovine bone and partly purified by batchwise adsorption to QAE-Sephadex in 0.15 M-NaCl/0.02 M-Tris/HCl (pH 7.4) followed by a stepwise elution with 1 M-NaCl, desalting, thermal decarboxylation and purification by preparative h.p.l.c. (Vermeer *et al.*, 1984). Decarboxylated sperm Gla-protein (from human sperm) was prepared as described earlier (Soute *et al.*, 1985). Descarboxyprothrombin fragment 13–29 was prepared by the proteolytic cleavage of purified bovine descarboxyprothrombin with subtilisin and purified with the aid of ion-exchange and size-exclusion chromatography (Soute *et al.*, 1981).

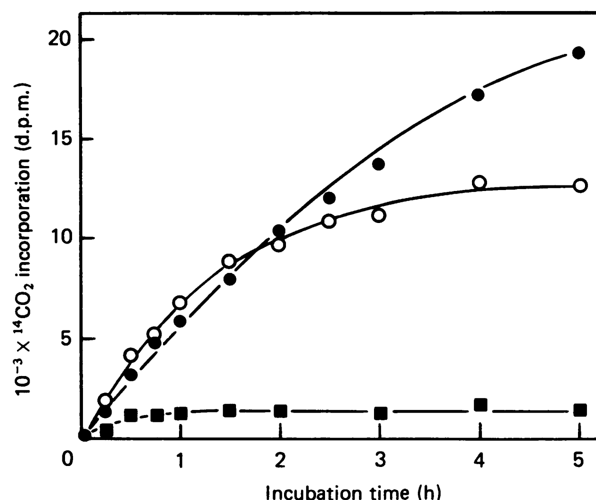

Fig. 1. Effect of temperature upon vessel wall carboxylase activity during time course studies

The incorporation of $^{14}CO_2$ was measured as described in the Materials and methods section in the presence of 8 mM-FLEEL after various incubation times at 25 °C (●), 30 °C (○) and 37 °C (■).

Determination of apparent K_m values

The reaction mixtures, containing various substrate concentrations, were incubated for different time intervals. For the determination of the kinetic constants, the period was chosen during which the reaction rates were linear. The data obtained in the absence of substrates were subtracted from those obtained in the presence of the substrate. The inverse of the initial reaction rates thus obtained was plotted against the inverse substrate concentrations. From the resulting Lineweaver–Burk plot, the apparent Michaelis constant (K_m) was calculated according to the method of Eisenthal & Cornish-Bowden (1974).

Other methods

Protein concentrations were determined as described by Sedmak & Grossberg (1977). Decarboxylation of Gla-containing proteins was performed according to Poser & Price (1979).

RESULTS

Once having succeeded in preparing in a reproducible way an active microsomal fraction from vessel wall, we wanted to characterize this vitamin K-dependent carboxylating enzyme system. In the first place we determined the optimal reaction conditions. These were similar for the hepatic and non-hepatic carboxylase (see the Materials and methods section). The carboxylase can be extracted from the crude microsomes of bovine aortae with the aid of detergents. The zwitterionic detergent CHAPS appeared to be a better detergent than the non-ionic Triton X-100 (results not shown). These findings are in agreement with the data obtained from earlier studies performed with liver carboxylase (Girardot & Johnson, 1982).

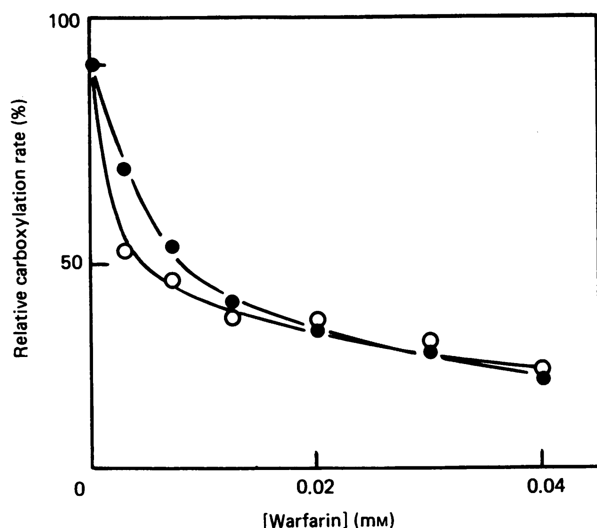

Fig. 2. Effect of warfarin on carboxylase/reductase from liver (●) and vessel wall (○)

The inhibition by warfarin of the enzyme systems was measured by performing the carboxylation reaction under standard conditions (see the Materials and methods section) except that vitamin KH_2 was replaced by 0.4 mM-vitamin K. FLEEL (8 mM) was used as an exogenous substrate and warfarin (in the form of its sodium salt) was mixed with vitamin K before it was added to the reaction mixture. The 100% enzyme activity values were 1054 d.p.m./min per mg of microsomal protein for liver carboxylase and 110 d.p.m./min per mg of microsomal protein for vessel wall carboxylase.

The carboxylating enzyme system from the vessel wall shows the highest activity at 25 °C and at this temperature the reaction rate was constant for 2 h (Fig. 1). On the other hand, carboxylase activity was hardly measurable at 37 °C, which indicates that *in vitro* the enzyme is quickly destroyed at this temperature. Because of these results all experiments described below were performed at 25 °C using an incubation time of 2 h.

In the next experiments we have compared the carboxylating enzyme system from the vessel wall with that from the liver. Besides vitamin K hydroquinone, also vitamin K quinone could be used as a coenzyme for carboxylase from both types of tissue (results not shown). This demonstrates that, like the liver enzyme, also vessel wall carboxylase is associated with a reducing enzyme system. Dithiols such as DTT proved to be the most effective cofactor *in vitro* for these reductases. Since it is well known that in liver the DTT-dependent reductase is the site of action of vitamin K antagonists like warfarin, we have compared the sensitivity for this drug of the carboxylase/reductase enzyme systems from liver and vessel wall, using vitamin K quinone as a coenzyme. As is shown in Fig. 2, both systems are, at least *in vitro*, inhibited to a similar degree.

We have also tried to detect the NADH-dependent vitamin K reductase in the vessel wall preparation, since its presence in a number of tissues has been reported (Wallin *et al.*, 1978). In these experiments again we used vitamin K quinone instead of the hydroquinone and DTT was replaced by NADH (2 mM). It turned out that under the assay conditions used here the NADH-mediated carboxylation was low relative to the dithiol-supported reaction, both in hepatic as well as in non-hepatic microsomes (less than 5%).

Table 1. Kinetic constants of various substrates for liver and vessel wall carboxylase

The apparent K_m values were calculated from the initial carboxylation rates at various substrate concentrations (see the Materials and methods section). The concentrations of the substrates employed in these experiments ranged from 1 to 8 mM for FLEEL, from 0.24 to 2.4 μM for d-osteocalcin, from 1.3 to 9.3 μM for d-fragment 13–29 and from 28 to 116 μM for d-sperm Gla-protein. N.m., not measurable.

Substrate	Apparent K_m (μM) Liver	Apparent K_m (μM) Vessel wall
FLEEL	1600	2300
d-Osteocalcin	0.4	1.8
d-Fragment 13–29	6	n.m.
d-Sperm Gla-protein	96	n.m.

Although we had not been able to detect differences between the vitamin K-binding sites of the two types of enzyme, it might still be possible that differences might be found at the substrate-binding sites. We have investigated this possibility by measuring the apparent kinetic constants of substrates of various origins in hepatic and non-hepatic microsomal fractions. Although we are aware of the complication that both, liver as well as vessel wall, carboxylases have not been purified completely, sufficient information can be obtained from these systems when the appropriate controls are made. For the determination of the kinetic constants, the reaction mixtures were incubated for 60 min. During this period the reaction rates were linear. The data obtained in the absence of vitamin K hydroquinone were subtracted from those obtained in the presence of vitamin K hydroquinone. The data obtained in the absence of substrates were subtracted from those obtained in the presence of the substrate. The following carboxylatable substrates were available: the pentapeptide FLEEL, decarboxylated osteocalcin (from bovine bone), decarboxylated fragment 13–29 (which is composed of the amino acid residues 13–29 in bovine descarboxyprothrombin) and decarboxylated sperm Gla-protein (from human sperm). The apparent K_m values for these four substrates were measured in the two carboxylating enzyme systems and the results of these experiments are summarized in Table 1. It is evident that d-osteocalcin is a good substrate for liver carboxylase and to a lesser degree also for vessel wall carboxylase. The apparent K_m values for both substrates are much lower than those observed for the pentapeptide, which are in the millimolar range. It was not possible to measure the apparent K_m values for fragment 13–29 and for d-sperm Gla-protein in vessel wall carboxylase, since even at high concentrations these substrates were carboxylated to a negligible degree.

It must be pointed out, however, that these studies were performed with equal amounts of microsomal protein. Since the enzyme activity per mg of protein is higher in microsomes from liver than in those from vessel wall, dissimilar amounts of carboxylase activity were

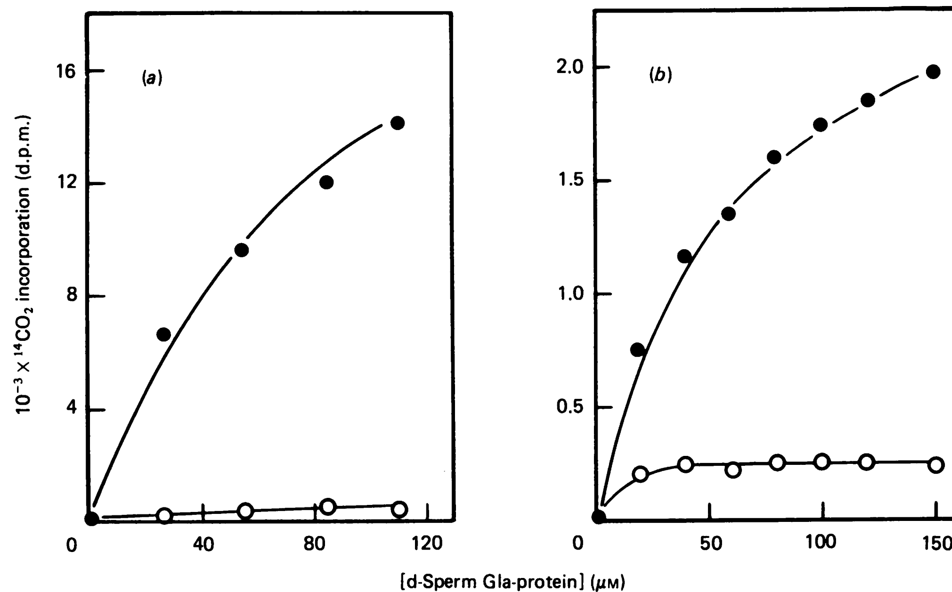

Fig. 3. Carboxylation of d-sperm Gla-protein at various concentrations of microsomal proteins

(*a*) The carboxylation of d-sperm Gla-protein in the presence of 1 mg of microsomal protein/ml from liver (●) and from vessel wall (○). (*b*) The carboxylation of d-sperm Gla-protein in the presence of similar amounts of peptide carboxylase. The amounts of microsomal proteins added were 0.125 mg (liver) and 1 mg (vessel wall) respectively. It was checked that under these conditions both preparations were able to carboxylate FLEEL at a similar reaction rate. The incorporation of $^{14}CO_2$ was measured after incubation periods of 60 min. Note the different scales for (*a*) and (*b*).

used. We also performed a control experiment in which we measured the carboxylation of d-sperm Gla-protein in both systems using similar amounts of carboxylase activity. Using an excess of the pentapeptide FLEEL (8 mM) as a substrate, we determined the relation between the carboxylation rate and the microsomal protein concentration. A linear relationship was found between 0.1 and 4 mg/ml and it appeared that 0.125 mg of hepatic microsomes contained a similar enzyme activity as 1.0 mg of the vessel wall preparation. Subsequently we compared the carboxylation of d-sperm Gla-protein under the conditions of similar protein concentration (Fig. 3*a*) as well as under those of similar carboxylase activity (Fig. 3*b*). It turned out that liver carboxylase was the only system to carboxylate d-sperm Gla-protein to a reasonable extent. These results therefore strongly favour the idea that vitamin K-dependent carboxylase is a group name for a series of enzymes, each with a different enzyme specificity and that vessel wall carboxylase is another member of this family.

DISCUSSION

In a previous study it has been demonstrated that the vitamin K-dependent carboxylases from the liver, kidney, lung and testis may be regarded as a group of isoenzymes, which mainly differ in their substrate specificity (Ulrich *et al.*, 1985). In this paper we report the isolation and partial characterization of the vitamin K-dependent carboxylase from bovine aortae. The enzyme is, as in other tissues, located in the microsomal fraction of the tissue homogenate. We have investigated whether the non-hepatic enzyme is comparable with hepatic carboxylase or whether also in this case differences might exist between both enzymes.

For the isolation of vessel wall carboxylase the general preparation procedure for tissue microsomes had to be slightly modified. It was found that, during the low-speed centrifugation at 4 °C of the collagen-rich vessel wall homogenate, carboxylase was co-precipitated with the collagen, resulting in a hardly detectable carboxylase activity in the microsomal fraction. By working quickly at 20 °C this problem could be solved. The resulting washed microsomes were solubilized and contained vitamin K-dependent carboxylase as well as DTT-dependent reductase.

In a first set of experiments we have compared the liver and vessel wall carboxylase/reductase systems with respect to their sensitivity towards warfarin. It turned out that both enzyme systems are inhibited *in vitro* by the drug to a more or less similar degree. This is in agreement with earlier experiments *in vitro* with other non-hepatic microsomal preparations (Soute *et al.*, 1982; Vermeer, 1984) and with experiments *in vivo*, in which it was shown that the oral administration of low doses of warfarin to rats induces the accumulation of non-carboxylated precursor proteins in the microsomal fraction of hepatic as well as of non-hepatic tissues (Roncaglioni *et al.*, 1983).

In a second set of experiments we have compared the affinity of the two carboxylating systems towards different substrates. Four substrates were available: the pentapeptide FLEEL, d-osteocalcin, d-fragment 13–29 and d-sperm Gla-protein. It turned out that the latter two substrates were not carboxylated to a significant extent by vessel wall carboxylase. In an earlier report, in which we have compared carboxylase from liver, lung, kidney and testis, we have shown that all substrates were carboxylated by these enzymes, but that the carboxylases had a preference for either d-fragment 13–29 or d-sperm Gla-protein (Ulrich *et al.*, 1985). Vessel wall

carboxylase is different from the former enzymes, because only FLEEL and d-osteocalcin are carboxylated, whereas the other substrates are not recognized at all.

Unfortunately the reaction product of vessel wall carboxylase has not yet been identified. It might be related to the Gla-containing protein found in hardened atherosclerotic plaque (Levy *et al.*, 1979, 1986), but this is still uncertain at the moment. Since many non-hepatic Gla-containing proteins are found in various calcified tissues, the product of vessel wall carboxylase might play a role in the deposition of calcium salts in the vessel wall during atherosclerosis. From our observations it seems likely that the vitamin K-dependent enzyme system from vessel wall is similarly sensitive to warfarin as is the liver enzyme and therefore it cannot be excluded that, in patients undergoing oral anticoagulant therapy, which is meant to reduce the degree of carboxylation of their blood coagulation factors, also the products of vessel wall carboxylase are synthesized in an undercarboxylated form. Whether this effect has any clinical relevance remains to be awaited, however.

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