A comparison between vitamin K-dependent carboxylase from normal and warfarin-treated cows

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A COMPARISON BETWEEN VITAMIN K-DEPENDENT CARBOXYLASE FROM NORMAL AND WARFARIN-TREATED COWS

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Detergent-solubilized microsomal preparations that catalyse the vitamin K-dependent γ-carboxylation of glutamic acid residues in peptide and protein substrates, have been obtained from the livers of normal and warfarin-treated cows. The preparations from warfarin-treated animals contained more endogenous substrate than those from normal cows, but otherwise the two preparations were indistinguishable. The enzymes vitamin K reductase and γ-glutamyl carboxylase, may function independently of each other in this system. They are, nevertheless, intimately linked in some way, so that the reduced vitamin K that is produced by the former enzyme can be used immediately by the latter.

Introduction

Vitamin K-dependent carboxylase has been obtained from the livers of various animals. Administration of warfarin to the animals leads to a considerable increase in the amount of endogenous substrate [1], and most studies dealing with the vitamin K-dependent carboxylase have utilized the endogenous substrate for the reaction [2]. The endogenous substrate, which accumulates in cow liver after the administration of warfarin, consists mainly of precursors of clotting factor X, and it co-purifies with the enzyme [3].

In this article, the properties of carboxylase preparations from normal and warfarin-treated cows have been compared in order to see if both types of carboxylase are similar, and whether conclusions obtained with the carboxylating enzyme system from normal animals can thus also be applied to that from warfarin-treated animals and vice-versa.

Abbreviation: DTT, dithiothreitol.

Materials and Methods

Chemicals. Vitamin K was obtained from Hoffmann-La Roche (Basel, Switzerland) and dihydroxyvitamin K was prepared as described previously [4]. Vitamin K-epoxide was prepared according to Fieser et al. [5]. The synthetic substrate Phe-Leu-Glu-Glu-Leu (F L E E L) was obtained from Vega Fox (Tucson, USA) and $NaH^{14}CO_3$ (40 Ci/mol) from New England Nuclear (Dreieich, FRG). Glucose oxidase, Triton X-100. warfarin and dithiothreitol (DTT) were purchased by Sigma (Saint Louis, USA), and Picofluor-15 by Packard Instruments (Warrenville, USA). All other chemicals were obtained from Merck (Darmstadt, FRG).

Preparation of carboxylase. Crude microsomes were prepared from the livers of normal and warfarin-treated cows as described earlier [3,4], and carboxylase was solubilized from the microsomal pellet by adding buffer containing 0.8 M KCl, 0.05 M Tris-HCl (pH 7.5), 0.5% Triton X-100 and 30% ethylene glycol. The obtained solution was used for all experiments described below.

Measurement of carboxylase activity. Unless indi-

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cated otherwise, the vitamin K-dependent incorporation of $^{14}CO_2$ was measured by incubating soluble carboxylase (5 mg protein) and 20 μCi $NaH^{14}CO_3$ in 0.25-ml reaction mixtures containing 0.4 M KCl, 0.05 M Tris-HCl (pH 7.5), 10 mM DTT, 12% ethylene glycol, 0.2% Triton X-100 and 10 mM F L E E L. The reaction was started by adding 0.2 mM dihydroxyvitamin K and the mixtures were incubated in parafilm-sealed tubes at 25°C. The reaction was stopped with 2 ml 10% trichloroacetic acid and the precipitates (containing the carboxylated endogenous substrate) were washed and counted in a Packard Tricarb scintillation counter using Picofluor-15 scintillation liquid. The trichloroacetic acid-supernatants (containing the carboxylated F L E E L) were degassed at 80°C and counted.

When the carboxylation reaction was performed in the absence of oxygen, the incubations occurred in Warburg-flasks. Before the reaction was started, 0.4 ml of a mixture containing glucose (0.1 M) and glucose oxidase (0.5 mg) in 0.1 M NaCl and 0.05 M Tris-HCl, pH 7.4 was applied in the centre well, vitamin K in the side-arm vessel and the other components of the reaction mixture in the outer well. The bulk of the oxygen was removed by flushing with nitrogen gas, the flasks were closed and the last traces of oxygen were removed by incubating the flasks for 1 h at 25°C. After this period the carboxylation reaction was started by adding the dihydroxyvitamin K to the reaction mixture in the outer well.

Statistical analyses. The significances of the differences between the means of the results for the normal and the warfarin-treated groups (four cows in each group) were analyzed by a one-tailed Wilcoxon rank-sum test.

Determination of dihydroxyvitamin K, vitamin K and vitamin K-epoxide. The various forms of vitamin K were extracted from the 1 ml reaction mixtures, by adding 10 ml hexane and 15 ml isopropanol and shaking the mixtures for 5 min. After this period, 5 ml water was added and the upper phase was removed. The solvent was evaporated and the remaining vitamin K was dissolved in 0.5 ml methanol and analyzed by means of high-performance liquid chromatography (HPLC) on a Lichrosorb 10 RP 18 column (Chrompack, 25 X 0.46 cm) in methanol. This technique was performed on a Spectrophysics SP 8000 liquid chromatograph with a flow rate of 2 ml/min. The three forms of vitamin K were well separated when the effluent was monitored at 254 nm (Fig. 1). The area under each peak was recorded automatically and was corrected for the relative absorbance at the detection wavelength.

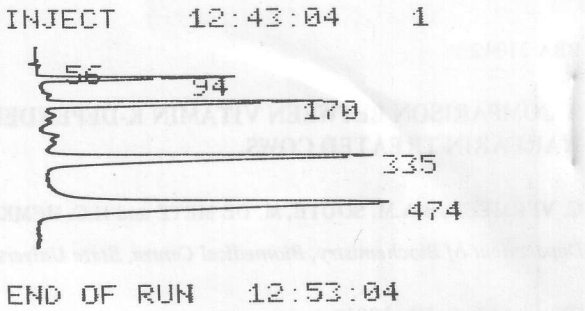

Fig. 1. Separation profile of vitamin K (474 s), dihydroxyvitamin K (170 s) and vitamin K epoxyde (335 s). The various forms of vitamin K (1 μg of each) were separated and detected by their absorbance at 254 nm. Calibration curves of each of the components were prepared at this wavelength and used for the determination of the absolute amounts of the various forms of vitamin K.

Results

We compared the properties of the vitamin K-dependent carboxylase present in eight different solubilized microsomal preparations. Four preparations were obtained from normal cows and the four other preparations from cows that had received warfarin. We first investigated the optimal conditions for the carboxylation reaction. They were equal for both types of carboxylase, and were as follows: 25°C, pH 7.5, 0.01 M DTT, 0.3–0.7 M KCl and 0.5% Triton X-100. The K_m values for dihydroxyvitamin K, CO_2 and F L E E L were determined and found to be equal for both groups (0.05, 0.17 and 11.5 mM, respectively). For technical reasons, we were unable to estimate and compare the K_m for O_2 of the different preparations. Both types of carboxylase were completely comparable, however, in their dependence upon O_2: in the absence of oxygen the incorporation of $^{14}CO_2$ amounted to 109 ± 8 dpm per mg protein and in the presence of oxygen 1472 ± 103 dpm (means of eight experiments ± S.E.).

The enzyme activities were compared by measuring the carboxylation reaction in the presence of 10

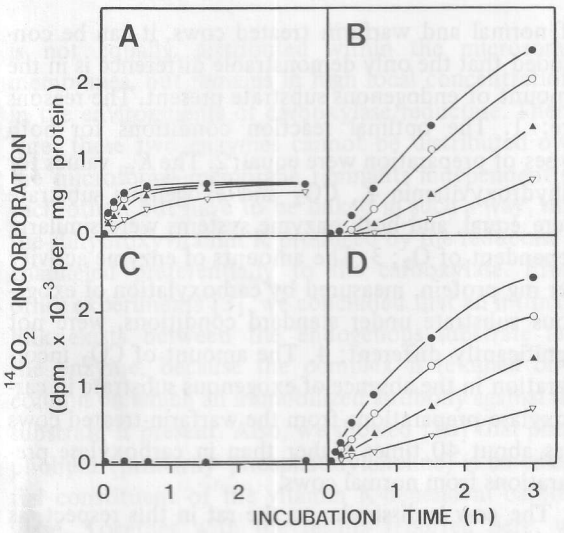

Fig. 2. Time course of the carboxylation at various vitamin K concentrations. The carboxylation was performed at 0.2 mM (●——●), 0.1 mM (○——○), 0.05 mM (▲——▲) and 0.025 mM (△——△) dihydroxyvitamin K. Panel A: carboxylase from warfarin-treated cows, endogenous substrate. Panel B: carboxylase from warfarin-treated cows, exogenous substrate. Panel C: carboxylase from normal coes, endogenous substrate. Panel D: carboxylase from normal cows, exogenous substrate.

mM FLEEL, because at concentrations greater than 20 mM a marked substrate inhibition of the carboxylation was observed. With 10 mM FLEEL and 0.2 mM dihydroxyvitamin K, the carboxylation rate, after a possible lag phase to be discussed later, was constant for at least 1 h and it was proportional to the amount of enzyme preparation added. When we compared the enzyme activity per mg protein of the different preparations under these conditions, no significant difference between the groups was found: the carboxylation rate in the preparations obtained from normal cows was 30.0 ± 3.2 dpm/min per mg protein and in preparations from the four warfarin-treated animals the rate was 35.2 ± 4.2 dpm/min per mg protein.

When CO_2 incorporation was determined in the absence of exogenous substrate, activity was observed only in carboxylase preparations from warfarin-treated animals (Fig. 2 A,C); this incorporation is interpreted as carboxylation of endogenous substrate. The initial rate of incorporation can be changed by varying the amount of dihydroxyvitamin K. This was also found when the carboxylation of exogenous substrate was measured (Fig. 2 B,D). The K_m values for dihydroxyvitamin K estimated from the initial rate of CO_2 incorporation in endogenous and exogenous substrate, were equal. The final level of incorporated CO_2 is proportional to the amount of the enzyme preparation added, and this level is a measure for the amount of endogenous substrate contained in this preparation. Carboxylase preparations from warfarin-treated cows contain significantly more endogenous substrate than the carboxylase preparations from normal cows (normal: 23.8 ± 1.6 dpm per mg protein, warfarin-treated: 900 ± 72 dpm per mg protein). The data in Fig. 2 show that, only when this endogenous substrate is present, a lag time occurs before the carboxylation of exogenous substrate starts. This is readily explained by a preferential carboxylation of the endogenous substrate.

We also investigated whether oxidized forms of vitamin K could be used for the carboxylation reaction. As shown in Fig. 3, both endogenous and exogenous substrates can be carboxylated in the presence of DTT, when vitamin K or vitamin K epoxide were

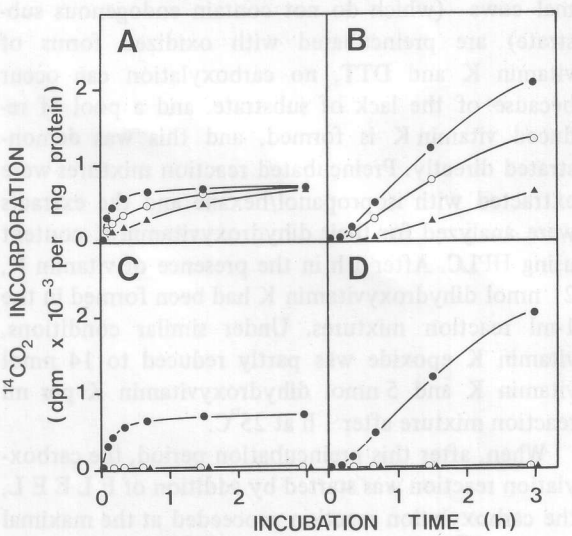

Fig. 3. The effect of various forms of vitamin K on carboxylase from wafarin-treated cows. The carboxylation was performed at 0.4 mM dihydroxyvitamin K (●——●), vitamin K (○——○) or vitamin K epoxide (▲——▲). Panel A: endogenous substrate. Panel B: exogenous substrate. Panel C: endogenous substrate + 20 μM warfarin. Panel D: exogenous substrate + 20 μM warfarin.

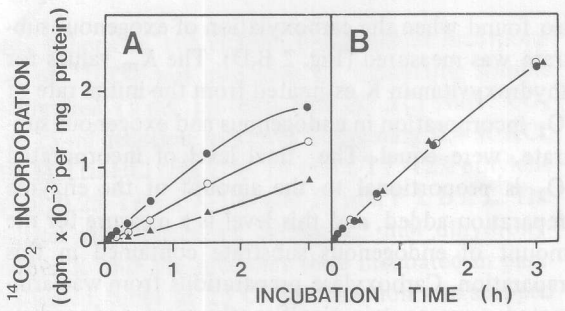

Fig. 4. The effect of various forms of vitamin K on carboxylase from normal cows. The carboxylation was performed at 0.4 mM dihydroxyvitamin K (●——●), vitamin K (○——○) or vitamin K epoxide (▲——▲). Panel A: without preincubation. Panel B: after 1 h preincubation at 25°C in the absence of F L E E L and $^{14}CO_2$.

used instead of dihydroxyvitamin K. This carboxylation was completely blocked by 20 μM warfarin. In the rat, this level of warfarin is known to strongly inhibit the reductase [2]. At equal concentrations of the coenzymes, the oxidized forms lead to lower carboxylation rates than that resulting from the reduced form. This indicates that the reduction step is rate-limiting. When carboxylase preparations from normal cows (which do not contain endogenous substrate) are preincubated with oxidized forms of vitamin K and DTT, no carboxylation can occur because of the lack of substrate, and a pool of reduced vitamin K is formed, and this was demonstrated directly. Preincubated reaction mixtures were extracted with isopropanol/hexane and the extracts were analyzed for their dihydroxyvitamin K content using HPLC. After 1 h in the presence of vitamin K, 21 nmol dihydroxyvitamin K had been formed in the 1-ml reaction mixtures. Under similar conditions, vitamin K epoxide was partly reduced to 14 nmol vitamin K and 5 nmol dihydroxyvitamin K per ml reaction mixture after 1 h at 25°C.

When, after this preincubation period, the carboxylation reaction was started by addition of F L E E L, the carboxylation reaction proceeded at the maximal rate for all three forms of vitamin K (Fig. 4).

Discussion

Upon comparison of the properties of the vitamin K-dependent carboxylase in microsomal preparations of normal and warfarin treated cows, it can be concluded that the only demonstrable difference is in the amount of endogenous substrate present. The reasons are: 1. The optimal reaction conditions for both types of preparation were equal; 2. The K_m values for dihydroxyvitamin K, CO_2 and exogenous substrate were equal, and both enzyme systems were similarly dependent of O_2; 3. The amounts of enzyme activity per mg protein, measured by carboxylation of exogenous substrate under standard conditions, were not significantly different; 4. The amount of CO_2 incorporation in the absence of exogenous substrate in carboxylase preparations from the warfarin-treated cows was about 40 times higher than in carboxylase preparations from normal cows.

The cow is dissimilar to the rat in this respect, as it has been shown by Shah and Suttie [6] and confirmed by us (unpublished data), that in rat liver the amount of carboxylase increases during warfarin treatment, whereas in cow-liver this treatment did not affect the enzyme level. As is known from the rat system [7–9], dihydroxyvitamin K is required for the carboxylation reaction. When vitamin K or vitamin K epoxide is used, the vitamin must be reduced by its reductase before the carboxylation reaction can proceed. With the endogenous, and also exogenous substrate, the reduction of vitamin K is the rate-limiting step, because the reduced form will always cause a substantially higher activity, if the carboxylase activities obtained with equal amounts of oxidized and reduced forms are compared.

The carboxylase can function independently of the reductase, with both exogenous and endogenous substrates, because 20 μM warfarin (which inhibits the reductase completely) will not inhibit the carboxylation if dihydroxyvitamin K is present. On the other hand, the reductase can function independently of the carboxylase, because vitamin K and its epoxide will be reduced when no substrate for carboxylation is present (carboxylase preparations from normal cows, tested without exogenous substrate). If substrate is added after preincubation with oxidized forms of vitamin K, the carboxylation proceeds as if dihydroxyvitamin K was present. The amounts of dihydroxyvitamin K that were formed amounted to 10–50% of the K_m value. Nevertheless, the carboxylation proceeded at its maximal reaction rate. This indicates that the newly-formed dihydroxyvitamin K

is not equally distributed within the microsomal membranes, but remains in high local concentrations in the environments of carboxylase/reductase. Therefore, these two enzymes cannot be distributed over the microsomal membrane remnants independent of each other, but have to be linked in such a way, that the dihydroxyvitamin K produced by the reductase is channeled preferentially to the carboxylase. From other experiments [3], we concluded that an intimate link exists between the endogenous substrate and the enzyme, because the complex is retained on a column on which an immobilized antibody against the substrate is present. Also, we showed [10] that phospholipid (primarily phosphatidylcholine) is an essential constituent of the vitamin K-dependent carboxylase. Together with the results reported here, we postulate the formation of an enzyme complex of carboxylase, reductase, endogenous substrate (if present) and phospholipid. The phospholipid moiety might function in the transport and/or storage of dihydroxyvitamin K, produced by the reductase. In our experiments, we have not yet been able to differentiate between the vitamin K-reducing enzyme and that reducing vitamin K-epoxide. Whether this means that both functions are fulfilled by the same enzyme, or enzyme system remains to be investigated.

Acknowledgment

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