

Characteristics of vitamin K-dependent carboxylating systems from human liver and placenta

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Bovine liver vitamin K-dependent carboxylase was compared with that obtained from human liver and placenta. Human liver microsomal preparations contained more endogenous substrate than did bovine preparations, but no differences were found between the two types of hepatic enzyme. This observation demonstrates that the bovine liver carboxylating enzyme system is a good model system which will help us to understand vitamin K action in man. Placental carboxylase differed from the liver systems because only vitamin K hydroquinone and not vitamin K quinone could be used as a coenzyme for the carboxylation reaction. Obviously, vitamin K reductase was absent in these preparations.

<i>Carboxylase</i>	<i>Vitamin K</i>	<i>Warfarin</i>	<i>Liver</i>	<i>Placenta</i>	<i>γ-Carboxyglutamic acid</i>
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1. INTRODUCTION

The four vitamin K-dependent clotting factors are synthesized in the liver, and during their synthesis they undergo a number of post-translational modifications, one of which is the vitamin K-dependent carboxylation of a number of glutamic acid residues [1,2]. The *in vivo* carboxylation reaction is inhibited by the oral administration of vitamin K-antagonists such as warfarin or dicoumarol. This kind of treatment (anticoagulant therapy) is frequently used in medical practice and results in the production of non-carboxylated clotting factors (descarboxy factors), which cannot be activated in the normal coagulation process [3]. Obviously, it became more interesting to investigate the mechanism of action of vitamin K as well as that of vitamin K-antagonists. *In vitro* model systems were developed from rat and bovine liver [4,5], from rat lung [6] and from horse spleen and kidney [7]. It is evident, that the results obtained with these model systems should be compared with carboxylating systems from human origin. A first attempt was made by Friedman et al. [8], who prepared carboxylase from human placenta. As compared with rat liver carboxylase, the activity of this

system was very low, however. Here, we describe the preparation of carboxylase from human liver and placenta and these two systems are compared with the formerly characterized bovine liver carboxylase [5].

2. MATERIALS AND METHODS

Vitamin K was obtained from Hoffmann-La Roche and vitamin K hydroquinone was prepared as in [5]. The synthetic substrate Phe-Leu-Glu-Glu-Leu (FLEEL) was obtained from Vega Fox and $\text{NaH}^{14}\text{CO}_3$ (40 Ci/mol) from New England Nuclear. Triton X-100, warfarin, β -mercaptoethanol, dithiothreitol (DTT) and dithioerythritol (DTE) were purchased by Sigma and NADH and NADPH by Boehringer Mannheim. Fragment Su was prepared from purified descarboxyprothrombin [9] and bovine carboxylase from normal cow liver [5]. Carboxylase from human liver was prepared in a similar way. Human livers were obtained from normal, healthy donors, who had died after traffic accidents and who had previously signed a donor-codicil. It was verified that they had not recently been under medical treatment, and that they had not received anti-vitamin K drugs. Carboxylase from term human placenta was prepared from the microsomal fraction of

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trophoblast in a similar way as was carboxylase from bovine liver, except that the microsomal suspension was solubilized with 0.12% Triton X-100. Carboxylase activity was measured in the presence of 10 mM FLEEL [5]. Unless stated otherwise, the carboxylation is expressed as the sum of the $^{14}\text{CO}_2$ incorporation into endogenous and exogenous substrate. Human prothrombin and clotting factor X were purified as in [10] and [11] and antibodies against these proteins were raised in rabbits.

3. RESULTS

The optimal reaction conditions for the 3 carboxylating systems were similar: pH 7.5, 25°C, 0.8 M KCl, 10 mM DTT and 10 mM MnCl_2 . In all 3 systems the presence of MnCl_2 stimulated the carboxylation ~2-fold. The K_m of the pentapeptide FLEEL, which was used as an exogenous substrate, was ~8 mM in all cases. Liver carboxylase could be solubilized with 0.5% Triton X-100, but placental carboxylase was rapidly inactivated at these detergent concentrations. The best results were obtained with 0.12% Triton X-100, which did not inactivate carboxylase and solubilized ~60% of the total amount of enzyme. The K_m values of vitamin K and vitamin K hydroquinone were measured in the various systems. These values were closely similar in bovine and human liver (table 1), but when we tried to measure the K_m of vitamin K in placental carboxylase, it resulted that this enzyme system was absolutely dependent on the presence of vitamin K hydroquinone. No activity could be detected in the presence of vitamin K quinone. Obviously the vitamin K reductase in these preparations was either absent or inactive. It

might also be assumed that vitamin K reductase is present in the placental system, but that it has a high specificity towards some reducing agent. Therefore we replaced DTT (which is normally present in our reaction mixtures) by either DTE, β -mercaptoethanol, NADH or NADPH, but none of these reducing agents were able to stimulate the vitamin K-dependent carboxylation. When we measured the amounts of endogenous substrate in the various preparations, a striking difference was observed between carboxylase from human liver and the other two preparations. Because all 3 tissues had been obtained from normal, non-anticoagulated subjects, it was to be expected, that hardly any endogenous substrate was present in either of these preparations. Nevertheless, it is obvious that the microsomal preparations from human liver contained substantial amounts of endogenous substrate (table 2), all of which was present in the trichloroacetic acid-precipitable fraction. We tried to identify this substrate using Sepharose-bound antibodies against human prothrombin and factor X. It resulted, that from the total amount of carboxylated product 45% could be bound to anti-factor X and 21% to antiprothrombin.

We also investigated the substrate specificities of the various systems for carboxylatable exogenous substrates such as FLEEL and fragment Su. Both substrates are derived from bovine descarboxyprothrombin: while the synthetic pentapeptide resembles the amino acid residues 5–9, fragment Su is similar to the residues 13–29. FLEEL contains the first 2 carboxylatable Glu-residues in descarboxyprothrombin and fragment Su the subsequent 6 ones. As is shown in table 3, the K_m of fragment Su ranged from 0.8–1.0 μM in the 3 dif-

Table 1
Characteristics of carboxylase from different tissues

Source of carboxylase	K_m (μM) of:		Warfarin concentration (μM) required for 50% inhibition in presence of:	
	Vitamin K	Vitamin KH_2	Vitamin K (25 μM)	Vitamin KH_2 (25 μM)
Bovine liver	13	10	2	400
Human liver	16	22	3	1500
Human placenta	—	16	—	1200

All tests were performed under standard conditions. Vitamin K hydroquinone is abbreviated as vitamin KH_2

Table 2

Amounts of enzyme and endogenous substrate in carboxylase from different tissues

Source of carboxylase	Carboxylase (dpm/min, mg protein)	Endogenous substrate (dpm/mg protein)
Bovine liver	31	0
Human liver	95	1120
Human placenta	12	0

The amount of carboxylase was measured as the carboxylation rate in endogenous + exogenous substrate (FLEEL, 10 mM) and is expressed as dpm per min and per mg protein. The amount of endogenous substrate is given as the total amount of $^{14}\text{CO}_2$ (dpm/mg protein) that could be incorporated into trichloroacetic acid-precipitable material. This way of quantitating carboxylase and its endogenous substrate is explained extensively in [5]

Table 3

Kinetic parameters in carboxylase from different tissues

Source of carboxylase	FLEEL		Fragment Su	
	K_m (mM)	V (dpm/min, mg)	K_m (μM)	V (dpm/min, mg)
Bovine liver	3.3	108	1.0	24.6
Human liver	2.8	99	0.8	35.5
Human placenta	7.1	14.3	0.9	13.5

The K_m and the maximal carboxylation rate V are calculated as in [9]; V is expressed as dpm per min and per mg microsomal protein

ferent types of preparations. The K_m of FLEEL on the other hand, was 2.5-times higher in placental carboxylase than in the liver systems, suggesting that the pentapeptide is recognized less efficiently by placental carboxylase than by the liver enzyme. In placental carboxylase the maximal rate of substrate carboxylation V was similar for both substrates. In the liver systems, however, the carboxylation rate of FLEEL was 3–4-times higher than that of fragment Su.

4. DISCUSSION

Here we describe the vitamin K-dependent carboxylation in microsomal preparations obtained from normal bovine liver, from human liver and from human placenta. When comparing the bovine and human liver systems, we observed that they were similar except that the human preparations contained substantial amounts of endo-

genous substrate. That both enzyme systems are similar, supports the idea that animal model systems will help us in understanding vitamin K action in man. The amounts of endogenous substrate in human liver were comparable to those in livers from warfarin-treated cows. This phenomenon was observed in 3 successive human livers, whereas in > 10 normal bovine livers we have never been able to detect comparable amounts of substrate [5]. We concluded, that in these human livers the production of endogenous substrate had occurred at a higher speed than had the carboxylation reaction. This might be due, either to a vitamin K deficiency or to a limiting amount of carboxylase. Vitamin K deficiency is not a common feature in man and moreover, it was not observed in placental carboxylase. We think, therefore, that the abnormal high level of clotting factor precursors in human liver is a result of the physical condition of the donors at the moment that the livers were ob-

tained: all donors had gone through a traffic accident, attended with severe brain damage, massive blood loss and diffuse intravascular and other coagulation processes. After hospitalization, they were ventilated for at least 4–8 h before excision of their livers. Because blood loss, decreased plasma concentrations of clotting factors and activation products of clotting factors [12,13] all have been reported to stimulate the *de novo* synthesis of clotting factors, it is likely that also in the human livers, described here, the production of clotting factors had been strongly enhanced. It is known that other post-translational enzymes (e.g., prolylhydroxylase, lysylhydroxylase, glucosyltransferase and galactosyltransferase) are present in only marginal quantities [14]. The same may be the case for carboxylase, and this would explain why during periods of an unusual high production of clotting factor precursors, the latter accumulate in the liver. In such a situation the carboxylation (or maybe the combined post-translational processes) is the rate-limiting step in their maturation. Since we do not have further evidence to support this theory, other explanations may be given for the high amounts of endogenous substrate in human liver. When we compared carboxylase in liver and placental microsomes the placental preparations seemed to contain carboxylase but no reductase: only vitamin K hydroquinone and not vitamin K quinone was able to stimulate the carboxylation reaction. Since the placental carboxylating system in [8] was only tested with vitamin K quinone, this explains why these authors observed such a low activity. It is not likely, that also *in vivo* the reducing enzyme system is absent, so we think that it is lost and/or destroyed somewhere during the preparation procedure. It is remarkable, that the differences in substrate specificity between the various carboxylases are so low. Only in the case of FLEEL we observed a slightly increased K_m in the placental system. It seems plausible, that a universal mechanism for substrate recognition is operative in carboxylase from various types of tissue. The differences between the maximal carboxylation rates in the 3 systems may partially be brought about by differences between the amounts of carboxylase/mg microsomal protein in the various tissues. On the other hand, the relation between V in liver microsomes and that in placental microsomes varies with the choice of the substrate. So it

seems, that the differences between the kinetic parameters in the 3 tissues indicate some substrate specificity of the various enzyme systems, but the differences are too small to draw definite conclusions as yet.

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REFERENCES

- [1] Suttie, J.W. (1980) *CRC Crit. Rev. Biochem.* 8, 191–223.
- [2] Gallop, P.M., Lian, J.B. and Hauschka, P.V. (1980) *New Engl. J. Med.* 302, 1460–1466.
- [3] Esmon, C.T., Suttie, J.W. and Jackson, C.M. (1975) *J. Biol. Chem.* 250, 4095–4099.
- [4] Suttie, J.W., Lehrman, S.R., Geweke, L.O., Hageman, J.M. and Rich, D.H. (1979) *Biochem. Biophys. Res. Commun.* 86, 500–507.
- [5] Vermeer, C., Soute, B.A.M., De Metz, M. and Hemker, H.C. (1982) *Biochim. Biophys. Acta* 714, 361–365.
- [6] Bell, R.G. (1980) *Arch. Biochem. Biophys.* 203, 58–64.
- [7] Vermeer, C. and Ulrich, M. (1982) *Thrombos. Res.* in press.
- [8] Friedman, P.A., Hauschka, P.V., Shia, M.A. and Wallace, J.K. (1979) *Biochim. Biophys. Acta* 583, 261–265.
- [9] Soute, B.A.M., Vermeer, C., De Metz, M., Hemker, H.C. and Lijnen, H.R. (1981) *Biochim. Biophys. Acta* 676, 101–107.
- [10] Butkowski, R.J., Bajaj, S.P. and Mann, K.G. (1974) *J. Biol. Chem.* 249, 6562–6569.
- [11] Mertens, K. and Bertina, R.M. (1980) *Biochem. J.* 185, 647–658.
- [12] Shah, D.V., Nyari, L.Y., Swanson, J.C. and Suttie, J.W. (1980) *Thrombos. Res.* 19, 111–118.
- [13] Graves, C.B., Munns, T.W., Carlisle, T.L., Grant, G.A. and Strauss, A.W. (1981) *Proc. Natl. Acad. Sci. USA* 78, 4772–4776.
- [14] Prockop, D.J., Berg, R.A., Kivirikko, K.I. and Uitto, J. (1976) in: *Biochemistry of Collagen* (Ramachandran, G.N. and Reddi, A.H. ed) pp. 163–273, Plenum, New York.