Characterization of vitamin K-dependent carboxylase from the livers of the adult ox and dicoumarol-treated calf

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The properties of the microsomal vitamin K-dependent carboxylase from the livers of the adult ox and dicoumarol-treated calf were investigated. The enzymes from both sources utilized glutamic residues of synthetic peptides as substrates and could be solubilized with Triton X-100 similarly to the enzyme from vitamin K-deficient rat liver. Under the optimal assay conditions, the microsomes from calf liver had peptide carboxylase activity comparable with that of the rat liver microsomes and 6.5-fold that of adult ox liver microsomes. The apparent K_m for reduced vitamin K and the ionic strength optima of the calf and adult ox enzyme clearly differ from those of the rat enzyme. Pyridoxal phosphate activated the adult ox carboxylase only slightly, whereas the calf enzyme was activated by pyridoxal phosphate as effectively as was the enzyme from the vitamin K-deficient rat. Mn^{2+} activated the adult ox enzyme 9-fold and calf enzyme 22-fold under optimal conditions (no KCl). Three other divalent metal cations (Ca^{2+} , Ba^{2+} , and Mg^{2+}) activated the adult ox and calf enzymes to about half the extent caused by Mn^{2+} . KCl inhibited this activation. The vitamin K-dependent carboxylase from the dicoumarol-treated calf is apparently more tightly bound to the microsomal membrane than is the adult ox enzyme. In many other respects (pH optimum, temperature optimum, K_m values for peptide substrate, substrate specificity, inhibitor effects), the properties of the adult ox and calf enzymes resemble closely those of the rat enzyme.

Vitamin K is a required cofactor for the post-translational carboxylation of specific glutamic residues in microsomal protein precursors to form γ -carboxyglutamic residues in prothrombin, other vitamin K-dependent plasma proteins, and uncharacterized proteins in other tissues (Suttie, 1978; Olson & Suttie, 1978). The microsomal carboxylase carrying out this reaction has been shown to require the reduced form of the vitamin, O_2 , CO_2 , and peptide-bound glutamic residues (Suttie, 1980*a,b,c*). The carboxylase obtained from the liver of vitamin K-deficient rats has been demonstrated to be active in detergent-solubilized preparations (Esmon & Suttie, 1976; Mack *et al.*, 1976) or acetone powders (Friedman & Shia, 1980) and has been partially purified (Houser *et al.*, 1978; Canfield *et al.*, 1980; Wallin *et al.*, 1980). The enzyme from other sources has not been characterized, although a survey of carboxylase activity in other species has been

presented (Shah & Suttie, 1979). Hemker and his co-workers (Vermeer *et al.*, 1976, 1978, 1980; De Metz *et al.*, 1980) have, however, reported an extensive characterization and partial purification from adult ox liver of an activity called prothrombin synthase which they regard as identical with the vitamin K-dependent carboxylase.

We have been unable to repeat the purification described for the adult ox preparations (Vermeer *et al.*, 1978; De Metz *et al.*, 1980) and decided to characterize the vitamin K-dependent carboxylase from this source. Because the conditions used in the assay of bovine prothrombin synthase (Vermeer *et al.*, 1976, 1978, 1980; De Metz *et al.*, 1980) differ considerably from those optimal for the rat liver carboxylase (Suttie *et al.*, 1979), we related the properties of the adult ox enzyme to those of the enzyme obtained from vitamin K-deficient rats. We have also determined some of the properties of the carboxylase obtained from the liver of a calf made hypoprothrombinaemic by dicoumarol treatment and compared these properties with those of the

Abbreviations used: Boc, *t*-butoxycarbonyl; -Bzl, benzyl; KH_2 , reduced vitamin K.

enzymes from the adult ox and vitamin K-deficient rats.

Materials and methods

Chemicals

KH₂ was prepared by reducing vitamin K₁ (phyloquinone) obtained from Sigma as previously described (Sadowski *et al.*, 1976). NaH¹⁴CO₃ (58 mCi/mmol) was purchased from Amersham/Searle and the pentapeptide Phe-Leu-Glu-Glu-Leu from Vega Biochemicals (Tucson, AZ, U.S.A.). Boc-Glu- α -Bzl was obtained from Sigma, Glu-Glu from Cyclo Chemical (Los Angeles, CA, U.S.A.), and Ser-Glu-Glu-Ala-Arg from Bachem (Torrance, CA, U.S.A.). Other peptide substrates were obtained from Dr. D. H. Rich, School of Pharmacy, University of Wisconsin-Madison. Triton X-100 (scintillation grade) was purchased from Research Products International (Elk Grove Village, IL, U.S.A.), octylglucoside and dithiothreitol from Calbiochem. Aquasol from New England Nuclear and warfarin from Endo (Garden City, NY, U.S.A.). Other chemicals were of the highest purity from local suppliers.

Preparation of liver

Adult ox liver was obtained immediately after slaughter from the Muscle Biology Laboratory of this university. A calf was made hypoprothrombin-aemic (10% normal one-stage clotting time) by dicoumarol treatment as previously described (Shah & Suttie, 1979). The liver tissue was cut into small pieces and then homogenized for 2 min at 0°C in a Waring Blender in 2 vol. of 25 mM-imidazole/HCl buffer, pH 7.2, containing 250 mM-sucrose and 1 mM-dithiothreitol. The homogenate was centrifuged at 10 000 g for 10 min, and the postmitochondrial supernatant thus obtained at 105 000 g for 60 min. The pellets (microsomal fraction) were surface-washed twice with the homogenization buffer and were then freshly used or stored (up to several months) in liquid nitrogen.

Determination of carboxylase activity

The microsomal pellets were resuspended by homogenizing in a Dounce homogenizer in 50 mM-imidazole/HCl buffer, pH 7.2, containing 1.0% Triton X-100 and 1 mM-dithiothreitol. The volume was made equal to that of the original post-mitochondrial supernatant, and insoluble material was removed by centrifuging at 105 000 g for 60 min. Alternatively, the solubilizing solution also contained 0.3 M-KCl and 0.25 M-sucrose which, in contrast with the former method, gave complete solubilization of the carboxylase from these sources. The solubilized supernatant was then dialysed overnight against an at least 500-fold excess of the

same solution without KCl and sucrose to make it possible to study the activities in the absence of KCl. This modification did not influence the results.

The carboxylase assay mixture contained 200–300 μ l of the solubilized preparation, 1.5 mM-Phe-Leu-Glu-Glu-Leu, 20 μ Ci of NaH¹⁴CO₃/ml and 100 μ g of KH₂ in a total volume of 450–500 μ l. Blank incubations contained all components except KH₂. If not otherwise stated, all assay tubes were incubated for 30 min at 17°C. Incorporation of ¹⁴CO₂ into peptide substrate was measured as described by Suttie *et al.* (1976). In some cases, the trichloroacetic acid supernatants remained turbid after the low-speed centrifugation used to remove protein precipitates and interference in the determination of radioactivity by liquid-scintillation spectrometry was noted. In these cases, 1 vol. of chloroform was added, and the tubes were mixed for 30 s on a Vortex mixer. The upper, radioactivity-containing, aqueous phase became clear within 15–30 min and was used for radioactivity determination.

Results

Effects of pH, temperature, and salt concentration on carboxylase activity

The highest activity of the adult ox liver carboxylase was observed at pH values slightly below 7.0 (Fig. 1a). Little activity was found above pH 8.0 and no activity at pH 8.5. The enzyme had significant activity at 0°C (results not shown) and the optimum temperature determined was 17°C (Fig. 1b). At temperatures above 24°C, the enzyme was unstable as indicated by the rapid levelling off of the incorporation curves. When the enzyme activity was measured as a function of the KCl concentration of the assay mixture, the adult ox liver carboxylase was activated slightly by low KCl concentrations, but inhibited at concentrations higher than 0.15 M (Fig. 1c). Additions of NaCl gave curves almost identical with those shown for KCl, indicating that the effect was not specific for K⁺ but was rather caused by changes in ionic strength. When a KCl activation curve was carried out for the enzyme obtained from a dicoumarol-treated calf, it was found (Fig. 1c) to be activated more than 3-fold by 0.3 M-KCl and to have significant activity above 0.5 M-KCl.

Metal activation

The rat liver carboxylase is activated by Mn²⁺ (Larson & Suttie, 1980), and the adult ox enzyme was activated nearly 10-fold by 5–10 mM-Mn²⁺ (Fig. 1d). Concentrations of Mn²⁺ above 10 mM were inhibitory. The apparent half-saturation concentration (*S*_{0.5}) for Mn²⁺ was high (1.5 mM) in the standard assay which contained 50 mM-imidazole, a known Mn²⁺ chelator (Sillén & Martell, 1971). The

Fig. 1. Dependency of the activity of vitamin K-dependent carboxylase from bovine liver on pH, temperature, KCl concentration, and Mn^{2+} concentration

(a) pH dependence; the buffers used were 50 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ (O) and 50 mM-Tris/HCl (●). (b) Temperature dependence; larger incubation volumes were used and aliquots were taken from the assay mixtures at the times indicated. The temperatures used are indicated in the Figure. (c) KCl concentration dependence; activity was determined in microsomal preparations from adult ox (●) and dicoumarol-treated calf (O). For comparative purposes the activity of both enzymes in 50 mM-imidazole/HCl, pH 7.2, 1% Triton X-100 and 1 mM-dithiothreitol has been set to 100. The actual activity of the calf enzyme under these conditions is higher than that of the adult ox enzyme. (d) Mn^{2+} concentration dependence; microsomes were resuspended in 1% Triton X-100 and 1 mM-dithiothreitol containing 50 mM-imidazole/HCl (O) or Hepes (●) buffer, pH 7.2. Mn^{2+} was added to the assay mixtures as MnCl_2 .

$S_{0.5}$ value for Mn^{2+} was, however, only slightly lower (1.2 mM) in Hepes [4-(2-hydroxyethyl)piperazine-ethane sulphonic acid] buffer, which does not bind Mn^{2+} (Good *et al.*, 1966). If dithiothreitol, a potential Mn^{2+} chelator, was also omitted the $S_{0.5}$ value for Mn^{2+} was still high (0.9 mM) but the activation by Mn^{2+} was less extensive (see below). The enzyme from the dicoumarol-treated calf was activated about 22-fold by Mn^{2+} . The optimal concentration of Mn^{2+} for this system was 8–15 mM and $S_{0.5}$ for Mn^{2+} in imidazole buffer was 2.5 mM. Although Mn^{2+} was the best activator, the effect of Mn^{2+} was not specific, as the enzyme from both sources was significantly activated by Ca^{2+} , Ba^{2+} , and Mg^{2+} (Table 1). Because the activities with these metals were compared with the activity in 50 mM-imidazole, pH 7.2, part of the activation was

probably a non-specific increase of the activity due to increase in ionic strength. However, this non-specific activation by 10 mM-divalent metal salt, which would correspond to 30 mM-KCl in terms of ionic strength, would be less than a 30% increase in activity compared with the few-hundred-fold activations caused by the addition of 10 mM salts of Ca^{2+} , Ba^{2+} , and Mg^{2+} (Table 1). The other metals tested, Zn^{2+} and Co^{2+} , did not activate the enzyme but were inhibitors at even relatively low concentrations. The activation of the enzyme by Mn^{2+} was also determined at different KCl concentrations (results not shown). The metal activation of both the adult ox and dicoumarol-treated calf enzyme was maximal when no KCl was present, and KCl inhibited the metal activation. Activation of the adult ox enzyme by 10 mM- Mn^{2+} was totally prevented by

Table 1. *Activation of adult ox and dicoumarol-treated calf liver carboxylase by divalent metal cations*

The microsomes were solubilized and activity assayed as described in the Materials and methods section except that no dithiothreitol was included. The activity without metal has been set to 100 for both enzymes. The unactivated activity of the calf enzyme was 2.5 times that of normal bovine enzyme.

Enzyme	[Metal ion] (mM)	Activity (% of control) with:					
		Mn ²⁺	Ca ²⁺	Ba ²⁺	Mg ²⁺	Zn ²⁺	Co ²⁺
Adult ox	1	400	228	190	194	33	60
Adult ox	10	865	553	439	394	2	27
Calf	1	518	166	185	213	60	90
Calf	10	1590	953	922	811	6	30

KCl concentrations above 0.2 M. The highest activity for both enzymes was found in 50 mM-imidazole buffer containing 10 mM-Mn²⁺ and no KCl, despite the fact that KCl activated the enzymes in the absence of Mn²⁺. EDTA inhibited the carboxylase, but greater than 5 mM-EDTA was needed for 50% inhibition; a residual activity of 30% still remained when the assays contained 10 mM-EDTA.

Effect of enzyme concentration: apparent K_m values

The dependence of enzyme activity on amount of the calf enzyme was determined in the presence and absence of Mn²⁺ (Fig. 2). Without Mn²⁺, the rate was linear with respect to the enzyme concentration to about 250 μ l of enzyme in the final volume of 450 μ l. In the presence of Mn²⁺ the rate levelled off earlier but was, however, linear over a broader enzyme concentration range when the incubation time was shorter (results not shown). The apparent K_m value for the pentapeptide Phe-Leu-Glu-Glu-Leu in the presence and absence of Mn²⁺ was determined from a double-reciprocal plot for the adult ox enzyme. Mn²⁺ increased only the maximum velocity of the reaction without any influence on the K_m, which was 3.5 mM (results not shown). The apparent K_m for KH₂ was 47 μ g/ml for the adult ox enzyme in the absence of Mn²⁺ (Fig. 3a) and 41 μ g/ml in the presence of 10 mM-Mn²⁺. For the calf enzyme in the absence of Mn²⁺, the apparent K_m for the pentapeptide was 4.0 mM and for KH₂ 40 μ g/ml (results not shown). The apparent K_m for NaH¹⁴CO₃ was 52 μ Ci/ml (0.9 mM) for both enzymes studied (Fig. 3b) in both the presence and absence of Mn²⁺. These values are rough estimates because of endogenous HCO₃⁻ in the assay solutions.

Activation of pyridoxal phosphate

Pyridoxal phosphate has previously been reported to activate the vitamin K-dependent rat liver carboxylase (Suttie *et al.*, 1979, 1980; Dubin *et al.*, 1979). The enzyme from the adult ox was, however, activated only slightly by pyridoxal phosphate (Fig. 4a), whereas the enzyme from a dicoumarol-treated calf was activated to the same extent (3-fold) as has

Fig. 2. *Dependency of the activity of vitamin K-dependent carboxylase from dicoumarol-treated calf liver on the enzyme concentration*

The microsomes were resuspended in 50 mM-imidazole/HCl, pH 7.2, 1% Triton X-100, 1 mM-dithiothreitol. The total volume of the assays was 0.45 ml; the portion of the incubation that was solubilized microsomes is shown. The suspension was diluted with 50 mM-imidazole/HCl, pH 7.2, and 1 mM-dithiothreitol but no Triton X-100. (●) No Mn²⁺; (O) 10 mM-MnCl₂.

been reported for the rat enzyme (Suttie *et al.*, 1979). Activation of the calf enzyme was maximum at about 2 mM-pyridoxal phosphate and decreased at higher concentrations. This response was not ob-

Fig. 3. Dependency of the activity of vitamin K-dependent carboxylase from adult ox liver on the concentration of reduced vitamin K (a) and dependency of the activity of the carboxylase from dicoumarol-treated calf liver on $NaH^{14}CO_3$ concentration (b)

(a) Activity was determined in the absence of Mn^{2+} . (b) Blank incubations without KH_2 at various $NaH^{14}CO_3$ concentrations are presented (●). These values have been subtracted from the total activity values to obtain the curve for the enzymic activity (○).

Fig. 4. Effect of pyridoxal phosphate concentration on the activity of the vitamin K-dependent carboxylase from the adult ox and dicoumarol-treated calf

(a) (●) Adult ox carboxylase, no Mn^{2+} present; (○) dicoumarol-treated calf carboxylase, no Mn^{2+} present; (□) dicoumarol-treated calf carboxylase in the presence of 10 mM- $MnCl_2$. For comparison, all three activities without pyridoxal phosphate have been set to 100%. (b) Effect of pyridoxal phosphate on the dicoumarol-treated calf carboxylase in the absence (●) and presence (○) of 10 mM- Mn^{2+} .

served in studies of the rat enzyme (Suttie *et al.*, 1980; Dubin *et al.*, 1979). In the presence of 10mM-Mn²⁺, pyridoxal phosphate did not activate the enzyme but rather acted as an inhibitor (Fig. 4a). When the actual activities rather than relative activities are plotted (Fig. 4b), it can be seen that the activity in the presence of both Mn²⁺ and pyridoxal phosphate was still much higher than in the presence of pyridoxal phosphate alone.

Effect of dithiothreitol and inhibitors

Dithiothreitol increased the non-Mn²⁺-stimulated activity of the enzyme slightly (10–15% at 1 mM and about 30% at 5–10mM-dithiothreitol). Mn²⁺ activation was also clearly dependent on free -SH groups. When an enzyme preparation which had been solubilized and stored at 4°C without thiol

addition was studied (Table 2), Mn²⁺ activation of the enzyme was markedly decreased even though there was little effect on the activity without Mn²⁺. Addition of dithiothreitol restored most of the original activation by Mn²⁺ (Table 2). Even when the calf enzyme was freshly used after solubilization, 1mM-dithiothreitol increased the activation by Mn²⁺ by about 30%. It is possible that the activation seen with various other metals (Table 1) would also be higher in the presence of dithiothreitol.

The effects of two -SH reagents, *p*-hydroxymercuribenzoate and *N*-ethylmaleimide, on the carboxylase were studied; they were found to give 50% inhibition at 0.2–0.5mM (Table 3). Later addition of an excess of dithiothreitol reversed the inhibition resulting from low *p*-hydroxymercuribenzoate concentrations and partially reversed the inhibition seen at high concentrations. *N*-Ethylmaleimide inhibition was not reversed when the activation of control enzyme by dithiothreitol is taken into account (Table 3). These results suggest that these reagents were indeed reacting with -SH group(s). The inhibition by -SH reagents was not significantly influenced by the presence of Mn²⁺ (Table 4), nor could addition of Mn²⁺ to the enzyme before the -SH reagents protect the enzyme from them.

Rather high concentrations of warfarin are required to inhibit the rat liver carboxylase (Suttie *et al.*, 1979), and a similar effect was seen with adult ox and calf preparations. No inhibition was seen at 0.1mM-warfarin, and 20–30% inhibition at 1.0mM. Some carboxylase activity remained at 10mM-warfarin, and the extent of inhibition was not dependent on the concentration of Mn²⁺ in the incubation. The enzyme from both the adult ox and calf was less sensitive to ethanol than was the carboxylase from

Table 2. Effect of dithiothreitol on the activation by Mn²⁺ of the dicoumarol-treated calf liver carboxylase

The carboxylase activity was measured immediately after solubilization of the microsomes, which was done as described in the Materials and methods section, except for omission of dithiothreitol, and after storage of the solubilized microsomal preparation for 40h at 4°C. When indicated, the activity assay mixture also contained 1mM-dithiothreitol and/or 10mM-MnCl₂.

Conditions in activity assay		10 ⁻² × Activity (d.p.m./ml) of:	
Dithiothreitol	Mn ²⁺	Fresh enzyme	Enzyme after storage
—	—	657	727
—	+	11050	3639
+	—	699	761
+	+	14920	12541

Table 3. Effect of -SH reagents on the dicoumarol-treated calf carboxylase

Microsomes were solubilized as usual except that no dithiothreitol was included. The solubilized enzyme was incubated for 30min at 0°C in mixtures containing 50mM-imidazole, pH 7.2, 0.7% Triton X-100 and the inhibitors at concentrations given below (70% of the incubation volume was enzyme; a control without inhibitor was included). Samples were taken for carboxylase activity assay (a 250μl sample to the normal assay mixture of 450μl, 20min incubation at 17°C). Dithiothreitol (8mM) was added to the rest of the incubation mixtures and incubation continued at 0°C for another 60min. Activities were then measured as before the addition of dithiothreitol. Blanks, 1150 d.p.m./ml for control incubations and slightly lower for the incubations containing the inhibitors, have been subtracted from the values. The carboxylase from adult ox liver gave similar results.

Addition	Before the addition of dithiothreitol		After the addition of dithiothreitol	
	Activity (d.p.m./ml)	Inhibition (%)	Activity (d.p.m./ml)	Inhibition (%)
Control (no addition)	80800	—	99400	—
0.1mM- <i>p</i> -Hydroxymercuribenzoate	66800	17	100500	0
0.5mM- <i>p</i> -Hydroxymercuribenzoate	11000	86	82900	17
0.2mM- <i>N</i> -Ethylmaleimide	66300	16	87400	12
1.0mM- <i>N</i> -Ethylmaleimide	21300	74	37000	63

Table 4. *Effect of Mn²⁺ on the inhibition of dicoumarol-treated calf carboxylase by p-hydroxymercuribenzoate*

The enzyme was incubated together with the controls for 30 min at 0°C and activity was assayed at 17°C as described in Table 3. In Expt. 1, neither the incubation nor the activity assay mixtures contained Mn²⁺ (similarly to the experiment of Table 3). In Expt. 2, incubation at 0°C was done as for Expt. 1, but the activity assay mixtures contained 10 mM-Mn²⁺. In Expt. 3, 10 mM-Mn²⁺ was added to the enzyme before the -SH reagent, and incubation then done as in the other experiments. The activity assay also contained 10 mM-Mn²⁺. A control was always included and treated in the same way as the samples containing inhibitor. Blanks, 1200 d.p.m./ml without Mn²⁺ and 1750 d.p.m./ml with 10 mM-Mn²⁺, have been subtracted from all values.

Expt.	Conditions	Control enzyme activity (d.p.m./ml)	Concn. of p-hydroxymercuribenzoate (mM)			
			0.2		0.5	
			Activity (d.p.m./ml)	Inhibition (%)	Activity (d.p.m./ml)	Inhibition (%)
1.	Incubation -Mn ²⁺ , assay -Mn ²⁺	61 700	39 570	36	12 170	80
2.	Incubation -Mn ²⁺ , assay +Mn ²⁺	920 810	646 290	30	200 770	78
3.	Incubation +Mn ²⁺ , assay +Mn ²⁺	731 500	540 020	26	167 120	77

Table 5. *Substrate specificity of the adult ox carboxylase*

The assay conditions were as described in the Materials and methods section. Substrate concentration was 1.5 mM. Besides Phe-Leu-Glu-Glu-Leu (see the text), *K_m* determination was done only for Boc-Glu-Glu-Leu-OMe (without Mn²⁺). The result was 5.0 mM. Blanks, 1200 d.p.m./ml without Mn²⁺, and 2040 d.p.m./ml with Mn²⁺, have been subtracted from all values.

Substrate	Activity			
	-Mn ²⁺		+10 mM-Mn ²⁺	
	d.p.m./ml	(%)	d.p.m./ml	(%)
Phe-Leu-Glu-Glu-Leu	24 080	100	131 600	100
Boc-Glu-Glu-Leu-OMe	21 300	88	99 300	75
Ser-Glu-Glu-Ala-Arg	11 550	48	47 200	36
Boc-Glu-α-Bzl	2600	11	2120	1.6
Leu-Glu-Glu-Leu	2600	11	16 100	12
Glu-Glu	70	0.3	600	0.5

vitamin K-deficient rats, which was inhibited 80% by 5% (v/v) ethanol (Suttie *et al.*, 1979). The adult ox enzyme was not inhibited by 5% ethanol, and the calf enzyme was only slightly (15%) inhibited. The adult ox enzyme was 50% inhibited by 12% (v/v) ethanol and the calf enzyme by 9% (v/v) ethanol.

Substrate specificity

The substrate specificity of the adult ox carboxylase was studied with a number of synthetic peptides (Table 5). Phe-Leu-Glu-Glu-Leu was the best substrate, closely followed by Boc-Glu-Glu-Leu-OMe. Leu-Glu-Glu-Leu was a much poorer substrate and Glu-Glu was inactive. Interestingly, in contrast with all the other peptides studied, the activity on Boc-Glu-α-Bzl was not stimulated by Mn²⁺.

Solubilization: effect of Triton X-100 and octylglucoside

The ability of different Triton X-100 concentrations to solubilize the adult ox enzyme is shown in

Fig. 5. Maximal solubilization of the enzyme activity was seen at about 0.7% Triton X-100, and inhibition was observed above 1.5% Triton X-100. Another neutral detergent, octylglucoside, was apparently not able to solubilize the carboxylase in active form. However, octylglucoside could be used to obtain about a 4-fold increase in specific activity of the carboxylase from the dicoumarol-treated calf, as many other microsomal proteins could be extracted with it while the carboxylase remained bound. The calf carboxylase was much more tightly bound to the microsomal membrane than was the enzyme from the adult ox and a 3-fold higher concentration of octylglucoside was needed to remove the former activity from the membrane.

Discussion

Hemker and his co-workers have reported a 10000-fold purification of an enzyme from normal bovine liver microsomes which they have called

Fig. 5. Effect of detergents on activity of the vitamin K-dependent carboxylase

(a) Solubilization of vitamin K-dependent carboxylase from adult ox liver by different concentrations of Triton X-100. After addition of Triton and incubation for 30 min at 0°C, the suspension was centrifuged at 105 000 *g* for 1 h, and the supernatant was assayed in the absence (●) and presence (O) of 10 mM-MnCl₂. The amount of enzyme was 300 μ l in the 450 μ l volume; the final Triton X-100 concentrations in the assays are, therefore, obtained by multiplying those shown in the Figure by 0.67. (b) Effect of octylglucoside on the vitamin K-dependent carboxylase activity remaining in microsomal pellets. Microsomes were resuspended in 25 mM-imidazole/HCl, pH 7.2, containing 1 mM-dithiothreitol and the indicated amounts of octylglucoside. The suspensions were shaken for 30 min at 0°C and then centrifuged at 105 000 *g* for 60 min. The pellets were resuspended in the original volume of the microsomes to 25 mM-imidazole/HCl, pH 7.2, 10% (w/v) glycerol, 10 mM-MnCl₂ and 0.7% Triton X-100, and dialysed overnight against the latter resuspension buffer. The residual activity in the pellets after the dialysis is shown for the enzyme from normal bovine (●) and dicoumarol-treated calf (O). The activities in the microsomal pellets treated similarly without octylglucoside have been set to 100% for both enzymes. The octylglucoside supernatants were also treated in the same way and contained less than 2% of the activity in the microsomes.

either carboxylase or prothrombin synthase (Vermeer *et al.*, 1976, 1978, 1980; De Metz *et al.*, 1980). This enzyme, which converts decarboxy forms of prothrombin into prothrombin, requires O₂, HCO₃⁻, and vitamin K. Added vitamin K did not, however, stimulate the reaction, presumably because of saturation of the enzyme by vitamin K under normal conditions. This enzyme activity was reported to be optimal at pH 7.8–8.5 and at 37°C (Vermeer *et al.*, 1978) which markedly differs from the optimal conditions for the rat carboxylase (Suttie *et al.*, 1979).

In the present paper we have studied the properties of the vitamin K-dependent carboxylase from the adult ox with soluble peptide substrates rather than endogenous protein precursors. These properties are markedly different from those previously described (Vermeer *et al.*, 1976, 1978, 1980; De Metz *et al.*, 1980). The pH optimum was about 6.8, the temperature optimum 17°C, and the enzyme had an absolute dependency on the addition of KH₂. In

these respects the adult ox carboxylase closely resembles the carboxylase from vitamin K-deficient rat (Suttie *et al.*, 1979). We were unable to repeat the purification described by Vermeer *et al.* (1978) and De Metz *et al.* (1980), and it seems clear that the activity they studied is different from the vitamin K-dependent carboxylase from adult ox liver that we have reported here. A more recent report of De Metz *et al.* (1981) appears to describe a system that is much more similar to the activities studied here and to the rat liver enzyme.

The properties of the adult ox carboxylase can also be compared with those of the enzyme from the vitamin K-deficient rat. The temperature and pH optima were similar, but ionic strength optima differed considerably. The rat enzyme has the highest activity in imidazole buffer containing 0.5 M-KCl (Suttie *et al.*, 1979), whereas the adult ox enzyme was most active in 0.1 M-KCl and was almost totally inhibited (Fig. 1) by 0.5 M-KCl. In a previous study (Shah & Suttie, 1979) of the liver

Table 6. Comparison of the apparent vitamin K-dependent carboxylase activity in the livers of vitamin K-deficient rat and dicoumarol-treated calf under different conditions

The microsomes were resuspended and activity determined for both rat and calf preparations as described in the Materials and methods section, except that the microsomal resuspensions were not recentrifuged. Incubation time at 17°C was 30 min. All assay mixtures contained 50 mM-imidazole/HCl, pH 7.2, 1% Triton X-100 and 1 mM-dithiothreitol, and the additional components shown in the table. Abbreviation used: n.d., not determined.

Conditions	10 ⁻² × Activity (d.p.m./g of liver)	
	Rat	Calf
50 mM-Imidazole/HCl, pH 7.2	1195	1291
+0.3 M-KCl	n.d.	4698
+0.5 M-KCl	9846	2441
+10 mM-MnCl ₂	19 127	29 840
+0.5 M-KCl + 10 mM-MnCl ₂	13 781	3826
Activities at optimal conditions		
I. -Mn ²⁺	9846 (100%)	4698 (48%)
II. +Mn ²⁺	19 127 (100%)	29 840 (156%)

carboxylase activity of a number of different species, the optimal assay conditions for vitamin K-deficient rats (i.e. 0.5 M-KCl) were chosen. The amount of carboxylase assayed in calf was, therefore, lower than it actually is, and the same may be true for some of the other species studied. The enzyme from the adult ox had approximately the same apparent K_m values for the peptide substrate Phe-Leu-Glu-Glu-Leu as has previously been determined for the enzyme from vitamin K-deficient rat, and the relative activity of a number of different substrates appeared to be similar (Rich *et al.*, 1981). The apparent K_m for KH₂ of the adult ox enzyme was about three times as high as previously reported (Suttie *et al.*, 1979) for the rat enzyme. The small activation of the adult ox enzyme by pyridoxal phosphate is another difference between the carboxylases from these two sources. The activation by Mn²⁺ of the adult ox enzyme was much higher than that reported for rat carboxylase (Larson & Suttie, 1980). However, the rat enzyme was studied only at high KCl concentrations, which were found to inhibit Mn²⁺ activation.

Properties of the carboxylase from a dicoumarol-treated calf were also determined. The apparent K_m values for peptide substrates and the effects of inhibitors (-SH reagents and warfarin) were not significantly different from those found for the adult ox enzyme. However, the calf enzyme had a higher optimal KCl concentration than did the enzyme from adult animal and resembled more closely the rat enzyme in this respect. The pyridoxal phosphate activation of the dicoumarol-treated calf enzyme was also more comparable with that of the enzyme from vitamin K-deficient rat (Dubin *et al.*, 1979; Suttie *et al.*, 1980) than with that of the adult ox enzyme. The activation by Mn²⁺ of the calf enzyme was more pronounced than that of the adult ox enzyme. The octylglucoside extraction studies (Fig.

7) suggest that the dicoumarol-treated calf enzyme is more tightly bound to the microsomal membrane than is the adult ox enzyme. The carboxylase from vitamin K-deficient rats is more tightly bound to the microsomal membrane (L. Uotila & J. W. Suttie, unpublished work) than is the enzyme in normal rats, and it seems probable that the apparent tighter membrane association of the carboxylase from hypoprothrombinaemic animals is due to higher microsomal precursor protein concentrations of the enzyme during hypoprothrombinaemia (Suttie, 1973).

Activation of the carboxylase by Mn²⁺ and pyridoxal phosphate apparently have something in common, as indicated by the disappearance of the pyridoxal phosphate activation in the presence of Mn²⁺, and the enhanced response of calf as compared with adult ox enzyme by both activators. Partially overlapping binding sites on the enzyme for both the metal and pyridoxal phosphate is one probable explanation for these findings. Huang *et al.* (1980) have also noted some interaction between Mn²⁺ and pyridoxal phosphate. Activation by Mn²⁺ was not specific and required a high Mn²⁺ concentration, suggesting that the carboxylase is a metal-activated enzyme rather than a metallo-enzyme (Mildvan, 1970). If the enzyme also contains metal(s) as a structural component, these are probably bound tightly and unrelated to the metal activation *in vitro* studied here. The lack of stimulation by Mn²⁺ of carboxylase activity when Boc-Glu- α -Bzl ester was used as a substrate suggests that Mn²⁺ might be bound to the Glu-Glu sequence contained in the other substrates. However, the apparent K_m for Phe-Leu-Glu-Glu-Leu was not influenced by Mn²⁺, nor were the K_m values for KH₂ or HCO₃⁻.

The enzymes from a dicoumarol-treated calf and an adult ox were found to have different ionic

strength optima, suggesting that the increase in carboxylase activity seen during vitamin K deficiency or anticoagulant treatment (Shah & Suttie, 1978; Dubin *et al.*, 1980; Shah *et al.*, 1981) might be an artifact caused by a change in the optimal assay conditions for the enzyme. However, the data in Fig. 1(c) demonstrate that the increased activity in the dicoumarol-treated calf preparation is independent of KCl concentration; when the optimum ionic strength is used for both assays and other assay conditions kept the same, the activity in the calf preparation will be 6.5-fold higher than that of the adult ox preparation. The activity of the rat liver vitamin K-dependent carboxylase has not been found to be greatly influenced by the age of the animal. It is possible, however, that some of the differences in activity between the adult ox and dicoumarol-treated calf enzyme may have been related to age differences. Table 6 further illustrates the extent to which assay conditions influence the relative activity of the enzyme from two different species. In the earlier report (Shah & Suttie, 1979) where only one set of conditions was used, only the vitamin K-deficient hamster among several species tested had an apparent carboxylase activity higher than that of the vitamin K-deficient rat, and the dicoumarol-treated and normal calf had low carboxylase activities. According to the present results (Table 6), both rat and calf enzymes give the highest carboxylase activity in the presence of 10 mM-MnCl₂ and the absence of KCl. Under these conditions, the activity in the calf is higher than that in the rat.

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