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CHARACTERISTICS OF AN ACETONE POWDER PREPARATION OF THE VITAMIN K-DEPENDENT γ-GLUTAMYL CARBOXYLASE

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Summary

An acetone powder, prepared from the liver microsomes of vitamin K-deficient rats, retains an active vitamin K-dependent γ-glutamyl carboxylase. While the basic requirements of the enzyme are similar to those of the carboxylase of either resuspended microsomes or detergent-solubilized microsomes, the acetone powder preparation reveals some additional properties of the carboxylase. Carboxylation of the synthetic pentapeptide substrate phenylalanyl-leucyl-glutamyl-glutamyl-valine can occur in the absence of nonionic detergent; however, when vitamin K hydroquinone drives the acetone powder carboxylation nonionic detergent is required for maximal activity. Experiments are described in which the acetone powder is incubated with the pentapeptide, pelleted by centrifugation, resuspended with fresh reactants, and incubated again. They suggest that the low V for the carboxylase, observed by all investigators, is, at least in part, not the result of irreversible enzyme inactivation nor depletion of reactants, but rather accumulation of a yet to be identified inhibitor(s). The acetone powder prepared from microsomes derived from livers of nutritionally normal cows contains vitamin K-dependent γ-glutamyl carboxylase. This enzyme can be solubilized from the powder using Triton X-100 and could provide a large supply of starting material for enzyme purification.

Abbreviations: γ-C-H bond, γ-carbon-hydrogen bond of glutamic acid; peptide I, the pentapeptide phenylalanyl-leucyl-glutamyl-glutamyl-leucine; peptide II, phenylalanyl-leucyl-glutamyl-glutamyl-valine; vitamin K-1, phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone); vitamin MK-2, menaquinone-2(2-methyl-3-geranyl-1,4-naphthoquinone); vitamin MK-3, menaquinone-3-(2-methyl-3-farnesyl-1,4-naphthoquinone); vitamin KH-2, hydroquinone of vitamin K-1 (2-methyl-3-phytyl-1,4-naphthoquinol); vitamin K-3, menadione (2-methyl-1,4-naphthoquinone); vitamin MK-1, methyllapachol.

Introduction

Vitamin K-dependent γ-glutamyl carboxylase, an intrinsic membrane protein(s) located in the endoplasmic reticulum [1], requires for its activity the hydroquinone form of vitamin K [2,3], bicarbonate or CO_2 [4], molecular O_2 [2,3], and an appropriate substrate containing glutamic acid [2—5]. The carboxylase can be solubilized in nonionic detergents, but has not been purified extensively. The detailed mechanism of carboxylation is unknown, but experiments suggest that vitamin K is required to cleave the gamma carbon-hydrogen bond of glutamic acid prior to the actual carboxylation event [6]. It has been suggested that the vitamin K hydroquinone in an O_2-dependent reaction is converted to a hydroperoxide [7] which ultimately provides the energy required to cleave the γ-C-H bond.

Several enzyme preparations have been used to study this carboxylation reaction. Microsomes derived from livers of vitamin K-deficient rats carboxylate endogenous substrates, the most studied of which is a precursor for prothrombin. When microsomes are treated with Triton X-100, the solubilized carboxylase will still carboxylate associated endogenous substrates and also synthetic substrates such as the pentapeptide phenylalanyl-leucyl-glutamyl-glutamyl-leucine (Peptide I) [8—10]. We have found that the carboxylase remains active after intact microsomes have been extracted with a number of organic solvents. This treatment results in a particulate carboxylase which differs in certain respects from the aforementioned preparations. The characteristics of acetone powder preparations of the carboxylase are the subject of this study.

Materials and Methods

Preparation of the vitamin K-dependent carboxylase. Male, 200—250 g CD strain rats were fed a vitamin K-deficient diet for 7—10 days in coprophagy minimizing wire-bottomed cages. After an 18 h fast the animals were exsanguinated and a liver microsomal pellet was obtained as described previously [4]. The pellet was extracted twice with a 40-fold excess (w/v) of cold acetone at 0°C in a Potter-Elvejhem homogenizer rotating at 2000 rev./min. Extraction was continued until a homogeneous suspension was obtained (about 2 min). After each extraction, the suspension was centrifuged at 7500 × *g* for 10 min at 4°C; residual acetone was removed from the final pellet as it was dried in vacuo. The stability to storage of the carboxylase in the acetone powder was evaluated. Freshly prepared powder was assayed for carboxylase activity as described below and then stored dessicated at either room temperature 4, —20, or —80°C. Over 90% of the activity of the fresh powder is retained after storage for 1 week at 4°C; 60—70% is retained after storage at either room temperature or at —20°C, while less than 50% remains after storage at —80°. Thus, the powder was routinely stored dessicated at 4°C *.

* The acetone powder retains between 60 to 90% of the vitamin K epoxidase activity and between 30 to 40% of the vitamin K epoxide reductase activity of resuspended microsomes. Epoxidase and carboxylase activities are preserved in the presence of nonionic detergents such as 1% Triton X-100 (see text), but epoxide reductase activity is lost under these conditions. The vitamin K quinone reductase which is present in microsomes and which converts quinone to active hydroquinone also is preserved in the powder since the quinone can be used to drive carboxylation.

Conditions for assay. Prior to assay acetone powder (25—50 mg/ml) was resuspended in ice-cold 0.25 M sucrose, 0.025 M imidazole-HCl, pH 7.2, 0.002 M dithiothreitol with (buffer 2) or without 1% Triton X-100 (buffer 1) with 5—10 passes in a Potter-Elvejhem homogenizer rotating at 2000 rev./min. Incubations were in a volume of 0.125 ml and contained 2.5 or 5 mg of the resuspended powder; 2 mM NADH when present; 0.5 mM pentapeptide substrate when present; either 0.01 ml ethanol or vitamin K (in the form and concentration indicated) in ethanol; 1 μCi $NaH^{14}CO_3$ (specific activity 50 mCi/mmol) was added last. The test tubes were capped and incubated at 28°C for 30 min unless otherwise indicated. Reactions were terminated by addition of either cold trichloroacetic acid (final concentration 10%) or perchloric acid (final concentration 7%); after centrifugation acid insoluble material was treated as has been described [4,10] to measure carboxylation of endogenous substrates, while the acid soluble material (supernatant) was assayed for carboxylation of pentapeptide [6]. Radioactivity was counted in a Searle Mark III liquid scintillation counter which was 85% efficient at counting ^{14}C. Assay of vitamin K-dependent tritium release into water, from Peptide I tritiated at the β and γ carbons of the glutamyl residues, was as described previously [6].

Clotting assays. Prothrombin two-stage activity and generation of thrombin using *Echis carinatus* venom were assayed as previously described [11].

Chemicals. The pentapeptide phenylalanyl-leucyl-glutamyl-glutamyl-valine (Peptide II) was prepared by solid phase peptide synthesis [5,6]. The finished peptide was cleaved from the resin with hydrofluoric acid and purified as has been described [6]. The pentapeptide Peptide I was purchased from Vega Biochemicals, Tucson, AZ. Peptide I tritiated at the β and γ carbons of both glutamyl residues was prepared as previously described [6]. *E. carinatus* venom, NADH, Triton X-100, phylloquinone (vitamin K-1), menadione (vitamin K-3), pyridoxal 5′-phosphate, and imidazole were obtained from Sigma. The vitamins menaquinone-2 (MK-2) and menaquinone-3 (MK-3) and the hydroquinone form of K-1 (KH-2) were prepared as described by Mayer and Isler [12].

Results

The carboxylase activity of microsomes resuspended in buffer 2 was compared to that of the acetone powder resuspended to a protein concentration equivalent to the microsomes from which the powder was derived (Table IA). The powder was nearly as active as were the microsomes when either carboxylation of endogenous or synthetic substrates was measured. When alkaline hydrolysis was performed after incubation in the presence of vitamin K and the hydrolysates were subjected to amino acid analysis [13], the radioactive product was γ-carboxyglutamic acid, whether the enzyme source was acetone powder or resuspended microsomes. Using microsomes solubilized with Triton X-100. Suttie et al. [14] have shown that the presence of pentapeptide does not inhibit carboxylation of endogenous substrate. This result also is obtained when the acetone powder is used, and carboxylation of endogenous substrate is measured at a number of concentrations of pentapeptide (Table II).

Vitamin K-dependent carboxylation of pentapeptide could be demonstrated in an acetone powder prepared from microsomes derived from normal bovine

TABLE I

A COMPARISON OF CARBOXYLASE ACTIVITY IN THE ACETONE POWDER WITH THAT IN RESUSPENDED MICROSOMES

Incubations were for 30 min at 28°C in a volume of 0.125 ml. Conditions were as described in Materials and Methods. Either microsomes (2.5 mg protein) or acetone powder (2.5 mg protein) provided the enzyme source and were resuspended in buffer 2. The vitamin was KH-2 (80 μg/ml) and NADH (2 mM) and Peptide II (0.5 mM) were present. Termination of the incubation and assay of carboxylation of endogenous and added pentapeptide substrates were as described in Materials and Methods.

	cpm	
	Acid insoluble	Acid soluble
(A) Rat liver		
Microsomal acetone powder		
—K	436	368
+K	1520	2170
Resuspended microsomes		
—K	125	320
+K	1390	2900
(B) Bovine liver		
Microsomal acetone powder		
—K	—	272
+K	—	1110
Resuspended microsomes		
—K	—	1330
+K	—	1400

liver (Table IB). Carboxylation does occur with the resuspended microsomes, but, as they were derived from normal bovine liver, the acetone extractions were necessary to remove endogenous vitamin K and reveal the dependence of the carboxylase on the vitamin.

The endogenous substrate is carboxylated to nearly the same extent as it is in resuspended microsomes. A portion of the substrate is precursor for prothrombin [2] and this has been shown to incorporate $^{14}CO_2$ at the gamma carbon of appropriate glutamyl residues during incubation with vitamin K, while simultaneously being converted to functional prothrombin [2]. This precursor can be converted to functional prothrombin as shown in Table III. The con-

TABLE II

EFFECT OF PENTAPEPTIDE SUBSTRATE CONCENTRATION ON CARBOXYLATION OF ENDOGENOUS SUBSTRATES

Conditions were as described in Table I using buffer 2. The pentapeptide Peptide II concentration was varied as indicated. The vitamin was KH-2 at 80 μg/ml.

Substrate Peptide II (mM)	cpm			
	Acid soluble		Acid insoluble	
	—K	+K	—K	+K
0	119	53	93	590
0.5	51	1320	90	680
1	123	2910	98	674
2	203	3380	125	728

TABLE III

GENERATION OF TWO-STAGE ACTIVITY

Aliquots from several incubations of acetone powder (conditions as described in Table I, omitting radioactive bicarbonate), were measured for their thrombin activity after a further incubation with either *E. carinatus* venom or in a two-stage prothrombin assay system. The units of thrombin (National Institutes of Health thrombin units) generated in these aliquots (diluted 1 : 25 from the carboxylation reaction) are shown, as are the actual clotting times (shown in parenthesis).

Initial incubation condition	Thrombin (units/assay)	
	Venom assay	Two-stage assay
0 time, +K-1	0.18 (22 s)	0.03 (67 s)
−K	0.19 (21.6 s)	0.02 (75 s)
+K-1	0.20 (21 s)	0.09 (32 s)
+MK-3	0.20 (21 s)	0.13 (25 s)

version of precursor to physiologically active prothrombin (measured in a two-stage prothrombin assay) requires prior incubation of the powder with vitamin. The generation of thrombin using *E. carinatus* venom depends neither on prior incubation nor on the presence of vitamin K; this would be expected since the venom will cleave prothrombin precursor(s), as well as normal prothrombin, to generate thrombin [15,16]. With MK-3 as the vitamin, almost 70% of the prothrombin precursor detected by the venom is converted to functional prothrombin.

Time courses of carboxylation by the acetone powder at 28°C are shown in Fig. 1. With KH-2, carboxylation of endogenous substrate is completed after 5 min (with K-1 or MK-3, this carboxylation is completed after 10—15 min).

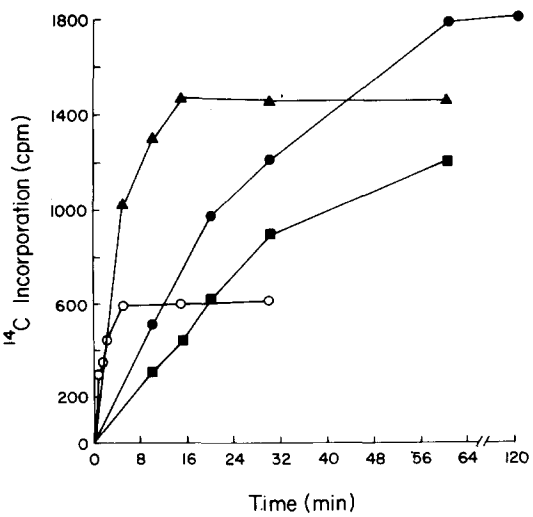

Fig. 1. Time courses. Incubations were for the times indicated. Vitamin concentrations were 20 μg/ml. ○———○, carboxylation of endogenous substrate, vitamin KH-2; ▲———▲, carboxylation of Peptide I, vitamin KH-2; ■———■, carboxylation of Peptide I, vitamin K-1; ●———●, carboxylation of Peptide I, vitamin MK-3. The acetone powder was resuspended in buffer 2 as described in Table I.

TABLE IV

ACTIVITY OF SEVERAL K VITAMINS

Reaction volume was 0.25 ml; incubation was for 10 min at 28°C. The acetone powder was resuspended in buffer 1. The vitamin source was varied as indicated. Conditions were otherwise as described in Table I.

Vitamin added (μg/ml)	cpm
0	326
0.1 MK-3	464
2 MK-3	2150
40 MK-3	2900
0.1 MK-2	318
2 MK-2	710
40 MK-2	1750
40 K-1	1120
40 K-3	331
40 MK-1	335
40 *cis* K-1	343

On the other hand, carboxylation of pentapeptide continues for about 15 min with KH-2 and for about 60 min using either K-1 or MK-3; in the last instance, after 30 min, 60—70% of total carboxylation has been achieved.

Stimulation of pentapeptide carboxylation by several K vitamins was studied (Table IV). Vitamins K-1, MK-2 and MK-3 are active, while K-3, MK-1 and the *cis* form of K-1 have little activity. These results agree with those we have reported previously [4], when carboxylation of endogenous substrate was assayed for microsomes in the presence of a variety of K vitamins.

Table IV also shows that with the acetone powder carboxylation of the pentapeptide can occur in the absence of Triton X-100 when the quinone form of the vitamin is used. Thus, while nonionic detergent is necessary to allow access of the pentapeptide to the carboxylase of intact microsomes, it is not required for carboxylation per se. On the other hand, as shown in Table V, nonionic detergent is required for maximal hydroquinone-driven carboxylation with the acetone powder as enzyme source. The nature of this requirement is unknown.

When the acetone powder is resuspended into buffer 1, about half its protein

TABLE V

EFFECT OF TRITON X-100 ON EXTENT OF CARBOXYLATION DRIVEN BY SEVERAL FORMS OF VITAMIN K

Vitamin used and concentrations were as indicated. When KH-2 was used, NADH was omitted. Conditions were otherwise as described in Table I.

Vitamin (μg/ml)	buffer 1 (—Triton X-100)	buffer 2 (+Triton X-100)
—	60	63
K-1 (4)	650	710
K-1 (20)	670	715
MK-3 (4)	1780	1770
MK-3 (20)	2020	1730
KH-2 (4)	180	1300
KH-2 (20)	220	1450

TABLE VI

MULTIPLE ASSAYS OF ACETONE POWDER ALIQUOTS

Reaction volume was 0.25 ml. Vitamin was MK-3 at 40 μg/ml. After a 30 min incubation, reactions were centrifuged (6000 × *g* for 20 min) and the resulting supernatants removed for assay. The pellets, containing the enzyme, were resuspended in fresh reactants and incubated again. This was repeated one time. (A) The pentapeptide was commercially prepared Peptide I. (B) The pentapeptide was Peptide I which was tritiated at the gamma carbons of both glutamyl residues. Tritium release was assayed.

	Incubation	1	2	3
(A) Carboxylation of Peptide I	—K	210	110	120
	+K	11 700	13 500	10 900
(B) Tritium release from ^{3}H-Peptide I	—K	380	360	350
	+K	3 230	4 480	3 600

is soluble, but virtually all the carboxylase is particulate and can be pelleted by centrifugation for 20 min at 6000 × *g*. This permits rapid separation of the carboxylase from the other reactants in incubations. Indeed, if after a 30 min incubation the carboxylase is pelleted and resuspended in fresh reactants and incubated again, the results obtained are shown in Table VIA. About the same amount of carboxylation of pentapeptide occurs during the second incubation. This can be repeated at least a third time. That this does not represent merely a continuing increase in specific activity of the $^{14}CO_2$ incorporated is shown by similar results, which are obtained if vitamin K-dependent tritium release from Peptide I containing β, γ-tritiated glutamyl residues is assayed for in multiple incubations (Table VIB). We have shown previously [6] that this tritium release reflects the vitamin K-dependent gamma carbon-hydrogen bond cleavage of glutamyl residues that precedes the addition of CO_2. Incubations carried out for 90 min give only about half the total carboxylation obtained if pelleted enzyme is provided every 30 min with fresh reactants. Addition of any or all of the reactants at 30 and 60 min to a 90-min incubation failed to stimulate the carboxylase. Preliminary experiments suggest that an unidentified inhibitor(s) accumulates during incubation.

Discussion

The acetone powder retains all four of the vitamin K-related enzymatic activities found in microsomes, a vitamin K reductase, the vitamin K epoxidase, the vitamin K epoxide reductase and the vitamin K-dependent γ-glutamyl carboxylase. When microsomes are exposed to 1% Triton X-100, only the epoxide reductase activity is lost; this is the case with the acetone powder as well. Endogenous substrate is preserved in the acetone powder, as well as in microsomes, and that portion that is precursor to prothrombin can be converted into functional prothrombin in a time and vitamin K-dependent incubation.

Apart from these similarities, the acetone powder has provided some new information about the vitamin K-dependent carboxylating system. While the hydroquinone form of the vitamin KH-2, drives carboxylation in microsomes resuspended in either buffer 1 or buffer 2, it is only minimally effective with the powder unless nonionic detergent is present in the incubation. If the acetone extractions effectively remove a required species (lipid?) from the micro-

some, the detergent then may be necessary to allow effective binding of the hydroquinone to a critical site of the carboxylase. Quinone forms of the vitamin are active, however, whether or not Triton X-100 is present. An awareness of the requirement for detergent in the KH-2-driven carboxylation may prove important in attempts to purify the carboxylase. Regardless of the nature of the starting material for purification, if the carboxylase is separated from all vitamin K reductase activity, the system becomes absolutely dependent on KH-2. Access of KH-2 to the enzyme may at some point become dependent on a certain concentration of detergent, and at the same time the detergent-to-protein ratio may be critical for the function of the carboxylase.

The particulate carboxylase in the acetone powder can be separated rapidly from soluble reactants in incubations by low speed centrifugation. This has allowed us to demonstrate that the low V for the carboxylase observed by all investigators is, at least in part, not the result of irreversible enzyme inactivation nor depletion of reactants, but rather accumulation of a yet to be identified inhibitor(s). By separating the powder from soluble molecules we have also been able to demonstrate the reversibility of inhibition of the carboxylase by the anticoagulant compounds tetrachloropyridinol and the imidazopyridines [17]. This characteristic of the powder should prove useful in future studies.

Further progress in understanding this very intriguing carboxylation is predicated on a successful purification of the carboxylase. This has proved a difficult task. Wallin et al. [18] report that about 80—90% of the rat liver microsomal protein can be removed from the carboxylase by differential extraction with detergents. A portion of the particulate enzyme, still a minor component of total protein, has been solubilized in Triton X-100 for further fractionation. An enzymatic activity termed prothrombin synthase, which converts the prothrombin precursor to prothrombin, has been extracted and partially purified from bovine liver microsomes [19,20]. Enzymatic incorporation of $^{14}CO_2$ into the precursor also occurs and both this reaction and the conversion of the precursor to prothrombin are vitamin K-dependent. The specific activity of this enzyme is quite low even when compared to the crude rat liver vitamin K-dependent γ-glutamyl carboxylase, and the two enzymes also appear to differ in other respects. For example, it is not yet evident that prothrombin synthase can carboxylate the synthetic substrates utilized by the rat liver enzyme. The demonstration that the acetone powder of bovine liver microsomes possesses a vitamin K-dependent γ-glutamyl carboxylase activity, which appears to be more like the rat liver carboxylase, is significant in that it offers the potential for large amounts of starting material for attempted purifications. This carboxylase can be solubilized from the acetone powder with a variety of nonionic detergents (Friedman, P.A. and Shia, M.A., 1979, unpublished observations). Whether this solubilized form of the carboxylase will be less resistant to purification than others and what its relationship is to prothrombin synthase remains to be determined.

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