VITAMIN K-DEPENDENT γ-CARBOXYGLUTAMIC ACID FORMATION BY KIDNEY MICROSOMES IN VITRO

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SUMMARY: γ-Carboxyglutamic acid has been identified as a constituent of renal tissue in chicken, rat, and rabbit and is depressed by vitamin K-deficiency or dicoumarol diets. Thorough perfusion of rat and rabbit kidneys to remove blood contamination does not remove the γ-carboxyglutamate containing protein(s), which appear to be localized in the cortex. Incubation of kidney microsomes with [^{14}C]$NaHCO_3$ *in vitro* results in the post-translational formation of protein bound [^{14}C]-γ-carboxyglutamic acid. Incorporation is stimulated 1.6- to 34-fold by addition of the active vitamin K 2-methyl, 3-farnesyl, 1,4-naphthoquinone. About 80% of incorporated, non-dialyzable ^{14}C is situated in the γ-carboxyl group of γ-carboxyglutamic acid.

INTRODUCTION: The central role of the kidney in calcium homeostasis is manifested through glomerular filtration and tubular reabsorption of Ca^{++} and phosphate and conversion of vitamin D metabolites to active forms which regulate dietary calcium absorption through intestinal Ca^{++}-binding proteins (1,2). Control of mineral ion equilibria between bone and body fluids is also achieved by Ca^{++}-dependent fluctuation of parathyroid hormone and calcitonin levels and renal regulation of blood pH (1-3). Pathological conditions such as renal osteodystrophy (3) attend the malfunction of any of these processes. Obviously there are mechanisms in kidney tissue for sensing Ca^{++} levels and thereby regulating appropriate metabolic activities. A most elegant system for protein-Ca^{++} interaction prevails in blood coagulation. Specific Ca^{++} binding sites in prothrombin (and perhaps in factors VII, IX, and X) are generated by post-translational vitamin K-dependent carboxylation of particular glutamic acid residues (4,5). The resulting new amino acid residues, γ-carboxyglutamic acid (Gla), serve as Ca^{++} binding sites and cause the proteolytic activation of prothrombin by factor X_a to be Ca^{++} and phospholipid-dependent (6). Gla has also been found in Factor X (7), in an acidic protein, osteocalcin, isolated from bone (8), and in a plasma protein of unknown function (9). This report establishes Gla as a constituent of one or more pro-

Abbreviations: Gla, γ-carboxyglutamic acid;
MK_3 2-methyl, 3-farnesyl, 1,4-naphthoquinone

teins synthesized in the kidney and the presence of a vitamin K-dependent protein carboxylation system in kidney.

MATERIALS AND METHODS: Day old Buff sex-linked male chicks were raised in heated brooders for 3 to 6 weeks on standard (Purina #5070) or vitamin K-deficient (Nutritional Biochemical Co.) diets supplemented with 800 mg/kg of dicoumarol (Sigma) or 10mg/kg vitamin K_1. New Zealand white rabbits were raised under standard conditions. Two stage prothrombin assays and maintenance of rats were as previously described (10). Perfusion of kidneys in anaesthetized rats and rabbits was achieved by pumping normal saline through the ligated abdominal aorta until the entire cortex was free of blood.

Microsomes were prepared from whole chick kidney, rat kidney cortex and liver by established methods (5,10). The microsomal pellet was suspended in 0.7 ml/gm original kidney wet weight. Incubation of microsomes with [^{14}C]-$NaHCO_3$ (50 mCi/m mole, New England Nuclear) was done as described by Friedman and Shia (10).

Synthetic Gla was a gift of Dr. R. Hiskey, University of North Carolina, Chapel Hill, and synthetic [^{14}C]-Gla was kindly provided by Dr. J. Suttie, University of Wisconsin, Madison. Gla was determined by amino acid analysis of alkaline and acid hydrolyzates as previously described (8), with the exception that pH 2.68 citrate was used as the starting buffer, thus adequately resolving Gla from all interfering compounds. A Gla color factor of 38.9% of that for glutamic acid was determined with synthetic Gla. Flow scintillation counting was achieved by mixing the effluent stream (8ml/hr) from the amino acid analyzer column with Aquasol (160 ml/hr). A Packard Tri-Carb scintillation counter equipped with a flow cell presented data both in graphical and digital format. The instrument background was 14 CPM with a counting efficiency of ∿90% for ^{14}C. Recovery of applied CPM was 95-100%.

RESULTS: Table 1 indicates that whole kidney from both rats and chicks contains low but significant levels of Gla. The kidney values are slightly higher than liver but much less than long bone. Kidneys from rats deprived of vitamin K and from chicks fed the vitamin K antagonist dicoumarol showed significant depression of Gla content. Addition of vitamin K to the K-deficient rat diet prevented this depression. Perfusion of kidney actually increases Gla levels in rabbit and possibly in rat (Table 2) by removing blood which has a very low Gla content (∿2-4 res Gla/10^5 amino acid residues). Furthermore,

Table 1. γ-CARBOXYGLUTAMIC ACID LEVELS IN VARIOUS TISSUES (res/10^5 amino acid res)

	Kidney	Liver	Long Bones	Prothrombin Titer
Rat (weanlings, 13d on diet)				
Control	11	7	62	100%
K-deficient	7	4	40	25
K-deficient (+ vit K)	10	8	68	102
Chick (3 weeks on diet)				
Control	12	8	65	100%
Dicoumarol	6	4	20	20

Table 2. DISTRIBUTION OF γ-CARBOXYGLUTAMIC ACID IN KIDNEY
(res/10^5 amino acid res)

Tissue	Treatment	Medulla	Cortex
Rabbit kidney	unwashed	<1.7	5.5
" "	saline washed	<1.7	8.7
" "	saline perfused	<1.7	9.9
Rat kidney	saline washed	3.1	9.1
" "	saline perfused	4.3	9.8
" "	saline perfused*	<2.0	7.0

*1 hr, pulsed perfusion on kidney from vitamin K-deficient rat

Table 3. VITAMIN K-DEPENDENT INCORPORATION OF [^{14}C]$NaHCO_3$ *IN VITRO*

	(CPM-background)		
	$-MK_3$	$+MK_3$	Ratio (+/-)
Chick - control diet			
Kidney - crude homogenate	185	208	1.12
microsomes	112	329	2.94
Liver - microsomes	148	201	1.36
Chick - dicoumarol diet			
Kidney - crude homogenate	204	256	1.25
microsomes	112	503	4.49
microsomes*	76	296	3.89
Liver - microsomes	123	966	7.85
Rat - K-deprived (7 days)			
Kidney microsomes	59	96	1.63

*From completely different group of chicks.

Reaction volumes were 240µl, of which 150µl was the microsomal suspension. The vitamin was MK_3 (20.8µg/ml). Incubations were otherwise identical to previous work, and were processed by TCA precipitation and centrifugation in scintillation vials as described (10).

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when separated into anatomically defined regions the perfused cortex is found to be the repository of the major portion of renal Gla.

The presence of Gla in kidney prompted investigation of its biosynthesis utilizing the *in vitro* microsomal assay system previously described for rat liver (5,10,11). As shown in Table 3, incorporation of radioactivity from [^{14}C]$NaHCO_3$ into TCA-precipitable material is stimulated in all cases by the presence of the active vitamin MK_3. While only small increases (12-25%) are observed in crude homogenates, microsomal preparations clearly exhibit a large

stimulation of ^{14}C incorporation by MK_3. The extent of MK_3 stimulation is increased by 1.5- to 2.4-fold in dicoumarol-fed chicks, compared to chicks fed control diet, in accordance with findings for rat liver (5) where vitamin K-deprivation increases the level of vitamin K-dependent carboxylation by accumulation of substrate for the reaction (non-carboxylated precursor proteins). Fig. 1 shows that 34 times as much [^{14}C]-Gla is synthesized in the presence of MK_3 as in its absence. Presumably the dialysis and alkaline hydrolysis procedures removed much of the normal ^{14}C background thus enhancing the MK_3 stimulation compared to Table 3. The fraction of total ^{14}C in the alkaline hydrolyzate present as Gla varied from 67%-86% in 7 separate experiments with a mean of 80%. Similar purity of the labeled reaction product from rat liver microsomes was previously reported (10).

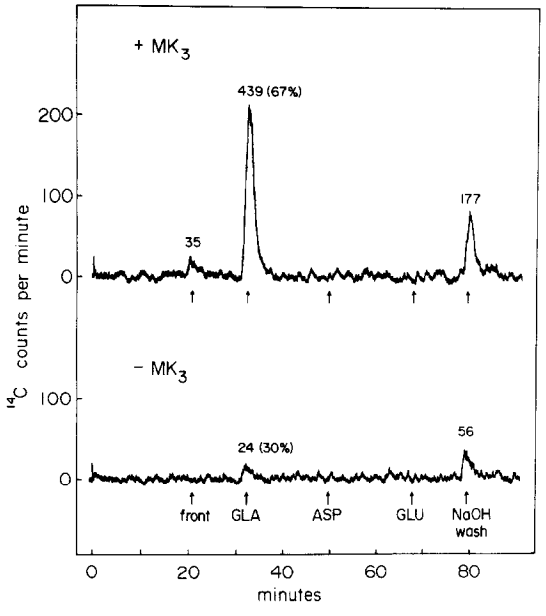

Figure 1: Elution profile of radioactive products from kidney microsomal alkaline hydrolyzates. Reaction volumes were 1.68 ml and included 1.05 ml kidney microsomes from dicoumarol-fed chicks (3 weeks), 16.8 µCi of [^{14}C]$NaHCO_3$, and other components as previously described (10). The vitamin, MK_3 (20.8µg/ml), was added only in the upper panel. After 30 min incubation at 37° Triton X-100 and acetic acid were added to final concentrations of 0.5% and 0.28M, respectively, the mixture was dialyzed exhaustively against 0.05M NH_4HCO_3 at 4°, lyophilized, and hydrolyzed in 2M KOH. Total CPM in the neutralized hydrolyzates were 16,160 (+MK_3) and 1060 (-MK_3). Fifty µl aliquots containing 680 CPM (+MK_3, 1/24 of total) or 90 CPM (-MK_3, 1/12 of total) were applied to the ion-exchange column. Total CPM in Gla in the entire incubation mixture: 10,800 (+MK_3, 67% of total) and 320 (-MK_3, 30% of total)--a 34-fold stimulation by the vitamin.

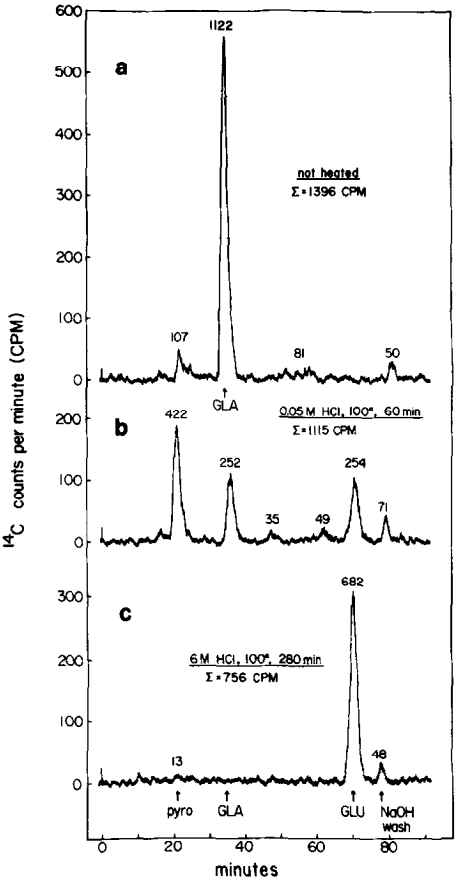

Figure 2: Elution profiles of putative [^{14}C]-Gla and the products of acid decarboxylation. Three large scale (6.68 ml) incubations of kidney microsomes in the complete system (+MK_3) were processed as described in Fig. 1. The pooled alkaline hydrolyzate was diluted and dispensed (400 µl) into 6 V-vials such that each contained 5500 CPM (4400 CPM in Gla). Equal volumes of H_2O (panel a, above), 0.1M HCl (b), or 12M HCl (c) were added to dupicate samples, the vials flushed with N_2, and heated. After evaporation to dryness 3 times under vacuum at 55° to remove $^{14}CO_2$, residues were dissolved in 200 µl 0.2M citrate buffer, pH 2.2, and 50 µl aliquots applied to the ion-exhange column. The CPM in each peak are indicated, as well as the total CPM summed over the entire chromatogram (recovery >95%). Duplicate runs showed less than 5% variation. Elution positions were established with synthetic radioactive standards. Conversion to [^{14}C]-glutamic acid with loss of equal CPM as $^{14}CO_2$ confirms the nature of the parent compound as [γ-carboxyl ^{14}C]-Gla. The major intermediate in the conversion (panel b) appears to be [^{14}C] pyro-γ-carboxy-glutamic acid (422 CPM). Although pyroglutamic acid also elutes at this position, insufficient total ^{14}C has been lost (281 CPM vs the required 676 CPM) to allow this peak to be [^{14}C]-pyroglutamic acid. Ninhydrin data on acid conversion of synthetic Gla to glutamic acid show identical kinetics to the above data and confirm cyclization as an intermediate step in decarboxylation.

Gla labeled in one of the two γ-carboxyl positions, the known product of the rat liver vitamin K-dependent carboxylase, decarboxylates randomly when heated in acid (5). Fifty percent of the radioactivity is lost as $^{14}CO_2$ and 50% retained in [^{14}C]-glutamic acid. These criteria are thorougly satisfied by the [^{14}C]-labeled reaction product of kidney microsomes (Fig. 2). The unheated material chromatographs in a position identical to synthetic [^{14}C]-Gla. Heating in 6M HCl at 100° for 280 min followed by flash evaporation results in the loss of 640 CPM (presumably as $^{14}CO_2$) and the appearance of 682 CPM at the glutamic acid position. The 682 CPM of glutamic acid represents 61% of the original 1122 CPM in [^{14}C]-Gla [or 52% if the small peaks 107 CPM and 81 CPM, which correspond to positions of label during intermediate stages of decarboxylation (e.g. Fig. 2b) are added to the parent 1122 CPM]. Heating in dilute acid (Fig. 2b) results in the loss of 281 CPM (as $^{14}CO_2$) and appearance of 254 CPM of [^{14}C]Glu.

DISCUSSION: The vitamin K-dependent carboxylase activity in kidney shares certain properties with the liver enzyme (5,10): 1) activity is present in microsomes; 2) addition of MK_3 stimulates incorporation of [^{14}C]$NaHCO_3$; 3) cycloheximide does not inhibit the reaction, confirming its post-translational nature; 4) about 80% of the fixed, non-dialyzable radioactivity is localized in the γ-carboxyl group of Gla; 5) activity is increased in microsomes of dicoumarol-fed animals compared to those from control animals suggesting an accumulation of non-carboxylated precursor protein (substrate).

Establishment of vitamin K-dependent protein carboxylation in kidney raises new possibilities for renal control of numerous biological processes involving calcium. 1) Might Gla-containing kidney protein(s) be involved in renal Ca^{++} transport? 2) Could the Gla-containing protein, by its interaction with Ca^{++}, modulate the rates of formation of 1,25-dihydroxyvitamin D_3 and 1,24,25-trihydroxyvitamin D_3 in kidney? 3) Is Gla-containing kidney protein translocated to other tissues such as bone or blood where it may perform other regulatory functions, or is it excreted in the urine? Gla-rich proteins are found in urine and kidney stones and may be etiologic factors in pathological calcification (11).

It should be noted that drugs which bear structural resemblance to the vitamin K-antagonist, dicoumarol, are known to have effects on renal function. Warfarin interferes with Ca^{++} transport in kidney cells (12); the bioflavonoid quercetin inhibits the Na^+ and K^+-dependent ATPase of sheep and dog kidney (13) and this inhibition is blocked by bicarbonate, a substrate for vitamin K-dependent carboxylation. Tetracyclines and their breakdown products, the spasmolytic drug methyl-3-chromone, and other bone seeking drugs cause proximal tubule lesions and an acute reversible Fanconi Syndrome (14), as well as

depressed growth rates of long bones and hypoplastic dentition (15). Dicoumarol and warfarin depress Gla levels of the Gla-rich osteocalcin in chicken bone (16) and affect human fetal bone development leading to nasal hypoplasia and stippled epiphyses (17).

Thus it seems possible that a variety of disorders involving kidney, bone and ectopic calcification might arise through perturbation of the vitamin K-dependent protein carboxylation system.

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