Vitamin K Dependent Carboxylation of Glutamate Residues to γ-Carboxyglutamate in Microsomes from Spleen and Testes: Comparison with Liver, Lung, and Kidney†

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ABSTRACT: Vitamin K dependent carboxylation of glutamate residues to γ-carboxyglutamate was demonstrated in proteins of spleen and testes microsomes. The rate of carboxylation in spleen microsomes was 0.9% and testes 3% of that in liver microsomes per milligram of microsomal protein. For comparison the rates of carboxylation in lung and kidney microsomes were 17 and 8%, respectively, of the rate in liver microsomes. The high rate in liver microsomes may be due to a high carboxylase level as indicated by the high rate of peptide carboxylation in liver microsomes. Protein carboxylation in liver microsomes was linear for only 15 min while carboxylation in microsomes from extrahepatic tissue persisted much longer so that the total protein carboxylation in lung microsomes was 60%, kidney 18%, testes 12%, and spleen 9% of that occurring in liver microsomes. Protein carboxylation was higher in microsomes from extrahepatic tissues of rats fed a vitamin K deficient diet as compared to animals fed a vitamin K sufficient diet. Protein carboxylation in microsomes from extrahepatic tissues was greatly stimulated by manganese ions and was dependent upon the addition of dithioerythritol. NADH could partially replace the dithiol in spleen, testes, and lung, but NADH-dependent carboxylation was relatively low in kidney and liver microsomes. Dithiol-dependent carboxylation was completely blocked by 10 μM warfarin, but NADH-dependent carboxylation was only slightly inhibited by 100 μM warfarin. Menaquinone-3 was much more active than vitamin K_1 in driving carboxylation. Solubilized microsomes catalyzed the carboxylation of glutamate residues to γ-carboxyglutamate in a pentapeptide Phe-Leu-Glu-Glu-Leu. The rate of carboxylation in lung microsomes was 22%, testes 3.3%, kidney 1.9%, and spleen 1.6% of the rate in liver microsomes.

Vitamin K may have a role in tissues other than the liver where the synthesis of the vitamin K dependent clotting proteins occur. Vitamin K dependent carboxylation of glutamate (Glu) residues to γ-carboxyglutamate (Gla) has been demonstrated in microsomes from liver (Esmon et al., 1975), bone (Lian & Friedman, 1978), kidney (Hauschka et al., 1976), placenta (Friedman et al., 1979), pancreas (Traverso et al., 1980), and lung (Bell, 1980). Gla formation also occurs in embryonic chorioallantoic membrane from chicks (Tuan, 1979). Gla has been found in cartilage (Lian et al., 1980), calcified atheromatous plaque (Lian et al., 1976; Deyl et al., 1979), tendons (Deyl et al., 1979), and spermatozoa (Gentsch & Martius, 1981), suggesting that vitamin K-dependent carboxylation also occurs in these tissues. Most of the tissues in which vitamin K dependent carboxylation has been found are tissues which accumulate significant amounts of vitamin K (Matschiner, 1970). Using this as a guide we have found that carboxylation and formation of Gla also occur in spleen and testes microsomes.

Materials and Methods

Male Sprague-Dawley rats (10–20 weeks old) from Charles River Laboratories were fed Purina rat chow except where indicated. Vitamin K deficiency was produced by feeding a vitamin K deficient diet prepared by Teklad Test Diet (Madison, WI) according to Matschiner & Taggart (1968) for at least 10 days. Sodium [^{14}C]carbonate (59 mCi/mmol) was obtained from Amersham/Searle (Chicago, IL) and γ-carboxyglutamate from Calbiochem, San Diego, CA. Menaquinone-3 was a generous gift from Dr. Paul Friedman, Center for Blood Research, Boston, MA.

Assay for Protein Carboxylation. A 25% homogenate (1 g of tissue plus 3 mL of SIKM buffer) of each tissue was prepared by homogenization with a Teflon–glass homogenizer driven by a motor. SIKM buffer contained 0.25 M sucrose, 0.005 M magnesium acetate, 0.1 M KCl, and 0.025 M imidazole hydrochloride, pH 7.2. The homogenate was centrifuged for 10 min at 15000*g*, and the supernatant was centrifuged at 100000*g* for 1 h. The microsomal pellet was rinsed with buffer and resuspended in buffer to a concentration of 0.2–0.5 g of original wet weight of tissue per mL. The incubation mixture contained 0.5 mL of microsomal suspension, 6.5 mM dithioerythritol (DTE), 0.1 M $MnCl_2$, and 2.5×10^7 dpm of $Na_2{}^{14}CO_3$. The reaction was initiated with 5 μg of menaquinone-3 in 0.01 mL of ethanol and incubated with shaking at 25 °C. Liver microsomes were routinely incubated for 15 min, spleen for 90 min, and the other tissue microsomes for 30 min except where otherwise indicated. ^{14}C-Labeled protein was determined by precipitation with trichloroacetic acid (Cl_3CCOOH) according to Esmon & Suttie (1976). The amount of microsomal protein obtained was liver 22.0, spleen 15.1, kidney 9.3, heart 5.4, testis 4.5, and lung 3.9 mg of microsomal protein per g of tissue (Lowry et al., 1951).

Assay for Peptide Carboxylation. Microsomes from all tissues were resuspended in SIKM buffer to an original tissue concentration of 0.4 g/mL except spleen which was resuspended to 0.2 g/mL. Triton X-100 was added to a concentration of 0.2% except for liver microsomes where the Triton concentration was 0.5%. The optimal amount of the pentapeptide Phe-Leu-Glu-Glu-Leu (Vega Corp., Tucson, AZ) was added: 1.5 mg (5 mM) for liver, 0.5 mg (1.7 mM) for lung, spleen, and testes, and 6 mg (20 mM) for kidney. The incubation mixture contained 0.5 mL of solubilized microsomes, 1 mg of NADH, 0.08 mg of DTE, 0.25 mg of pyridoxal phosphate, pentapeptide, and 2.5×10^7 dpm of $Na_2{}^{14}CO_3$. The reaction was initiated with 5 μg of MK-3 and incubated with shaking at 25 °C for 30 min. There was no detectable car-

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Table I: Vitamin K Dependent Protein Carboxylation in Microsomes from Vitamin K Deficient Rats[a]

microsomes	cpm in Cl_3CCOOH precipitate from incubation: no vitamin	cpm in Cl_3CCOOH precipitate from incubation: vitamin K added	cpm of ^{14}C-labeled protein produced min^{-1} (g of tissue)$^{-1}$	% of liver activity per: g of tissue	% of liver activity per: mg of microsomal protein
spleen	111	820	80 ± 20	0.6	0.9
testes	62	778	87 ± 24	0.7	3.2
heart	37	126	13 ± 2	0.1	0.4
lung	56	3000	397 ± 100	3.0	17
kidney	141	3820	440 ± 196	3.3	7.9
liver	231	46700	13200 ± 2100	100	100

[a] Incubations were carried out as described under Materials and Methods. The results are the averages for three to six preparations of microsomes ± SEM.

boxylation with heart microsomes when 0.5 mg of peptide was added. After incubation the ^{14}C-labeled peptide produced was measured by determining ^{14}C in the Cl_3CCOOH supernatant according to Suttie et al. (1976).

Results

Vitamin K Dependent Protein Carboxylation in Microsomes of Extrahepatic Tissues. Microsomes were prepared from vitamin K deficient rats because of the possibility that protein substrates would accumulate which could be carboxylated in vitro upon the addition of vitamin K. When microsomes were suspended in buffer containing DTE and $Na_2{}^{14}CO_3$, MK-3 stimulated the incorporation of ^{14}C into the trichloroacetic acid-insoluble fraction (Table I). Lung, kidney, and liver were studied for comparison, as vitamin K dependent carboxylation was known to occur in these tissues. In spleen and testes, MK-3 stimulated an 8–12-fold increase in the amount of ^{14}C in the Cl_3CCOOH precipitates while in heart there was a 3-fold increase.

Protein carboxylation was approximately linear for at least 30 min in microsomes of extrahepatic tissue, but in liver microsomes carboxylation was linear for only 15 min (Figure 1). There was little carboxylation after 30 min in kidney microsomes but carboxylation continued after 30 min in microsomes from the other extrahepatic tissues. Carboxylation in spleen microsomes was linear for 3 h. The time course of carboxylation in heart microsomes was not studied because of low activity.

The rates of protein carboxylation in spleen and testes were about 20% of the rates in lung and kidney microsomes per gram of tissue (Table I). All the tissues had less than 4% of the activity of liver microsomes per gram of tissue. Lung microsomes had 17% and kidney 8% of the activity of liver per milligram of microsomal protein. However, the total amount of protein carboxylation in lung, kidney, testes, and spleen microsomes was 60, 18, 12, and 9%, respectively, of that occurring in liver microsomes (Figure 1).

Identification of [^{14}C]Gla in ^{14}C-Labeled Protein from Spleen and Testes Microsomes. To determine if [^{14}C]Gla was formed in spleen microsomes, we hydrolyzed an extract of incubated microsomes with 2 N KOH and separated the amino acids by ion-exchange chromatography (Figure 2). The ^{14}C eluted in the same chromatographic zone as authentic Gla. When the peak fractions were combined and heated with 6 N HCl, 60% of the ^{14}C was lost. The theoretical loss of ^{14}C from [^{14}C]Gla labeled in one of the carboxyl groups would be 50%. The ^{14}C-labeled protein produced by testes microsomes was analyzed with similar results. When the putative [^{14}C]Gla was heated with HCl, 48% of the ^{14}C was lost. We concluded that vitamin K induced the formation of [^{14}C]Gla residues in proteins of spleen and testes microsomes.

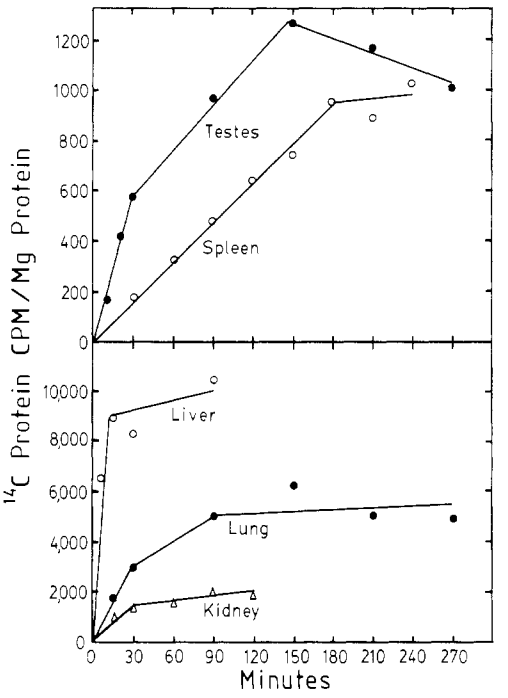

FIGURE 1: Vitamin K dependent protein carboxylation in microsomes from liver and extrahepatic tissues. Microsomes from vitamin K deficient rats were incubated as described under Materials and Methods for the indicated times. The results are the averages for two or more preparations of microsomes.

Table II: Stimulation of Protein Carboxylation by Mn^{2+} [a]

	% control activity at $MnCl_2$ concn of: 0.02 M	0.1 M	0.5 M
liver	112	227	230
lung	238	240	
spleen	171	178	
kidney	285	300	
testes	398	580	
heart		117	

[a] Microsomes from vitamin K deficient rats were incubated as described under Materials and Methods except that the indicated concentrations of $MnCl_2$ were added to SIKM buffer. The control was the amount of carboxylation obtained without the addition of $MnCl_2$.

Vitamin K Dependent Protein Carboxylation in Microsomes from Normal Rats. The rate of carboxylation in microsomes from rats fed a vitamin K sufficient diet was significantly lower than in microsomes from K-deficient rats (Figure 3). The decrease ranged from 43% in testes to 77% in lungs.

Stimulation of Protein Carboxylation by Mn^{2+}. Since Mn^{2+} stimulated protein carboxylation in liver microsomes (Larson & Suttie, 1980), we tested the effects of $MnCl_2$ on carbox-

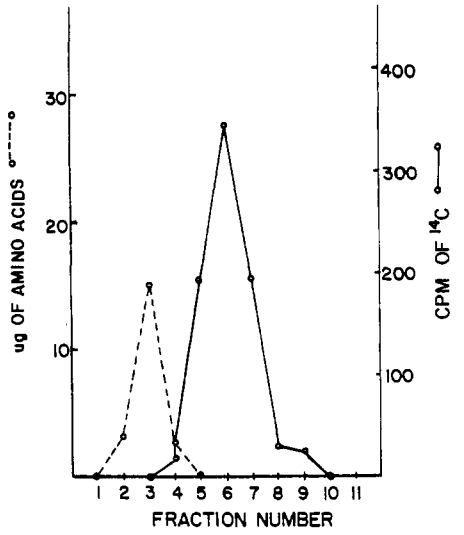

FIGURE 2: Ion-exchange chromatography of alkaline hydrolysate of extract from spleen microsomes. Spleen microsomes from a vitamin K deficient rat were suspended in 6 mL of SIKM buffer containing 1 mg/mL DTE, 2 μg/mL MK-3, and 10^8 dpm/mL $Na_2{}^{14}CO_3$. After incubation for 90 min at 25 °C the mixture was centrifuged at 105000*g* for 60 min. The microsomal pellet was resuspended in 2 mL of buffer containing 0.45% Triton and centrifuged at 105000*g* for 1 h. The supernatant was dialyzed against three changes of 1-L portions of 0.05 M NH_4HCO_3 over 3 days. The dialyzed solution which contained 3400 dpm of ^{14}C and 1.4 mg of protein was hydrolyzed in 2 N KOH at 110 °C for 16 h. The hydrolysate was neutralized with 70% perchloric acid, and the precipitate was removed by centrifugation. The supernatant was chromatographed on Dowex 1-X8 according to Gundberg et al. (1979). The elution pattern of buffer C is shown. Six-milliliter fractions were collected and assayed for ^{14}C by liquid scintillation spectrometry and for amino acids with ninhydrin reagent using casamino acids as the standard (Moore, 1968). Fractions 5–7 were combined, lyophilized, and dissolved in 1 mL of water. One milliliter of 12 N HCl was added and the sample heated at 110 °C for 16 h, resulting in the loss of 60% of the ^{14}C.

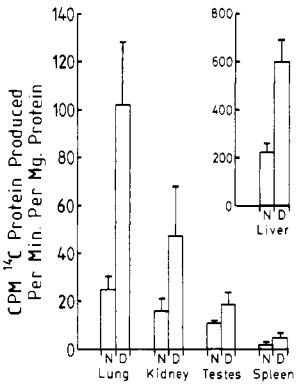

FIGURE 3: Comparison of vitamin K dependent protein carboxylation in microsomes from normal and vitamin K deficient animals. Incubations were carried out as described under Materials and Methods. N represents microsomes from rats fed Purina rat chow diet, and D represents microsomes from rats fed a vitamin K deficient diet for 10–20 days. The results are the averages for three to six preparations of microsomes ± SEM.

ylation in microsomes from extrahepatic tissues (Table II). Mn^{2+} stimulated carboxylation in all tissues except heart. The activation was due to Mn^{2+} since increasing the concentration of chloride ions with KCl had little effect on carboxylation. Mn^{2+} at 0.1 M increased protein carboxylation by about 2-fold in lung, spleen, and liver, 3-fold in kidney, and 6-fold in testes.

Vitamin K Requirement for Protein Carboxylation. The optimal concentration of MK-3 for carboxylation was between 0.1 and 1.0 μg/mL for spleen and lung microsomes and between 1 and 10 μg/mL for kidney, testes, and liver microsomes

Table III: MK-3 Hydroquinone and Protein Carboxylation[a]

vitamin	reducing agent	cpm in ^{14}C-labeled protein/g of tissue				
		spleen	kidney	testes	lung	liver
MK-3	none	210	1 300	90	0	37 600
MK-3	DTE	2070	16 000	3450	11 000	204 000
MK-3 hydroquinone	none	290	4 150	2180	3 790	71 400
MK-3 hydroquinone	DTE	2330	17 300	3290	12 400	198 000

[a] Microsomes were prepared from vitamin K deficient rats, and incubations were carried out as described under Materials and Methods except that the concentrations of MK-3 and MK-3 hydroquinone were 5 μg/mL. The latter was prepared just before use by the method of Sadowski et al. (1976).

Table IV: Warfarin Inhibition of Protein Carboxylation[a]

microsomes	% inhibition by warfarin	
	DTE-dependent 10 μM warfarin	NADH-dependent 100 μM warfarin
spleen	96	17
testes	76	6
lung	78	0
kidney	95	14
liver	87	0

[a] Microsomes were prepared from vitamin K deficient rats, and incubations were carried out as described under Materials and Methods except that either 1 mM DTE or 2 mM NADH was provided as the reducing agent. Warfarin was added as indicated. The results are the average of duplicate incubations.

(Figure 4). Vitamin K_1 could also drive carboxylation, but its activity was substantially less than MK-3 in all tissues.

Requirements for Reducing Agents for Protein Carboxylation. If DTE[1] was left out of the incubation mixture, carboxylation was reduced by 90% or more in extrahepatic microsomes and by 82% in liver microsomes (Table III). If MK-3 hydroquinone was substituted for MK-3, substantial carboxylation occurred in testes, lung, kidney, and liver without DTE. However, the hydroquinone was inactive in spleen microsomes. MK-3 hydroquinone had about the same activity as MK-3 plus DTE in all tissues.

NADH could be substituted for the dithiol and drive carboxylation in lung, spleen, and testes (Figure 5). The activity of the pyridine nucleotide was low relative to DTE in liver and kidney microsomes. Optimal activity was obtained with DTE in all tissues and was not increased by the addition of NADH.

Inhibition of Protein Carboxylation by Warfarin. Warfarin inhibited vitamin K dependent protein carboxylation by 76–96% when the dithiol was the reducing agent (Table IV). However, there was little or no inhibition by warfarin at a concentration 10 times higher (100 μM) when NADH was the reducing agent.

Vitamin K Dependent Peptide Carboxylation. MK-3 induced carboxylation of the pentapeptide Phe-Leu-Glu-Glu-Leu in microsomes from kidney, spleen, testes, and lung but not in heart. Peptide carboxylation was linear for at least 30 min in extrahepatic microsomes (Figure 6) as it is in liver microsomes.

We previously demonstrated that Glu residues in the pentapeptide were carboxylated to Gla by lung microsomes (Bell, 1980). To determine if [^{14}C]Gla was produced after incu-

[1] Dithiothreitol, the isomer of dithioerythritol (DTE), could replace DTE in incubation mixtures with no significant differences in activity.

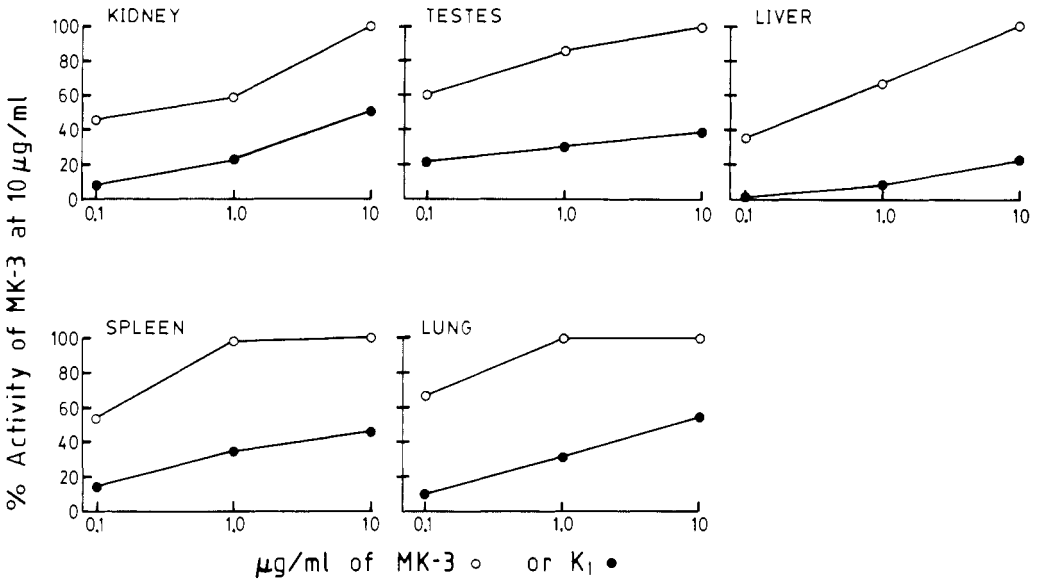

FIGURE 4: Vitamin K requirement for protein carboxylation. Incubations were carried out as described under Materials and Methods except that the indicated concentrations of MK-3 (○) or K_1 (●) were used. Each point is the average for two or more preparations of microsomes.

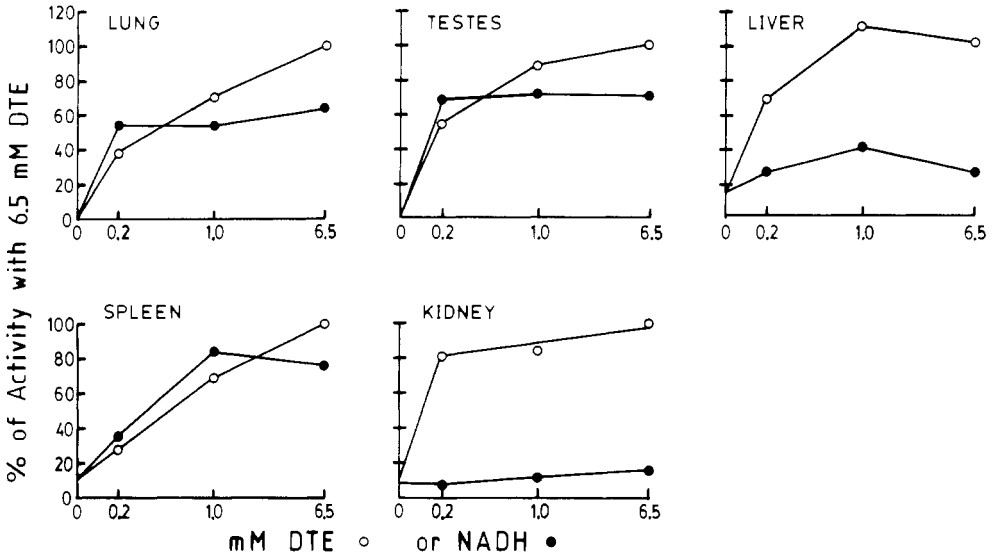

FIGURE 5: Requirement of reducing agents for protein carboxylation. Incubations were carried out as described under Materials and Methods except that the indicated concentrations of DTE (○) or NADH (●) were used. Each point is the average for two or more preparations of microsomes.

bation with kidney, testes, and spleen microsomes, we examined the Cl_3CCOOH supernatants, which would contain the ^{14}C-labeled peptide. The Cl_3CCOOH supernatants from the incubations with kidney and testes microsomes were hydrolyzed with KOH and the alkaline hydrolysates chromatographed on an ion-exchange column according to Gundberg et al. (1979). In each case there was a peak of ^{14}C in the chromatographic zone where authentic [^{14}C]Gla is eluted. When aliquots of the combined peak fractions were heated with 6 N HCl at 110 °C for 18 h, approximately half of the ^{14}C was lost, indicating that [^{14}C]Gla was present.

The alkaline hydrolysate of the Cl_3CCOOH-soluble ^{14}C from spleen was analyzed by thin-layer chromatography according to Suttie et al. (1976). About 70% of the chromatographed ^{14}C migrated with authentic Gla. Another sample of the alkaline hydrolysate was heated with 6 N HCl at 110 °C for 18 h resulting in the loss of 56% of the ^{14}C. When an aliquot of the solution treated with HCl was analyzed by thin-layer chromatography, all of the radioactivity migrated with authentic glutamic acid. We concluded that [^{14}C]Gla was formed in the carboxylation of the peptide.

The optimal concentration of peptide was determined for microsomes from each tissue (see Materials and Methods). When the rate of carboxylation was compared by using these concentrations, lung microsomes had 22% of the activity of liver microsomes, but the other tissues had less than 4% of the liver activity per milligram of microsomal protein (Table V). The lower rate in microsomes from extrahepatic tissues is not surprising since the amino acid sequence in the peptide corresponds to a sequence in the precursor of prothrombin, a protein synthesized in liver.

Discussion

When physiological amounts of ^{14}C-labeled vitamin K_1 were fed to rats for 4 days, liver accumulated the highest amount of radioactivity per weight of tissue (Matschiner, 1970). However, spleen had only slightly less and testes and heart had about a third as high a level of ^{3}H-labeled K_1 and metabolites as liver. For this reason we examined these tissues and found that indeed vitamin K dependent carboxylation of Glu residues to Gla occurs in microsomes from spleen and testes. There also appeared to be vitamin K dependent car-

Table V: Comparative Peptide Carboxylation[a]

microsomes	cpm of ^{14}C-labeled peptide produced min^{-1} (g of tissue)$^{-1}$	cpm of ^{14}C-labeled peptide produced min^{-1} (mg of microsomal protein)$^{-1}$	% liver activity per g of tissue	% liver activity per mg of microsomal protein
spleen	943	63.0	1.1	1.6
testes	567	126	0.7	3.3
lung	3230	827	3.9	22
kidney	690	74.0	0.8	1.9
liver	83300	3800	100	100

[a] Microsomes from vitamin K deficient rats were incubated as described under Materials and Methods. Each value is the average for three preparations of microsomes.

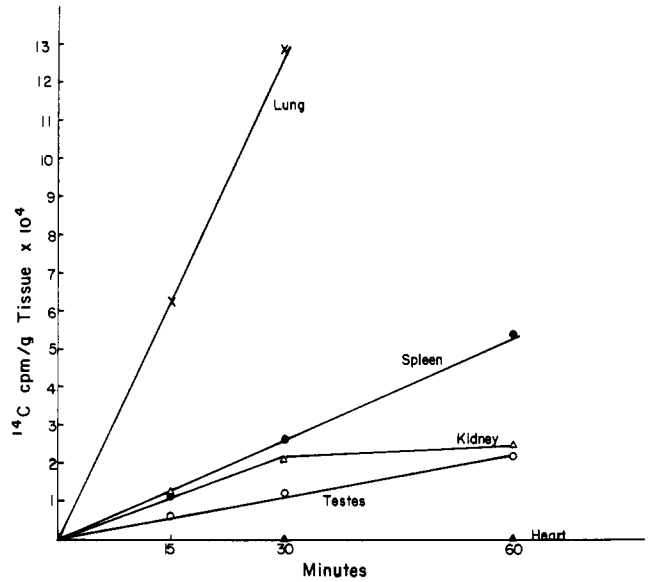

FIGURE 6: Vitamin K dependent peptide carboxylation in microsomes from extrahepatic tissues. Peptide carboxylation was carried out as described under Materials and Methods. Each point is the average of two incubations.

boxylation in heart microsomes, but the activity was so low that we did not attempt to identify [^{14}C]Gla as a product of the carboxylation.

Protein carboxylation was greatly stimulated by manganese ions in microsomes from liver and extrahepatic tissue except for heart. Carboxylation was dependent on the addition of a reducing agent such as DTE. NADH can replace the dithiol in lung, spleen, and testes, but in kidney and liver the NADH-dependent carboxylation was relatively low. The highest activity was obtained with DTE in all microsomes, suggesting that the most important physiological reducing agents are thiols. When vitamin K was replaced by vitamin K hydroquinone, significant carboxylation was observed without the addition of a reducing agent in all tissues except spleen. However, MK-3 hydroquinone had less activity than MK-3 plus DTE in microsomes from all tissues, suggesting that the dithiol is required for more than simply the formation of the hydroquinone.

Vitamin K deficiency increases protein carboxylation in liver because of the accumulation of a precursor in microsomes which can be carboxylated in vitro upon addition of vitamin K (Suttie, 1972). Vitamin K deficiency also caused an increase in carboxylation in spleen, testes, heart, lung, and kidney microsomes, suggesting that these tissues accumulate a substrate for carboxylation. The rate of protein carboxylation was much faster in liver microsomes than in extrahepatic microsomes. Peptide carboxylation is a measure of carboxylase activity since it does not depend on the amount of endogenous substrate present. The rapid rate of protein carboxylation in liver microsomes was probably due to its high carboxylase activity (Table V). However, *total* protein carboxylation in extrahepatic microsomes ranged from 9% in spleen to 60% in lung of the carboxylation occurring in liver microsomes.

The function of Gla in clotting proteins is to bind calcium and phospholipids, which is required for their conversion to biologically active clotting factors. Osteocalcin, the Gla–protein in bone, may be involved in calcium binding for calcification of bone, or it may be a regulatory molecule for calcification (Hauschka et al., 1978; Price & Williamson, 1981). The function of Gla–protein in lung, spleen, kidney, or testes is not clear. With regard to testes, it is interesting that a Gla–protein has recently been found in cock spermatazoa (Gentsch & Martius, 1981). It is likely that Gla–proteins play a role in these tissues since both the vitamin K dependent carboxylase and protein substrate(s) for carboxylation are found in microsomes from these tissues. We are investigating the nature of the Gla–protein formed in the in vitro incubations. Deyl et al. have reported the presence of peptides very similar to or identical with osteocalcin in atheromatous aorta from rats, turkey tendon (Deyl et al., 1979), and rat kidney cortex (Deyl et al., 1980). Vermeer et al. have recently detected vitamin K dependent carboxylation in 16 different bovine tissues, indicating the widespread occurrence of this reaction (Vermeer et al., 1982).

Acknowledgments

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Registry No. NADH, 58-68-4; H-Phe-Leu-Glu-Glu-Leu-OH, 69729-06-2; γ-carboxyglutamic acid, 53445-96-8; menaquinone-3, 860-25-3; vitamin K_1, 11104-38-4; manganese, 7439-96-5; dithioerythritol, 6892-68-8; menaquinone-3 hydroquinone, 84193-97-5; vitamin K, 12001-79-5; glutamic acid, 56-86-0.

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Retention of Allosteric Properties in an Inactive, Proteolyzed Form of Phosphofructokinase†

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ABSTRACT: Treatment of rabbit skeletal muscle phosphofructokinase by subtilisin resulted in inactivation as a consequence of the cleavage of an approximately 10000 dalton equiv from each protomer of the enzyme. Although the initial cleavage rapidly removed a 12 amino acid peptide from the carboxyl terminus without loss of activity [Kemp, R. G., Foe, L. G., Latshaw, S. P., Poorman, R. A., & Heinrikson, R. L. (1981) *J. Biol. Chem. 256*, 7282–7286], most or all of the residual cleavage was from the amino terminus. The cleaved products did not remain associated with the remaining protein. The inactive, 74000-dalton protein remained tetrameric and displayed much of the association–dissociation behavior of the native enzyme. The inactive protein associated to higher polymers in the presence of fructose 1,6-bisphosphate and dissociated in the presence of high concentrations of citrate, but not with low concentrations of citrate plus MgATP as seen with native enzyme. The inactive, digested protein also underwent ligand-induced conformational changes as indicated by changes in the reactivity of thiol groups with 5,5′-dithiobis(2-nitrobenzoic acid). The subtilisin-digested protein retained all of the reactive thiol groups and, as observed with native enzyme, AMP blocked the reactivity of two highly reactive thiol groups. On the other hand, fructose 6-phosphate was not capable of blocking these thiol groups as observed with native phosphofructokinase. MgATP did not block the reactivity of the thiol groups of phosphofructokinase as observed with native enzyme. These data suggested that the inactive protein retained binding sites for AMP, fructose-1,6-P_2, and citrate but had lost binding sites for the two substrates and for MgATP at the inhibitory site. These conclusions were confirmed by direct binding studies which showed only one binding site for adenine nucleotides in contrast to the three observed with native enzyme. Cyclic AMP was bound by the proteolyzed product with the same affinity as observed with the native enzyme. Fructose-2,6-P_2 enhanced the affinity of both proteins for cyclic AMP. Equilibrium binding studies confirmed the integrity of the fructose-1,6-P_2 binding site and the virtual absence of fructose-6-P binding. The data suggest a relatively discreet domain essential to the catalytic activity but structurally distinct from several allosteric regulatory sites.

Rabbit skeletal muscle phosphofructokinase has a complex regulatory behavior that is the consequence of its interaction with metabolites at specific binding sites [see Uyeda (1979) for review]. Found on the 84000-dalton protomer are a catalytic site for ATP and fructose-6-P,[1] a citrate site that is also capable of binding phosphoenolpyruvate and phosphoglycerates, a sugar bisphosphate site, an adenine nucleotide activating site, an ATP inhibitory site, and an undetermined number of sites for the interaction of inorganic phosphate and several cations. Of further consideration in the overall topography of the enzyme, one must include regions of interaction among protomers for the formation of the active tetramer and for the display of allosteric regulatory behavior, regions that interact in the formation of higher polymers, and the site of phosphorylation (Kemp et al., 1981). In an earlier study of limited proteolysis of phosphofructokinase (Riquelme & Kemp, 1980), we have shown that the enzyme is inactivated upon subtilisin cleavage of an approximately 10000 dalton

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NaDodSO_4, sodium dodecyl sulfate; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; fructose-6-P, fructose 6-phosphate; fructose-2,6-P_2, fructose 2,6-bisphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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