VITAMIN K-DEPENDENT CARBOXYLASE: EFFECT OF Mn^{2+} AND OTHER DIVALENT CATIONS

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1. Introduction

Vitamin K catalyses the post-translational carboxylation of specific glutamyl residues in microsomal precursor proteins to form γ-carboxyglutamyl residues in prothrombin and other vitamin K-dependent proteins. The carboxylation of these precursors, and of low molecular weight substrates of this carboxylase can be demonstrated in detergent-solubilized microsomal preparations, and reviews of the current understanding of this field are available [1–3]. The molecular role of vitamin K in this unique O_2-dependent carboxylation has not been established, and this report presents evidence to support our observation [4] that the carboxylase activity can be stimulated by divalent cations, and addresses the possible physiological significance of this stimulation.

2. Materials and methods

Liver microsomal pellets from rats fed a vitamin K-deficient diet for 7–10 days were prepared as in [5], solubilized in 0.025 M imidazole, 0.25 M sucrose, 0.5 M KCl (pH 7.2) (SIK buffer) with 1.0% Triton X-100 (Research Prod., Elk Grove Village, IL) and centrifuged at 105 000 × *g* for 60 min to remove insoluble material. 'Low detergent' microsomes were prepared the same way except that 0.2% Triton was used and the solubilized microsomes were not recentrifuged. 'EDTA extracted' microsomes were solubilized in 0.25 M sucrose, 0.025 M imidazole, 1.0% Triton, and 5 mM EDTA at pH 6.5, centrifuged as before, dialyzed against the same buffer for 1 h at 4°C, and then against sucrose–imidazole–Triton at pH 7.2 (without EDTA) for 1 h. Incubations were at 17°C for 30 min and consisted of 0.4 ml microsomal preparation and 0.1 ml SIK buffer containing 2.5 mM substrate Phe–Leu–Glu–Glu–Leu (Vega Biochem., Tucson, AZ). Vitamin K hydroquinone, 50 μg/ml for carboxylase assays and 20 μg/ml for epoxidase assays was added in 5 μl ethanol. All metals were dissolved in water immediately prior to addition to the incubations. Assays for carboxylation of peptide and endogenous protein substrates and for vitamin K epoxide formation have been described [6–8]. Manganese deficiency was induced by feeding 21-day-old (weanling) rats a manganese-deficient diet for 6–7 weeks, followed by this diet without menadione for 1 week. The diet consisted of 18% vitamin-free casein, 72.9% glucose, 0.25% vitamin mix, 4.8% salt mix, 0.05% choline–Cl, and 4% cod liver oil. The vitamin mix and salt mix were made as in [9] except that manganese was omitted from the salt mix. Manganese deficiency was assured by assaying liver arginase by the method in [10].

3. Results and discussion

Addition of $MnCl_2$ to the standard Triton-solubilized microsomal preparations markedly stimulated peptide carboxylase activity (fig.1A). At 10 mM, the only other divalent metal chlorides to increase carboxylase activity were $MgCl_2$ (24%), $CaCl_2$ (29%), and $BaCl_2$ (12%). The percent stimulations are relative to the ~100% stimulation seen with manganese. Other metals tested include $FeCl_3$, $FeSO_4$, $SnCl_2$, $CoCl_2$, $ZnCl_2$, $CdCl_2$ and Na_2SeO_3. Preparation of 'EDTA-extracted' microsomes or solubilization of microsomes

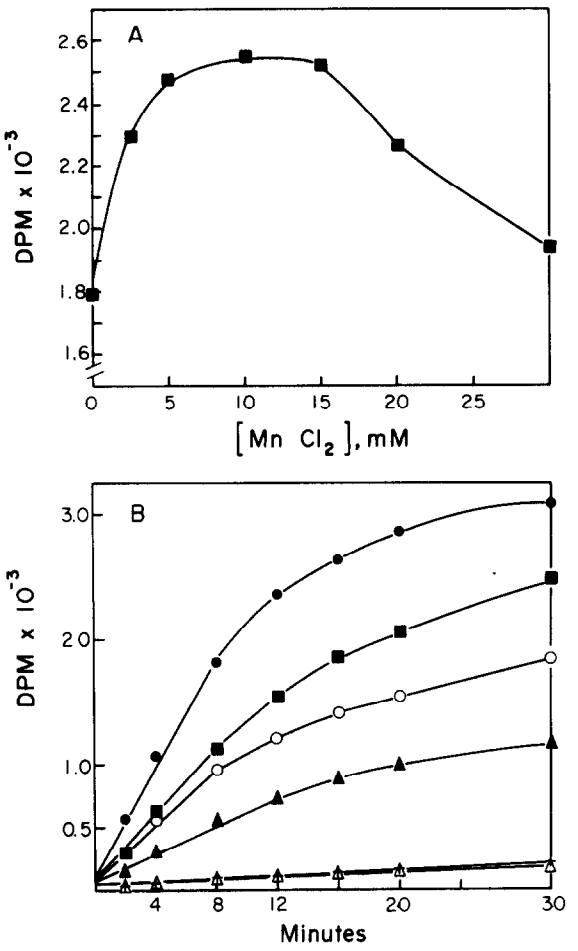

Fig.1. (A) Effect of manganese on carboxylase activity in SIK–Triton-buffered microsomes. Incubation conditions are in section 2. (B) Effect of variation in incubation conditions on manganese stimulation of carboxylase activity: (○——○) SIK buffer; (●——●) SIK buffer + 10 mM Mn^{2+}; (△——△) SI buffer; (▲——▲) SI buffer + 10 mM Mn^{2+}; (+——+) 'EDTA-extracted' microsomes; (■——■) EDTA-extracted microsomes + 10 mM Mn^{2+}. Incubation conditions are in section 2.

in sucrose–imidazole buffer without KCl resulted in an almost total loss of vitamin K-dependent carboxylase activity which could be partially restored by the addition of $MnCl_2$ (fig.1B). Manganese stimulated both the initial rate and the extent of the reaction. When EDTA-treated microsomes were dialyzed against SIK–Triton buffer some activity was restored and the stimulatory effect of manganese was reduced. Dialysis of SIK–Triton-solubilized microsomes against the same buffer also resulted in a preparation which was only slightly stimulated by manganese. Dialysis against KCl-containing buffers appeared to result in an incorporation of KCl into the detergent micelles in such a way as to block subsequent entry by manganese or other divalent metals. Dialysis against sucrose–imidazole buffer containing both KCl and manganese resulted in about the same activity as the addition of 10 mM $MnCl_2$ to undialyzed SIK–Triton-solubilized microsomes.

Carboxylase activity is located primarily on the inner surface of the microsomes [11] and carboxylation of exogenous peptide substrates cannot be assayed in the absence of detergent. Neither EDTA nor manganese would be likely to penetrate the sealed microsomal membranes. When the microsomal pellet was resuspended in the presence of manganese, to incorporate the metal into the microsomes, both the rate and extent of protein carboxylation were stimulated. Carboxylation of endogenous microsomal precursor proteins was stimulated 112% under these conditions. Manganese stimulated peptide and protein carboxylation activity equally well in a 0.2% as in 1.0% Triton-solubilized preparation, and in an EDTA-treated low detergent preparation. Manganese also stimulated carboxylation to essentially the same extent in two other systems: the acetone powder preparation in [12]; and the partially purified 'Complex A' system developed in [13]. Conversion of vitamin K to its 2,3-epoxide, or 'epoxidase' activity, was also stimulated by manganese (table 1). EDTA extraction resulted in ~80% loss of epoxidase activity, which could be partially restored with manganese.

To determine if manganese had a role in the carboxylase system other than its apparent effect on reconstitution, the carboxylase activity of manganese-deficient rats was investigated. Prothrombin concen-

Table 1
Effect of manganese on vitamin K epoxidation

Conditions	Vitamin K 2,3-epoxide (nmol/ml)	
	Control	10 mM Mn^{2+}
Low carboxylase substrate	4.0	6.0
High carboxylase substrate	7.7	10.9

Incubations were for 20 min at 17°C with 20 μg vitamin KH_2/ml. Epoxidase activity is dependent on carboxylation [18] and 1 mM (low) or 10 (high) mM Phe–Leu–Glu–Glu–Leu was added as a substrate for the carboxylase. Values are means of duplicate incubations

Table 2
Effect of dietary manganese deficiency on vitamin K-dependent carboxylase

Mn^{2+} status	Study I		Study II	
	$-Mn^{2+}$	$+Mn^{2+}$	$-Mn^{2+}$	$+Mn^{2+}$
Control	3310 ± 450	5220 ± 650	2800 ± 560	4500 ± 410
Mn^{2+}-deficient	2760 ± 860	4480 ± 1310	1240 (2)	2140 (2)
Deficient + Mn^{2+}	–	–	3500 ± 830	5610 ± 1000

Weanling male rats were fed the respective diets for 7 weeks, then a vitamin K-deficient diet (±Mn^{2+}) for 1 week. Vitamin K-dependent carboxylation of Phe–Leu–Glu–Glu–Leu was carried out in the presence (+Mn^{2+}) or absence (–Mn^{2+}) of 10 mM $MnCl_2$. In study II a third group was fed the Mn^{2+}-deficient diet for 6 weeks and 4 days, the control diet 3 days and the +Mn^{2+}, vitamin K-deficient diet 7 days. The values, expressed as dpm/mg microsomal protein, are mean ±SD for 5 rats in study I and 4 rats in study II. Manganese deficiency was more apparent in study II, and 2 of the 4 Mn^{2+}-deficient rats died before 7 weeks.

tration (two-stage assay) was not decreased in manganese deficient rats which had 20% of normal liver arginase activity. Results of assays of carboxylase activity in manganese deficient rats were equivocal. Initial studies utilizing pooled livers suggested a 50% drop in carboxylase activity in manganese-deficient rats. Two subsequent studies (table 2) showed a decrease which was statistically significant only in study II where the manganese deficient rats had only 10% of normal liver arginase levels. These animals were grossly sick and may have had a general impairment of protein metabolism. In a third study (not shown) normal carboxylase values were seen in both manganese-deficient rats and rats fed the control diet at the intake of the deficient rats. In all studies the carboxylase activity was stimulated by the addition of manganese in vitro.

Although it is possible that there is a specific manganese requirement for this enzyme, it appears unlikely from these data that the vitamin K-dependent carboxylase is a manganese metalloenzyme. The lack of a consistent effect with manganese deficient rats does not however prove this. Normal activity of pyruvate carboxylase, a manganese protein, has been observed in manganese-deficient chickens. In this case, magnesium substitutes for the manganese [14]. The stimulation of the vitamin K-dependent carboxylase by 4 different metals in vitro is more consistent with a metal-activated system such as the glycosyl transferases [14]. Although much of the data can be explained on the basis of the known effects of manganese on membrane reconstitution, the Mn^{2+}/Mn^{3+} redox potential is favorable for interaction with O_2, $O_2^{\cdot-}$, or hydroperoxide species [15–16] and manganese could also be involved in the binding of oxygen and its reaction with vitamin K. If manganese were involved with the oxygenation reaction, carboxylation would have to be directly linked to the oxygenation to explain the manganese stimulation of carboxylation. Another possible role for manganese is chelation of the carboxyl groups of the Glu–Glu residues of the peptide similar to the mechanism proposed for pyruvate carboxylase [17]. The metal would thus be expected to pull electron density from the carboxyls and stabilize the often proposed γ-carbanion. It has not been possible to do detailed kinetics on this system because of the reconstitution effect, and although the significance of manganese in the mechanism of vitamin action is still unclear, the metal requirement should be considered in any purification of the system.

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