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STIMULATION OF VITAMIN K-DEPENDENT CARBOXYLATION BY PYRIDOXAL-5'-PHOSPHATE*

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SUMMARY

This paper presents evidence that the approximately two-fold increase in vitamin K-dependent carboxylation of the pentapeptide PheLeuGluGluLeu, but not of endogenous protein substrate, brought about by pyridoxal-5'-phosphate, is due to binding of the pyridoxal-5'-phosphate to microsomal enzyme(s), rather than to the pentapeptide. Pyridoxine inhibits this peptide carboxylation, while pyridoxal, pyridoxamine, and pyridoxamine-5'-phosphate have no effect on the reaction.

INTRODUCTION

Amino acid transaminases, decarboxylases, and several other enzymes require pyridoxal-5'-phosphate as a coenzyme, which is bound through the formation of Schiff's base with the ε-amino group of a specific lysine residue of the enzyme protein. It is also known that Schiff's bases are formed in an aqueous solution between pyridoxal-5'-phosphate and amino acids, amines (1), several peptides (2), and bovine serum albumin (3). In the vitamin K-dependent carboxylation system, pyridoxal-5'-phosphate has been found to stimulate the carboxylation of pentapeptide, but not of endogenous substrate (4). This

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stimulation can be due to either reaction of the pyridoxal-5'-phosphate with the enzyme(s) or with the pentapeptide. In this communication, we provide evidence that a pyridoxal-5'-phosphate enzyme complex accounts for this stimulation of peptide carboxylation.

MATERIALS AND METHODS

Materials: Pyridoxine HCl (vitamin B_6), pyridoxal HCl, pyridoxal-5'-phosphate, pyridoxamine diHCl, pyridoxamine-5'-phosphate·HCl, Sephadex G-10-120, and Sephadex G-25-150 were obtained from Sigma Chemical Co., St. Louis, Mo. Triton X-100 and sodium dithionite were purchased from Eastman Kodak Co., Rochester, N.Y. Vitamin K_1 as a Tween suspension was obtained from Merck, Sharp and Dohme, Rahway, N.J. and sodium [^{14}C] carbonate (specific activity, 59.9 mCi/mmol) from Amersham-Searle. Warfarin was obtained from Endo Laboratories, Inc., Garden City, N.Y.

The pentapeptide (PheLeuGluGluLeu) was synthesized using the Merrifield technique as described by Stewart and Young (5), cleaved with HF, and purified by countercurrent distribution by Mr. Craig Ferris.

All other compounds were high-purity preparations obtained from commercial sources.

Methods: Vitamin K deficiency was produced in 10 to 14 days by maintaining Sprague-Dawley male rats on a vitamin K-deficient diet (6) in coprophagy-preventing cages (7). Hypoprothrombinemic rats were prepared by treatment with warfarin (5 mg/Kg injected intraperitoneally) 18 hrs before the experiments (8).

Vitamin K hydroquinone was prepared by reduction of vitamin K with sodium dithionite (9).

The Triton X-100 soluble system from normal, warfarin-treated, and vitamin K-deficient rat liver microsomes, was prepared by a modification of the method of Mack *et al.* (12).

Carboxylation activity was determined as follows. One hundred µl of incubation mixture were used per assay. Each assay tube contained 60 µl of soluble system (1 mg protein), 20 µl pentapeptide solution (7.5 mM in 0.25 M potassium phosphate buffer, pH 7.4), 10 µl sodium [^{14}C] carbonate (2.5 µCi), and 5 µl 0.05 M potassium phosphate buffer, pH 7.4, or 5 µl vitamin B_6 derivative solution (50 mM in this buffer). The reaction was initiated by the addition of 5 µl of vitamin K hydroquinone (25 µg). The samples were incubated in a constantly shaking water bath at 25° for 30 min. The reaction was stopped by adding 1 ml ice-cold solution of 10% ($^{W}/_{V}$) trichloroacetic acid. After the addition of 100 µl of bovine serum albumin solution (10 mg/ml), the protein precipitate was left for 30 min at room temperature and removed by low speed centrifugation. One ml of the supernatant was gassed for 30 min with air to remove unreacted [^{14}C]-CO_2, then counted in 5 ml of Scintisol (Isolab Inc., Ohio) in a Packard 3950 liquid scintillation counter. The trichloroacetic acid pellet was dissolved twice in 2% sodium carbonate solution and reprecipitated with 10% trichloroacetic acid and counted in the same way.

Modification of enzyme(s) or pentapeptide by reacting with pyridoxal-5'-phosphate was carried out as follows. The Triton X-100 soluble system, from vitamin K-deficient rat liver microsomes (10 mg protein) or pentapeptide

solution (10 mg), was incubated 24 hrs at 0° with pyridoxal-5'-phosphate (final concentration 2.5 mM), and applied to a column (1 x 30 cm) of Sephadex G-25-150 equilibrated with 0.05 M potassium phosphate buffer, pH 7.4, containing 2% ($^{V}/_{V}$) Triton X-100 or Sephadex G-10-120 equilibrated with 0.05 M potassium buffer, pH 7.4. Void volume fractions containing enzyme(s) or pentapeptide bound to pyridoxal-5'-phosphate were yellow in color and were used to estimate activity.

Protein was estimated by the method of Esen (11) with bovine serum albumin as the standard. Pentapeptide and pyridoxal-5'-phosphate-bound pentapeptide were determined by amino acid analysis.

RESULTS AND DISCUSSION

Dose Response of Pyridoxal-5'-phosphate Stimulation of Carboxylation:

Figure 1 shows that the stimulation of carboxylation of the pentapeptide PheLeuGluGluLeu, by pyridoxal-5'-phosphate, occurs both when vitamin K-deficient and when warfarin-treated rat liver microsomes are used as a source of soluble system. The soluble system derived from normal rat liver microsomes is much less active. The stimulation reaches a plateau for all three microsomal systems at 2.5 mM pyridoxal-5'-phosphate. The soluble system, derived from vitamin K-deficient microsomes, also shows the most endogenous protein carboxylation activity.

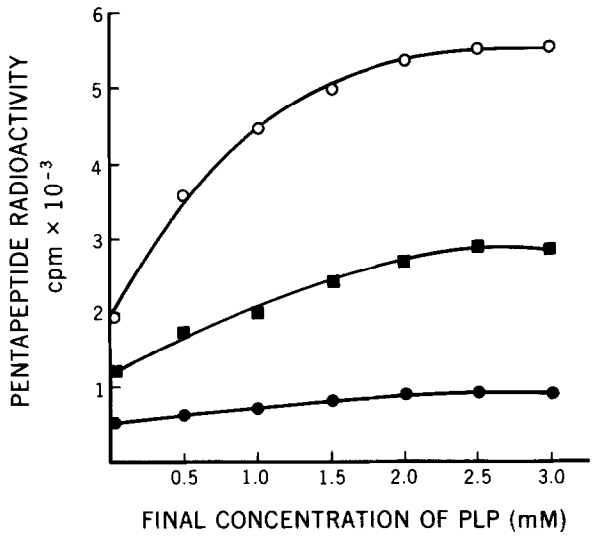

Figure 1. Stimulation of vitamin K-dependent pentapeptide carboxylation by pyridoxal-5'-phosphate. ●——●, soluble system from normal rat liver microsomes. ■——■, soluble system from warfarin-treated rat liver microsomes. O——O, soluble system from vitamin K-deficient rat liver microsomes.

TABLE I

EFFECT OF VITAMIN B_6 DERIVATIVES ON VITAMIN K-DEPENDENT PENTAPEPTIDE AND ENDOGENOUS SUBSTRATE CARBOXYLATION

	Carboxylation % of Control System[1]					
	Normal Rats[2]		Warfarin-treated Rats[3]		Vitamin K-deficient Rats[4]	
Compounds Added (2.5 mM)	Pentapeptide	Endogenous Substrate	Pentapeptide	Endogenous Substrate	Pentapeptide	Endogenous Substrate
Pyridoxine	37	94	39	88	38	91
Pyridoxal	71	94	64	95	91	99
Pyridoxal-5'-phosphate	160	102	224	101	252	103
Pyridoxamine	86	108	80	102	87	110
Pyridoxamine-5'-phosphate	75	87	81	106	102	105

[1]Control system is defined as the incubation mixture of solubilized microsomes with no added vitamin B_6 derivatives. All values expressed are the averages of three experiments after subtracting the minus vitamin K values which were 100 cpm for pentapeptide and 30 cpm for endogenous substrate. The conditions of incubation are described in Materials and Methods.

[2]100% is equivalent to 500 cpm for pentapeptide, 170 cpm for endogenous substrate.

[3]100% is equivalent to 1,000 cpm for pentapeptide, 1,000 cpm for endogenous substrate.

[4]100% is equivalent to 2,300 cpm for pentapeptide, 2,000 cpm for endogenous substrate.

Effect of Vitamin B_6 Derivatives on Vitamin K-dependent Carboxylation: The effect of various vitamin B_6 compounds on vitamin K-dependent carboxylation is shown in Table I. The endogenous protein carboxylation is changed very little by adding any of the pyridoxine related compounds. Stimulation of the carboxylation of pentapeptide by pyridoxal-5'-phosphate is demonstrated in all three carboxylation systems, while pyridoxal, pyridoxamine, and pyridoxamine-5'-phosphate do not have significant effects. Inhibition (61 to 63%) is caused by added pyridoxine. The stimulation by pyridoxal-5'-phosphate varies from 1.6- to 2.5-fold, the amount being related to the original carboxylation activity of the system. The data suggest that some integral part of the carboxylation system may be altered to facilitate pentapeptide binding, but not to influence endogenous protein carboxylation. The inhibition by pyridoxine may indicate that it binds to the same site as pyridoxal-5'-phosphate and prevents the binding of pentapeptide.

Interaction of Pyridoxal-5'-phosphate with Enzyme or Substrate: Table II shows that the stimulation of carboxylation of pentapeptide can be observed by

TABLE II

EFFECT OF PYRIDOXAL-5'-PHOSPHATE ASSOCIATE WITH ENZYME(S) OR SUBSTRATE ON PENTAPEPTIDE CARBOXYLATION[1]

Enzyme System	No PLP[2]	Plus PLP[3]
Soluble System[4] plus Pentapeptide (1.5 mM)	100	250
Soluble System plus PLP bound to Pentapeptide	25	40
PLP-bound Soluble System plus Pentapeptide	240	240
PLP-bound Soluble System plus PLP-bound Pentapeptide	23	25

[1]Averages of six separate experiments (different microsomes), each run in duplicate.

[2]Pyridoxal-5'-phosphate

[3]2.5 mM

[4]From vitamin K-deficient rat liver microsomes (10 mg protein/ml)

adding the protein-bound pyridoxal-5'-phosphate fraction to pentapeptide. On the other hand, pyridoxal-5'-phosphate-bound pentapeptide shows only 25% of the activity of that with free pentapeptide. This solution supplied the same amount of pentapeptide as used in the experiments in which the peptide was not reacted with pyridoxal-5'-phosphate. This clearly demonstrates that stimulation of pentapeptide carboxylation is not due to pyridoxal-5'-phosphate interaction with the pentapeptide, but must be due to an alteration of a solubilized protein of the carboxylation system. The modified pentapeptide serves as a very poor substrate. The nature of the interaction between pyridoxal-5'-phosphate and the enzyme is presumably the reaction of the pyridoxal-5'-phosphate with a lysine ε-amino group of a protein of the carboxylation system.

The relationship of the pyridoxal-5'-phosphate to the vitamin K-dependent carboxylation of the pentapeptide appears to indicate a change in conformation at the carboxylation site, which enhances the availability of the pentapeptide.

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