REVERSAL BY VITAMIN K OF CYCLOHEXIMIDE INHIBITED BIOSYNTHESIS OF PROTHROMBIN IN THE ISOLATED PERFUSED RAT LIVER

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SUMMARY

Cycloheximide inhibition of prothrombin synthesis from isotopic leucine in the isolated perfused rat liver can be reversed by high doses of vitamin K_1 without affecting the blockade of synthesis of non vitamin K-dependent proteins. A partially competitive type of inhibition was observed which suggests that cycloheximide affects the association of vitamin K with a regulatory protein bound to the ribosome. Puromycin, on the other hand, inhibited both general and vitamin K-dependent factor synthesis, regardless of the dose of vitamin K in the system.

Vitamin K has been shown to stimulate the appearance of prothrombin (factor II) and three other vitamin K-dependent coagulation proteins (factors VII, IX, and X) in the plasma of birds and mammals deficient in this vitamin. In 1964, Mattii, et al. (1) demonstrated that the isolated livers of rats pretreated with vitamin K would elaborate prothrombin in vitro. J. P. Olson, et al. (2) confirmed this observation but noted that the action of vitamin K was not significantly altered by the presence of puromycin in their system. They concluded that vitamin K acted to convert a preformed peptide to the active coagulation factor. R. E. Olson, et al. (3), using a totally artificial plasma, reported a similar action of vitamin K in

the isolated perfused liver from both normal and vitamin K-deficient rats, but contrary to J. P. Olson, et al. (2), observed that puromycin completely blocked the action of the vitamin. They also reported the incorporation of leucine-1-^{14}C into prothrombin synthesized under the influence of vitamin K by precipitation of the product with specific antibody.

The purpose of this communication is to report some novel effects of cycloheximide upon the biosynthesis of prothrombin in the isolated perfused rat liver including the reversal of its inhibitory action in the presence of high concentrations of vitamin K.

MATERIALS AND METHODS

Sprague-Dawley rats were fed a vitamin K-deficient diet for 10 days at which time their plasma prothrombin was reduced to less than 6% of normal. These rats were on a phenobarbital anesthesia and perfused by the basic technique of Miller, et al. (4) as modified by R. E. Olson, et al. (3). The perfusion medium was by combining Krebs-Ringer bicarbonate, 3.2% bovine serum albumin, 300 mg% of glucose, 20% washed rat red blood cells and an amino acid mixture containing suitable concentrations of amino acids with radioactive leucine-1-^{14}C at 10 to 50 μc. Vitamin K_1 (as Aquamephyton^R) was added two hours after initiation of perfusion and the perfusion continued for 4 hours. The perfusate was analyzed for prothrombin and total protein radioactivity at hourly intervals during the entire experiment. Antibiotic inhibitors of protein synthesis were added initially in some experiments. The concentrations of puromycin used was 200 μg per ml and of cycloheximide, 10 and 100 μg per ml. In some experiments, the liver was homogenized at the end of the experiment and the radioactivity of the total TCA-precipitable

protein measured. The radioactivity of prothrombin itself was determined by precipitation with specific antibody as previously described (3). Prothrombin was assayed by the one stage clotting method of Hjort (5). All measurements of radioactivity were made on a Packard scintillation spectrometer.

RESULTS AND DISCUSSION

Studies of the dose-response curve for vitamin K_1 and prothrombin in the isolated perfused vitamin K-deficient rat liver showed typical saturation kinetics with an apparent V_{max} of 5% (or normal rat plasma values) in four hours. The actual biosynthetic rate was comparable to that of the intact vitamin K-deficient rat given vitamin K since the perfusion volume has 10 times the rat's blood volume. Since the response to vitamin K in this system is essentially linear for four hours, the final content of prothrombin obtained in four hours has the dimensions of velocity and is treated throughout in that way. Although vitamin K_1 is a modifier, and not a substrate, the typical Michaelis-Menten kinetic pattern obtained in this system (albeit with whole cells), suggests strongly that vitamin K is combining with a regulatory protein which is responsible for the typical saturation response. The apparent K_m for vitamin K_1 in the vitamin K-deficient rat liver was 2 nanograms per ml.

Figure 1 shows on a semilogarithmic curve the three dose-response curves for vitamin K_1 and prothrombin in this system when (a) there is no inhibitor, (b) cycloheximide at 10 µg per ml is added and (c) cycloheximide at 100 µg per ml is added. The curves are typical of competitive inhibition of an enzyme-substrate interaction. The apparent K_m for vitamin K_1 in the presence of 10 µg per ml of cycloheximide was 100 nanograms per

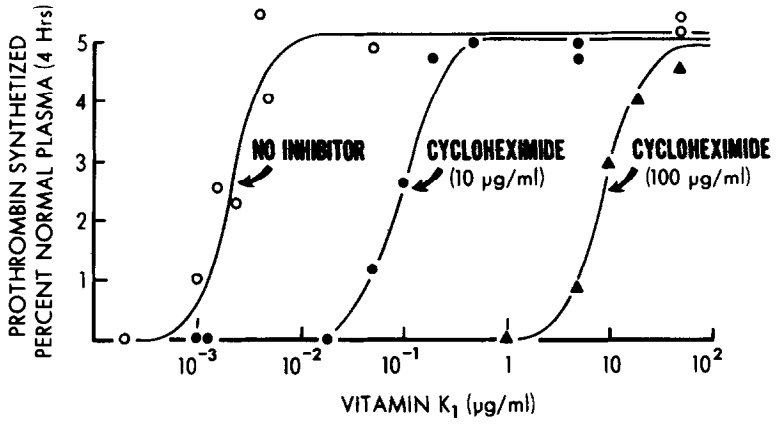

Figure 1. Dose-response curve for vitamin K_1 and prothrombin in the isolated perfused vitamin K-deficient rat liver. The curve to the left shows the response of the uninhibited liver. The two curves to the right show the response in the presence of two concentrations of cycloheximide.

ml and for 100 µg per ml was 10.0 µg per ml. Since the K_i varied with concentration of inhibitor, this type of competitive inhibition falls into the partial competitive type in which it appears that the inhibitor affects the association constant for substrate (in this case modifier) with a biologically active protein (6). The reversal of the action of cycloheximide for prothrombin synthesis (as measured with the clotting assay) was complete at high vitamin K_1 concentrations and the apparent V_{max} of the system was not changed in the presence of inhibitor. Measurement of prothrombin synthesized in the cycloheximide containing system at reversing vitamin K concentrations by use of a specific precipitin for rat prothrombin showed comparable radioactivities in the antigen-antibody precipitates, i.e., 500 ± 100 DPM per ml perfusate for studies in which 10 µc of leucine-1-^{14}C was given.

The incorporation of leucine-1-^{14}C into liver and total perfusate protein in the isolated perfused rat liver was not affected by the presence or absence of either vitamin K_1 or

TABLE I

INCORPORATION OF 10 μCi OF LEUCINE-1-^{14}C INTO LIVER AND PLASMA PROTEIN BY THE ISOLATED PERFUSED RAT LIVER IN 6 HOURS

Exp.	Vitamin K_1 μg/ml	Cycloheximide μg/ml	Perfusate Protein DPM/ml*	Liver Protein DPM/mg
1	±2	0	25,200 ± 1610	1770 ± 204
2	±2	100	508 ± 89	70 ± 9
3	50	100	801 ± 65	228 ± 18

*Each ml of perfusion fluid contains about 35 mg of protein of which 30 is bovine serum albumin. Variance is given as standard error of the mean. Each value represents 4-6 determinations.

warfarin (7). On the other hand, cycloheximide had a marked effect upon general protein synthesis in this system, as shown in Table I. In the presence of 2 μg/ml of vitamin K_1 in the perfusate, 100 μg per ml of cycloheximide inhibited the incorporation of labeled amino acid into liver and perfusate protein 96-98%. In the presence of 50 μg per ml of vitamin K_1, however, significantly more label appeared in both perfusate and liver protein, with a relaxation of the inhibition of general protein synthesis to 88-95% of control values. The small difference in perfusate radioactivity caused by vitamin K was essentially equivalent to that precipitated with anti-prothrombin antibody, and detected by the clotting assay for prothrombin. Thus the change in total protein synthesis stimulated by vitamin K in the presence of cycloheximide is associated with the biosynthesis of vitamin K-dependent clotting proteins.

The contrast in interactions of cycloheximide and puromycin with vitamin K in the isolated perfused liver is

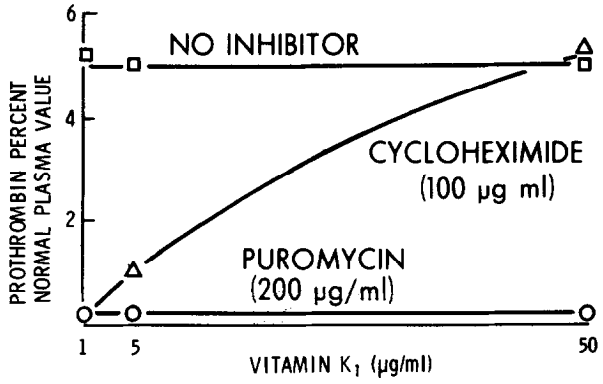

Figure 2. Effect of puromycin and cycloheximide upon the biosynthesis of prothrombin in the isolated perfused vitamin K-deficient liver at various vitamin K_1 concentrations. The response of uninhibited livers is shown at the top (□); that of cycloheximide inhibited livers in the middle (Δ) and puromycin inhibited livers at the bottom (O).

shown in Figure 2. No reversibility of the effect of puromycin by vitamin K was noted over the same range of vitamin K_1 concentrations used to demonstrate reversibility of cycloheximide blockade. As shown in Figure 2, the control values for prothrombin constitute a maximum plateau in the designated range of vitamin K_1 concentrations used in these experiments.

The action of vitamin K to reverse the effect of cycloheximide on prothrombin but not general protein synthesis provides an important clue as to the mode of action of vitamin K. It has been demonstrated by Siegel, et al. (8) and Rao, et al. (9) that cycloheximide binds to the 60 S particle of the mammalian ribosome and arrests the peptide synthetase required to elongate the peptide chain. It tends to freeze polysomes undergoing protein synthesis in a fixed state of aggregation (10) and prevent completion of polypeptide chains. Felicetti, et al. (11) observed that cycloheximide is a reversible inhibitor of the peptide synthetase in reticulocytes and could be washed out with restoration of protein synthesis. Munro, et al. (12)

noted that cycloheximide inhibited both chain initiation and chain elongation in ribosomes prepared from rat liver and that the block in elongation could be reversed by addition of glutathione in high concentration, consistent with a site of action for the drug on transferase II (13).

From these observations plus the experiments reported here, we should like to suggest that cycloheximide and a regulatory protein which binds vitamin K interact with the 60 S particle of the ribosome at a common site and that the presence of cycloheximide modifies the association between vitamin K_1 and its regulatory protein so as to provide partial competitive inhibition. The fact that the action of vitamin K to reverse cycloheximide inhibition of protein synthesis is restricted to only vitamin K-dependent proteins suggests that the regulatory protein for vitamin K is available to combine with only a small percent of total hepatic ribosomes.

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