In vitro prothrombin synthesis from a purified precursor protein

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IN VITRO PROTHROMBIN SYNTHESIS FROM A PURIFIED PRECURSOR PROTEIN

II. PARTIAL PURIFICATION OF BOVINE CARBOXYLASE

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Summary

In this paper, we describe the isolation and partial purification of an enzyme system that converts bovine decarboxyfactor II (PIVKA-II) into prothrombin (factor II). It is shown that the increase in factor II activity occurs in parallel with $^{14}CO_2$ incorporation into $BaSO_4$ adsorbable proteins. The system is not strictly vitamin K-dependent because it is obtained from the livers of normal healthy cows. By preincubating the enzyme(s) with an excess of warfarin, an absolute vitamin K_1-dependence can be obtained. The reaction is inhibited by its own product, factor II.

Introduction

The final step in the biosynthesis of prothrombin (factor II) is the modification of 10 glutamic acid residues into γ-carboxyglutamic acid residues [1]. This carboxylation takes place in a postribosomal enzymatic reaction, which is vitamin K-dependent [2]. Several authors have reported the development of a rat-liver cell-free system from vitamin K-deficient rats which is able to produce prothrombin by an O_2-dependent carboxylation reaction [3—8]. In vitamin K-deficient rats, however, the prothrombin precursors accumulate in the liver and do not reach the blood [9]. Hence, the rat-liver cell-free system contains both the enzyme and the substrate and this hinders further purification of the enzyme system.

In human and cattle, the precursors of the vitamin K-dependent clotting factors reach the blood when vitamin K antagonists are administered. We developed a factor II-synthesizing enzyme system by which factor II activity is generated from separately purified prothrombin precursor (decarboxyfactor II), obtained from coumarin-treated cows [10]. The enzyme system, which we call

factor II-synthetase, is obtained from normal healthy cows. In this communication we report the partial purification of the system and some of its characteristics.

Materials and Methods

Reagents, buffers and chemicals

Buffer A: 50 mM KCl, 200 mM sucrose, 20 mM Tris · HCl, pH 7.8. Buffer B: 50 mM KCl, 20 mM Tris · HCl, pH 7.8.

Vitamin K_1 was obtained from Hoffmann-La Roche, Switzerland. Warfarin (3-(α-acetonyl-benzyl)-4-hydroxycoumarin), ATP, NADH, and Triton X-100 were obtained from Sigma, U.S.A. $NaH^{14}CO_3$ (60 Ci/mol) and [^{14}C]-formaldehyde (15 Ci/mol) were obtained from The Radiochemical Centre, Amersham, England and Aquasol-2 from New England Nuclear, U.S.A.

All other chemicals were obtained from Merck, G.F.R.

Coagulation factors, assays and definition of units

Factor II was purified according to Owen et al. [11] and decarboxyfactor II was described earlier [12]. Coagulation tests were performed as described by Vermeer et al. [13]. The amount of each clotting factor present in bovine reference plasma [13] was arbitrarily taken as 1 U/ml. In a similar way, 1 U/ml of decarboxyfactor II was defined as the amount of precursor that gives the same clotting time in the *Echis carinatus* assay [13] as the bovine reference plasma.

Labelling of decarboxyfactor II

Decarboxyfactor II was labelled with [^{14}C]formaldehyde [12].

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed in gels containing 7% polyacrylamide [14] in 5 mM Tris · HCl (pH 8.4)/38.4 mM glycine/1.5 mM $CaCl_2$. This buffer was used both in the gels and in the reservoirs.

Measurement of factor II synthesis

Microsomal protein fractions (see below) were incubated in the presence of decarboxyfactor II (0.4 U/ml), vitamin K_1 (0.2 mM) and $NaHCO_3$ (1 mM) in buffer B. In our routine tests, reaction mixtures of 0.1 ml, containing 0.01 units of absorbance (A_{280}) of microsomal proteins were incubated at 37°C for 1.5 h and subsequently diluted 4 times with cold buffer B. Factor II synthetase was defined as the activity able to decrease the one-stage clotting time (factor II assay) of the reaction mixture during incubation due to the production of prothrombin from decarboxyfactor II. The difference of the clotting times before and after incubation was used to measure the amount of factor II that had been synthesized during the reaction.

Measurement of carboxylase

The introduction of new carboxyl groups in decarboxyfactor II was measured under similar conditions as factor II synthesis, except that unlabelled

$NaHCO_3$ was replaced by 20 μCi $NaH^{14}CO_3$. The reaction was stopped by adding 1 ml ice-cold buffer containing 10 mM $NaHCO_3$ (pH 8.0). Subsequently, we adsorbed the reaction mixtures with 20 mg $BaSO_4$ which was washed 4 times with 10 ml $NaHCO_3$. The $BaSO_4$ was brought into Aquasol-2 and counted as such. Carboxylase was defined as the activity that is able to increase the amount of $BaSO_4$-adsorbable counts. The difference between the amounts of adsorbed counts before and after the reaction was taken as a measure of the activity of carboxylase in our reaction mixture.

The preparation of bovine liver microsomes

About 100 g fresh bovine liver was taken immediately after slaughtering and cut into 5-g pieces, rinsed with ice-cold buffer A and mixed in a Waring Blendor with 300 ml buffer A. The slurry was homogenized further in a Potter homogenizer with a tight-fitting Teflon pestle and centrifuged twice for 15 min at 12 000 $\times g$. The supernatant was centrifuged for 30 min at 150 000 $\times g$. The pellet was washed 4 times by resuspending in 300 ml buffer B and centrifuging for 30 min at 150 000 $\times g$. Finally, the pellet was dissolved to 320 A_{280} units and referred to as "crude microsomes".

Partial purification of factor II synthetase

Crude microsomes from 30 g liver were supplemented with Triton X-100 to a final concentration of 2%, incubated at 0°C for 30 min and centrifuged for 1 h at 150 000 $\times g$. The supernatant was applied to a Sepharose 4B column in buffer B. The elution profile is shown in Fig. 1A. Factor II synthetase activity was eluted at 2 positions, with different apparent molecular weights.

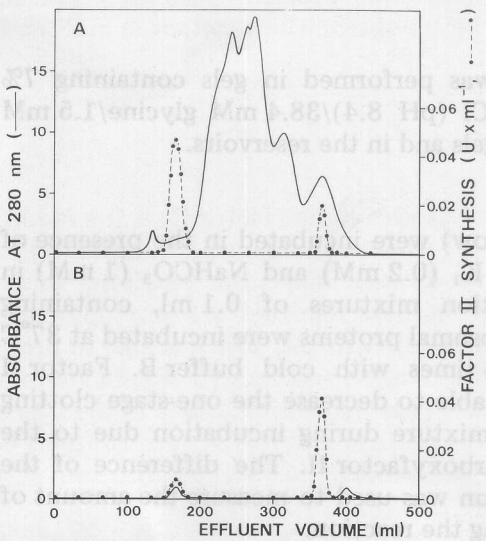

Fig. 1. Fractionation of crude microsomal proteins on Sepharose 4B. (A) Crude microsomes were supplemented with Triton X-100 to a final concentration of 2% (v/v), centrifuged for 1 h at 150 000 $\times g$ and 6 ml of the supernatant were applied to a Sepharose 4B column (2.5 $\times$ 100 cm) in buffer B. (B) The high molecular weight activity peak was pooled, concentrated and once more filtered through the same column.

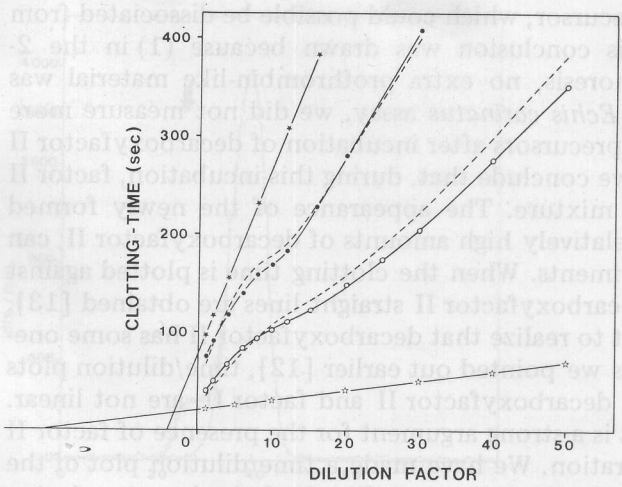

Fig. 2. *t-D* plots of standard reaction mixtures (see Materials and Methods) during prothrombin synthesis. ★———★, before incubation; ●———●, after 45 min at 37°C; ○———○, after 90 min at 37°C; ☆———☆, factor II alone (0.4 U/ml). The dotted lines represent the values obtained when mixtures of factor II and decarboxyfactor II were analyzed (for details, see text).

When we rechromatographed the high molecular weight fraction on the same column, the main part of the factor II synthetase was dissociated and eluted at the low molecular weight position (Fig. 1B). In this way, we obtained a purification of at least 10 000 fold. The molecular weight as determined on Sephadex G-100 was about 60 000. After sodium dodecyl sulphate polyacrylamide gel electrophoresis, the preparation, which was called "semi-purified synthetase" still contained three protein bands.

Results

(A) Evidence for the generation of prothrombin

When the partially purified microsomal proteins were incubated in the presence of decarboxyfactor II, the clotting time in the one-stage coagulation assay decreased dramatically. This shortening of the clotting time is not caused by a proteolytic degradation of decarboxyfactor II into thrombin or a thrombin-like molecule for the following reasons:

(1) When decarboxyfactor II was omitted from the reaction mixture and replaced by factor II, no decrease of the clotting time was observed, indpendent of the amount of factor II that was used in the experiment.

(2) In the clotting assay no coagulation occurred in the absence of Ca^{2+}.

(3) Known thrombin inhibitors, such as diisopropylfluorophosphate, benzamidinium chloride and hirudin did not inhibit the acceleration of the clotting time during incubation of the reaction mixture.

(4) After chromatography on Sephadex G-100 columns, the newly formed factor II activity eluted at the same position as decarboxyfactor II and factor II and not at that of thrombin.

The shortening of the reaction mixtures clotting time was neither due to

an increase of prothrombin precursor, which could possible be dissociated from the microsomal proteins. This conclusion was drawn because (1) in the 2-dimensional immunoelectrophoresis, no extra prothrombin-like material was discovered, and (2) with the *Echis carinatus* assay, we did not measure more prothrombin or prothrombin precursors after incubation of decarboxyfactor II with microsomal proteins. So we conclude that, during this incubation, factor II is introduced in the reaction mixture. The appearance of the newly formed factor II, in the presence of relatively high amounts of decarboxyfactor II, can be visualized by kinetic experiments. When the clotting time is plotted against the dilution of factor II or decarboxyfactor II straight lines are obtained [13]. In this context, it is important to realize that decarboxyfactor II has some one-stage prothrombin activity. As we pointed out earlier [12], time/dilution plots of mixtures, containing both decarboxyfactor II and factor II, are not linear. So a curved time/dilution plot is a strong argument for the presence of factor II in a decarboxyfactor II preparation. We have made a time/dilution plot of the reaction mixture after varying incubation times (Fig. 2). It is clear that during incubation, the time/dilution plot flattens and becomes slightly curved. After 30 min, the line resembles the plot of a mixture containing 0.391 U/ml decarboxy-factor II and 0.009 U/ml factor II (dotted line). After 120 min, it resembles the plot of a mixture containing 0.378 U/ml PIVKA-II and 0.022 U/ml factor II.

The presence of factor II in the reaction mixtures after incubation can also be deduced from experiments in which we adsorbed incubated and non-incubated reaction mixtures to varying amounts of $BaSO_4$ (Fig. 3). The decarboxyfactor II in these mixtures adsorbs only to large amounts of $BaSO_4$, whereas the factor II in the incubated mixtures adsorbs to small amounts of $BaSO_4$. Therefore, the incubated mixtures show a biphasic adsorption curve. The non-incubated mixtures on the other hand show a linear relationship between the amount of added $BaSO_4$ and the adsorption of the "apparent" factor II activity caused by decarboxyfactor II.

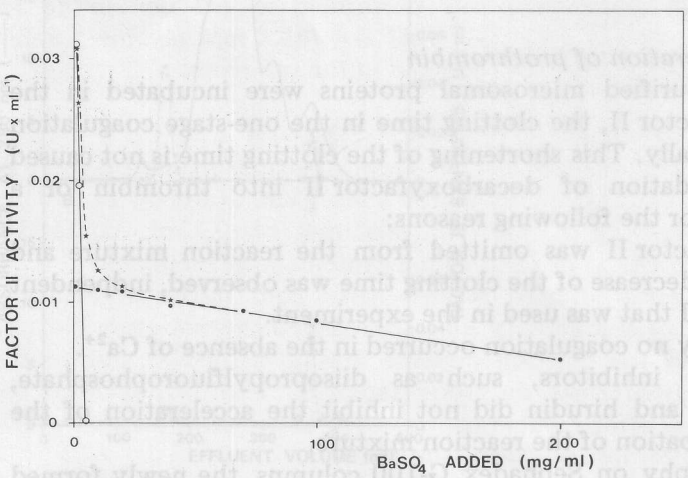

Fig. 3. $BaSO_4$ adsorption of factor II from standard reaction mixtures. ●———●, before incubation; ★———★, after incubation for 1.5 h at 37°C; ○———○, 0.03 U/ml of purified factor II. The mixtures were diluted four times with buffer B and shaken for 2 min on a Vortex mixer with the indicated amount of $BaSO_4$. The latter was removed by centrifugation and factor II was assayed in the supernatant.

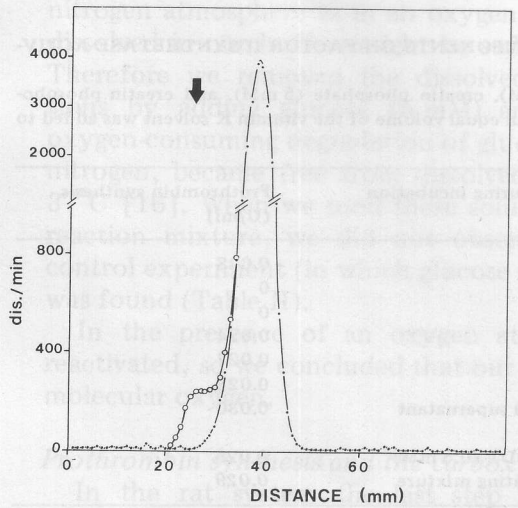

Fig. 4. Polyacrylamide gel electrophoresis of standard reaction mixtures containing ^{14}C-labelled decarboxyfactor II. ●——●, before incubation; ○——○, after 90 min at 37°C. The position of factor II (in a parallel gel) is marked with an arrow.

(B) Evidence for the synthesis of prothrombin from externally added decarboxyfactor II

Now it was proven that extra factor II is introduced in the reaction mixture, one may question whether this factor II arises either by supersession of existing factor II by PIVKA-II from microsomal fragments, or by synthesis from a substrate, which is already present in the microsomal fragments or by synthesis from the decarboxyfactor II which was added to the reaction mixture. The proof that externally-added decarboxyfactor II was converted into factor II was given by introducing ^{14}C-labelled decarboxyfactor II into the reaction mixtures. Before and after incubation the mixtures were submitted to polyacrylamide gel electrophoresis, whereafter the gels were sliced and counted. A new peak arises after incubation at the position of factor II (Fig. 4) and the new peak was observed only when electrophoresis was performed in the presence of 1.5 mM $CaCl_2$.

(C) The influence of several variables on the synthesis of factor II

The synthesis of factor II by crude microsomes depends on the temperature, the ionic conditions and pH [10]. In our more purified system, the following optima were found: 37°C, 50 mM KCl, 10 mM Tris · HCl and pH 7.8—8.5. When we investigated the dependence of the reaction on cofactors such as vitamin K_1 or HCO_3^-, it appeared necessary to work with limiting amounts of factor II synthetase. The best results were obtained when the reaction velocity was about 30% of its maximal value. When high amounts of factor II synthetase were added to the reaction mixtures, the formation of factor II was not significantly dependent on any cofactor. Earlier experiments with crude microsomes [10] showed that the factor II-synthesizing system was dependent on microsomal supernatant, ATP, Mg^{2+}, HCO_3^- and oxygen. When we tried to

TABLE I

THE INFLUENCE OF SEVERAL REACTION COMPONENTS ON FACTOR II SYNTHETASE ACTIVITY

The ATP-generating mixture contained ATP (2 mM), creatin phosphate (5 mM), and creatin phosphokinase (50 μg/ml). When vitamin K_1 was omitted, an equal volume of the vitamin K solvent was added to the reaction mixture. DTT, dithiothreitol.

Ommission during incubation	Addition during incubation	Prothrombin synthesis (U/ml)
None		0.028
Decarboxyfactor II (0.4 U/ml)		0
Synthetase (2 μg/ml)		0
Vitamin K_1 (0.2 mM)		0.024
$NaHCO_3$ (1 mM)		0.027
O_2 *		0.028
	microsomal supernatant (10% v/v)	0.030
	NADH + DDT (0.5 mM)	0.025
	ATP generating mixture	0.029

* The normal atmosphere was replaced by gassing the tubes for 1 min with nitrogen.

repeat those experiments with the semi-purified preparation, the results were at variance with our earlier report (see Table I). In agreement with the observations of Esmon and Suttie [15], purification of factor II synthetase results in a loss of the energy dependency of the reaction. This means that ATP, creatin phosphate, creatin phosphokinase and Mg^{2+} may be omitted. Neither we were able to demonstrate any dependence of the synthesis of factor II on microsomal supernatant, NADH or reducing agents such as dithiothreitol. On the other hand the reaction appeared to be dependent on the presence of molecular oxygen, bicarbonate and vitamin K_1. Although this cannot be concluded from Table I, more detailed investigations lead to the conclusion that these three cofactors are required for the conversion of decarboxyfactor II into factor II.

Prothrombin synthesis and molecular oxygen

When we observed that the synthesis of factor II proceeded equally well in a

TABLE II

THE INFLUENCE OF DISSOLVED O_2 ON FACTOR II-SYNTHETASE ACTIVITY

Glucose oxidase was added to all tubes. After adding glucose (as indicated) the tubes were gassed with nitrogen for 1 min, closed and incubated at 37°C for 10 min. Decarboxyfactor II + vitamin K_1 and factor II synthetase were incubated separately in this way. After 10 min the solution containing PIVKA-II was added to that containing factor II-synthetase. The tubes were again gassed, either with nitrogen or oxygen (as indicated) and incubated at 37°C. For further details see text.

Glucose oxidase (mg/ml)	Glucose (mM)	Atmosphere	Prothrombin synthesis (U/ml)
0.5	5	N_2	0
0.5	5	O_2	0
0.5	0.5	N_2	0.009
0.5	0.5	O_2	0.021
0.5	0	N_2	0.026
0.5	0	O_2	0.026

nitrogen atmosphere as in an oxygen atmosphere, we realized that the oxygen dissolved in our buffers might be sufficient to give a normal reaction velocity. Therefore we removed the dissolved O_2 from our buffers and protein solutions by adding glucose (5 mM) and glucose oxidase (0.5 mg/ml). By the oxygen-consuming degradation of glucose our solutions, which were kept under nitrogen, became free from dissolved oxygen after incubation for 10 min at 37°C [16]. When we used these solutions to compose a factor II synthesizing reaction mixture, we did not observe any synthetase activity, whereas in a control experiment (in which glucose was omitted) a normal synthetase activity was found (Table II).

In the presence of an oxygen atmosphere factor II synthetase could be reactivated, so we concluded that our enzyme system is dependent on dissolved molecular oxygen.

Prothrombin synthesis and the carboxylation of decarboxyfactor II

In the rat system the last step in the synthesis of prothrombin is the carboxylation of ten glutamic acid residues at the N-terminal position of decarboxyfactor II. The source of the attached carboxyl group is HCO_3^- [17]. Therefore we investigated whether also in the bovine system prothrombin is synthesized from decarboxyfactor II and HCO_3^-. As was shown in Table I, simple omission of HCO_3^- from the reaction mixtures does not result in any decrease of factor II synthesis. However, in this case, HCO_3^- could be formed by the solution of CO_2 gas into our buffers and protein solutions. We therefore added to the incubation mixtures ^{14}C-labelled $NaHCO_3$ instead of unlabelled $NaHCO_3$ and we measured the incorporation of label into $BaSO_4$-adsorbable proteins (see Materials and Methods). A time-course of the reaction is given in Fig. 5.

Unlike the increase in factor II activity the reaction has a lag-time of about 15 min. This can be explained by the presence of unlabelled CO_2 which is fixed

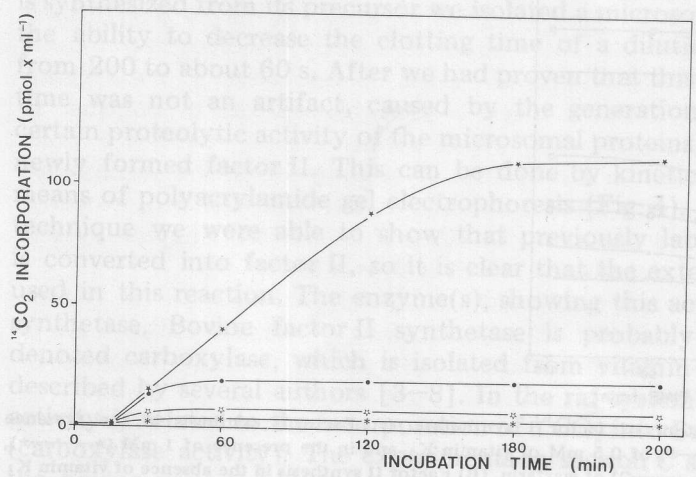

Fig. 5. Incorporation of $^{14}CO_2$ into $BaSO_4$ adsorbable proteins during incubation of varying reaction mixtures. 1 pmol/ml corresponds to 136 dpm/ml. ★———★, complete system; ☆———☆, complete system in which vitamin K_1 is replaced by warfarin (5 mM); ●———●, microsomal proteins alone; *———*, decarboxyfactor II alone.

TABLE III

STIMULATION AND INHIBITION OF FACTOR II SYNTHETASE AND CARBOXYLASE

Reaction mixtures were incubated for 1.5 h and the prothrombin synthesis was measured and expressed in U/ml and in pmol/ml. The calculation of pmol/ml is based on the assumption that 1 litre of normal bovine plasma (1 U/ml) contains 100 mg or 1.4 μmol of factor II [2]. The $^{14}CO_2$ incorporation of $BaSO_4$ adsorbable counts is expressed in dpm/ml and in pmol/ml. When vitamin K_1 was omitted, an equal volume of vitamin K solvents was added to the reaction mixtures.

Compound added to reaction mixture	Prothrombin synthesized		$^{14}CO_2$ incorporation	
	U/ml	pmol/ml	dpm/ml	pmol/ml
None	0.026	37.4	11 900	88
Vitamin K_1 (0.2 mM)	0.033	46.2	14 600	107
Warfarin (5 mM)	0.003	4.2	1 200	9
Marcoumar (5 mM)	0.003	4.2	1 600	12

already in the decarboxyfactor II converting enzyme system. Both reactions, synthetase and carboxylase, are completely inhibited by 5 mM warfarin (see also below).

Prothrombin synthesis and vitamin K_1

It is generally accepted that vitamin K_1 plays a role in the biosynthesis of factor II. Moreover, it was shown by Shah and Suttie [17] that this vitamin is

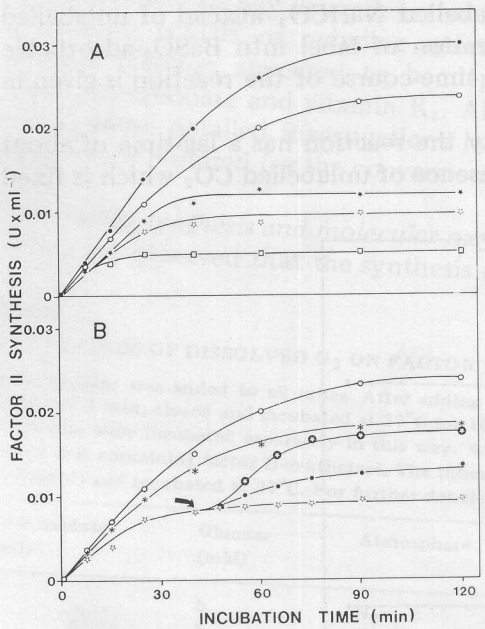

Fig. 6. Inhibition and stimulation of factor II synthetase. (A) Factor II synthesis in the presence (●———●) and absence (○———○) of 0.5 mM of vitamin K_1 and in the presence of 1 mM (★———★), 2 mM (☆———☆) and 10 mM (□———□) of warfarin. (B) Factor II synthesis in the absence of vitamin K_1 (○———○), in the presence of 2 mM of warfarin (☆———☆), and in the presence of 2 mM warfarin + 0.5 mM vitamin K_1 (*———*). After 40 min of incubation at 37°C the inhibited reaction mixture was reactivated (arrow) by adding either 0.5 mM of vitamin K_1 (●———●) or 0.15 mM of vitamin K_1 (●———●).

required during the carboxylation of rat decarboxyprothrombin. In our system we observed factor II synthesis in the absence of vitamin K_1. As is shown in Table III, the addition of vitamin K_1 to the reaction mixture increases the factor II synthesis only to a small extent. This is to be expected because we used normal healthy cows for the preparation of the microsomal proteins, so vitamin K may still be present in the enzyme system. We therefore tried to inhibit the reaction with known vitamin K inhibitors such as warfarin and marcoumar. In Table III it is shown that these inhibitors are able to decrease both, the factor II synthetase and the carboxylase activity in parallel.

A time-curve of the inhibition of factor II synthetase by warfarin is shown in Fig. 6A. Up to about 10 min the reaction rate is independent of the presence of inhibitor, thereafter the reaction is strongly inhibited and after 40 min no residual factor II synthesis occurs. After this period of time the activity of the inhibited system can be partially restored by adding vitamin K_1 (Fig. 6B). A lag-time of about 10 min was observed before the reaction was started again.

(D) Feed-back inhibition of prothrombin synthesis

Looking at the time-course of factor II synthesis (Figs. 5 and 6) we see that the increase of factor II activity gradually slows down and finally stops after approximately 90 min. As only 5% of the added precursor is consumed at this stage, the question arose: why does the reaction stop? The answer was found, when we added purified factor II to the reaction mixture, prior to incubation. The synthesis of factor II, and in parallel, the incorporation of $^{14}CO_2$ label, stops immediately after adding 0.025 U/ml purified factor II. When less factor II is added, the ratio between factor II and decarboxyfactor II determines the amount of additionally synthesized factor II.

Discussion

In our efforts to develop a bovine-liver cell-free system in which prothrombin is synthesized from its precursor we isolated a microsomal protein fraction with the ability to decrease the clotting time of a dilution of decarboxyfactor II from 200 to about 60 s. After we had proven that this reduction of the clotting time was not an artifact, caused by the generation of thrombin (e.g. by a certain proteolytic activity of the microsomal proteins) we tried to visualize the newly formed factor II. This can be done by kinetic analysis (Fig. 2) and by means of polyacrylamide gel electrophoresis (Fig. 4). With the aid of the latter technique we were able to show that previously labelled decarboxyfactor II is converted into factor II, so it is clear that the externally added precusor is used in this reaction. The enzyme(s), showing this activity we called factor II synthetase. Bovine factor II synthetase is probably similar to the enzyme denoted carboxylase, which is isolated from vitamin K-deficient rat livers, as described by several authors [3—8]. In the rat system the factor II synthetase activity is related to the incorporation of $^{14}CO_2$ into $BaSO_4$ adsorbable proteins (carboxylase activity). The experiments in section C show that in this respect the bovine system is quite similar to the rat system. It should be noted that each factor II molecule contains 10 γ-carboxyglutamic acid residues so we expect the $^{14}CO_2$ incorporation to be 10 times higher than the prothrombin

synthesis (on pmol basis). The fact that we observed a $^{14}CO_2$ incorporation of only 3 pmol per pmol of synthesized factor II may be due to the presence of dissolved (unlabelled) CO_2 gas in our buffers. Two observations argue in favor of this explanation: (a) the synthesis of factor II proceeds without addition of any $NaHCO_3$, although $NaH^{14}CO_3$ is incorporated, if added, and (b) the amount of dpm incorporated into $BaSO_4$ adsorbable proteins varied somewhat from one experiment to the other although the generated factor II activity was fairly constant in all our experiments. Whether the carboxylation of decarboxy-factor II is the only step required for its conversion into factor II remains to be investigated. In fact, in contrast to the rat system, bovine carboxylase (factor II synthetase) lends itself extremely well for investigations of the precise reaction mechanism of factor II synthetase, because the enzyme and the substrate (decarboxyfactor II) can be purified separately and analyzed before and after reaction. The product of the reaction (factor II) can be isolated and compared with decarboxyfactor II and normal factor II.

The main difference between this study and previous ones on rat carboxylase lies in the fact that we purify the enzyme in a low molecular weight form from non-deficient animals, and free from its substrate. The rat carboxylase is obtained from vitamin K deficient rats, in which prothrombin precursors are accumulated in the liver and in which the carboxylase activity is nihil. Therefore rat carboxylase is highly dependent on the addition of vitamin K. On the other hand, bovine factor II synthetase is isolated from the livers of normal healthy cows (purification about 10 000 times). In these livers and probably also associated with the enzyme itself, vitamin K is still present in normal amounts. Therefore addition of the vitamin causes only a small increase in the synthesis of factor II. Nevertheless, we conclude that factor II synthetase requires vitamin K for its proper action, because inhibitors of vitamin K such as warfarin were able to block the generation of factor II activity and the incorporation of $^{14}CO_2$. In agreement with the observations of Esmon and Suttie [15] and Helgeland [5] we found that warfarin is not a good inhibitor. Rather large amounts (5 mM) were required to block all factor II synthesis. This is probably due to the fact that vitamin K_1 readily dissolves in non-polar solvents and warfarin not. As vitamin K_1 must be associated to carboxylase (factor II synthetase), the binding place is probably apolar. In contrast to vitamin K, warfarin is water-soluble, which means that it requires a polar solvent, and will hardly bind to the vitamin K binding site in the carboxylase enzyme system. When the appropriate amount of warfarin is added, however, no factor II is synthesized anymore. At that moment the enzyme system becomes highly vitamin K_1 dependent, which is shown in Fig. 6B.

When we try to compare our results with those obtained with the rat system, we are hampered by the fact that the conclusions of various authors are at variance with each other. Our result are closely similar to those obtained by Lowenthal and Jaeger [8], which means that our system does not require ATP [5,17], NADH [4,5], or a reducing agent [4,5,8]. Of course it is possible that several of these cofactors, like vitamin K_1, are still bound to the enzyme. The fact that limiting amounts of synthetase are required in order to measure the dependency of the system on vitamin K and oxygen is interesting in this respect. Only a fraction of the total enzyme population might be complexed

with one or more cofactors (vitamin K_1) or gases (CO_2, O_2). When a limiting amount of synthetase is incubated with an excess of decarboxyfactor II, addition of these cofactors may increase the reaction rate whereas an excess of synthetase would be inhibited by its own product (factor II) before one of the cofactors has run out. It is obvious that further purification of factor II synthetase is required in order to perform more detailed studies on the mechanism of the conversion of decarboxyfactor II into factor II.

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