A New Carboxylation Reaction

THE VITAMIN K-DEPENDENT INCORPORATION OF HCO₃⁻ INTO PROTHROMBIN*

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CHARLES T. ES MON,‡ JAMES A. SADOWSKI, AND JOHN W. SUTTIE§

From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

The bovine plasma zymogen prothrombin contains a number of γ-carboxyglutamic acid residues which are not found in an abnormal prothrombin produced when cattle are given the vitamin K antagonist dicoumarol. These modified glutamic acid residues appear to be formed post-translationally by a reaction which requires vitamin K. It has been shown that postmitochondrial supernates from vitamin K-deficient rats incorporate added HCO₃⁻ into microsomal proteins upon the addition of vitamin K. This incorporation is dependent upon the presence of the prothrombin precursor in the microsomal preparations, and upon factors which are present in the postmicrosomal supernatant. Most of the radioactive protein which can be obtained from the microsomal pellet by extraction with 0.25% Triton X-100 has been identified as prothrombin and it can be shown that all of the radioactivity is in the amino-terminal activation fragment of prothrombin. This portion of the protein has previously been shown to contain the γ-carboxyglutamic acid residues. Hydrolysis of the purified radioactive prothrombin resulted in a loss of 50% of the radioactivity and subsequent chromatography of the amino acid hydrolysate demonstrated that the remaining radioactivity was entirely in glutamic acid. These results are consistent with the hypothesis that all of the HCO₃⁻ was incorporated into the carboxyl groups of γ-carboxyglutamic acid residues.

Vitamin K is required for the synthesis of four blood-clotting zymogens: prothrombin, factor X, factor IX, and factor VII. The vitamin appears to function post-translationally (1) by modifying a precursor protein. This precursor has been identified (2) in microsomal preparations from anticoagulant-treated rats, and has now been isolated and partially characterized (3, 4). Although it is inactive in prothrombin assay systems, this precursor is activated to thrombin by several snake venoms (4), suggesting that the vitamin K-dependent modification is required for the physiological activation of prothrombin rather than in the activity of the thrombin generated.

The liver precursor is in many ways similar to the biologically inactive form of prothrombin (abnormal prothrombin) which appears in the plasma of the bovine following administration of the vitamin K antagonist, dicoumarol. Unlike prothrombin, the abnormal prothrombin does not bind Ca²⁺ ions (5, 6) and this defect is presumably responsible for its failure to activate in the bioassay. As it was possible to isolate (7) a low molecular weight calcium-binding peptide from normal, but not abnormal, prothrombin, it appeared that the vitamin-dependent alteration involved a chemical modification of a specific region of the polypeptide chain.

The chemical difference in the abnormal and normal prothrombin has been shown by Stenflo et al. (8) to be the presence of a number of γ-carboxyglutamic acid residues in normal prothrombin but not in abnormal prothrombin. This residue has also been identified by Nelsestuen et al. (9) and the characterization has been confirmed by Magnusson et al. (10). These observations suggest that vitamin K functions as part of the metabolic system responsible for the γ-carboxylation of specific glutamic acid residues of the liver prothrombin precursor.

We have recently described (11) an in vitro system which converts the rat liver microsomal precursor protein to biologically active prothrombin in response to the addition of vitamin K. This system should serve to test the hypothesis that the vitamin K-dependent, post-translational modification of the precursor involves the carboxylation of glutamic acid residues.

MATERIALS AND METHODS

Treatment of Animals—Male 250-g rats of the Holtzman strain were housed in coprophagy-preventing cages (12) and fed a diet low in vitamin K (13) for 7 to 8 days. The animals were fasted for 18 hours prior to killing.

Incubation Conditions—Livers from vitamin K-deficient rats were homogenized in 0.25 M sucrose, 0.025 M imidazole-HCl, pH 7.2 (2 ml/g of liver), and the homogenate was centrifuged at 12,800 × g for 10 min.
to obtain a postmitochondrial supernatant which was incubated under the conditions previously described (11) for the in vitro synthesis of prothrombin. Cycloheximide (100 μg/ml) and H^14CO_3^- (5 μCi/ml of approximately 59.5 mCi/mmol of Na^14CO_3 (Amersham/Searle)) were included in the incubation medium, and prothrombin synthesis was initiated by the addition of vitamin K, (20 μg/ml). After incubation for 15 min at 37°C, the suspension was cooled, and the microsomes were removed by centrifugation at 105,000 × g for 60 min. The microsomal pellet was extracted with calcium-free Krebs-Ringer bicarbonate buffer containing 0.015 M potassium oxalate and 0.25% Triton X-100. The Triton X-100 extract was adsorbed with BaSO_4 (25 mg/ml). The BaSO_4 was removed by centrifugation and the pellet was washed and eluted as described earlier (14).

### Determination of Radioactivity
Bovine serum albumin (2 mg) was added to 0.2 ml of the Triton X-100 microsomal extract, or 0.2 ml of the BaSO_4 adsorbed extract and the proteins were precipitated by the addition of 5 ml of 10% trichloroacetic acid. The BaSO_4 eluate (0.1 ml) was precipitated after the addition of 4 mg of albumin. The precipitates were washed at 4°C for 30 min and then collected by centrifugation at 3000 × g for 20 min. The supernatant was discarded, the pellet was dissolved in 1 ml of 0.2 M NaOH, and precipitated with 5 ml of 10% trichloroacetic acid. After 30 min at 4°C, the suspension was centrifuged as before, the supernatant was discarded, and the pellet was dissolved in 1 ml of NCS (Amersham-Searle) before transferring the sample to 10 ml of Econofluor (New England Nuclear). The distribution of radioactivity in sodium dodecyl sulfate electrophoretic gels was determined following combustion of the dried gel slices. Radioactivity was determined.

### Isolation of Clotting Factor and Bioassay
Factor Xa and factor V were prepared as described previously (15). Phospholipid was fractionated II prepared as described by Folch (16).

Prothrombin was assayed by the two-stage method of Ware and Seegers as modified by Shapiro and Waugh (17). Factor X was assayed by the one-stage method of Bachman et al. (18).

### RESULTS

**Correlation of Prothrombin Synthesis and Carboxylation**—The possible existence of a vitamin K-dependent protein carboxylation and prothrombin synthesis was investigated by incubating (11) postmitochondrial supernatants prepared from livers of vitamin K-deficient rats in the presence of vitamin K and in the absence of vitamin K and in the presence of both vitamin K and a vitamin K antagonist, chloro-K. During these incubations, de novo protein synthesis was inhibited with cycloheximide. The data (Table I) indicated that vitamin K was required for optimal H^14CO_3^- incorporation into the Triton X-100 extractable microsomal proteins as well as for prothrombin synthesis. Furthermore, chloro-K, which inhibited prothrombin synthesis, also inhibited H^14CO_3^- incorporation.

| Treatment | Triton X-100 microsomal extract | BaSO_4 adsorbed Triton X-100 extract | BaSO_4 Eluate |
|-----------|-------------------------------|-----------------------------------|-------------|
| Control (no added vitamin K) | 1,600 | 16 | >50 | 1.8 |
| + Vitamin K | 19,512 | 82 | 60 | 4,928 | 51 |
| + Vitamin K + chloro-K | 7,244 | 30 | 51 | 1,035 | 6.3 |

*All values are corrected to 20 ml (equivalent to 8 g of liver).*

Determined by the two-stage method of Ware and Seegers as modified by Shapiro and Waugh (17).

20 μg/ml of Triton X-100 extract with BaSO_4 indicated that a high percentage of the radioactivity was incorporated into the BaSO_4 adsorbable proteins. Adsorption of the extract with BaSO_4 removed 56% of the radioactive protein from the Triton X-100 microsomal extract and elution from the BaSO_4 resulted in an increase in specific activity of 14C protein from 65 dpm/μg to 44,390 dpm/μg in the BaSO_4 eluate. These results suggest a highly specific incorporation of bicarbonate into the vitamin K-dependent clotting proteins. The nature of the non-BaSO_4 adsorbable proteins has not yet been determined.

If bicarbonate was being incorporated primarily into protein precursors of the vitamin K-dependent clotting factors, then conditions which prevent synthesis of the active clotting factors from these precursors should also prevent H^14CO_3^- incorporation into protein. Previous experiments in our laboratory have indicated that microsomal prothrombin synthesis requires some factor(s) present in the soluble portion of the cell. When microsomes were prepared from the postmitochondrial supernatant and resuspended in buffered sucrose without the addition of cytosol (Table II, A), both H^14CO_3^- incorporation and prothrombin synthesis were inhibited.

If the protein carboxylation observed does represent the specific postribosomal incorporation of H^14CO_3^- into the vitamin K-dependent clotting factors, it should require the presence of the precursors of these proteins. Microsomes from normal, vitamin K-sufficient rats contain very little prothrombin precursor, when compared to microsomes from vitamin K-deficient rats, and because of the low precursor level, microsomes from these rats would be expected to incorporate less H^14CO_3^- than microsomes from the vitamin K-deficient rats. When the extent of in vitro carboxylation was compared to systems derived from normal or vitamin K-deficient rats (Table II, B), the data indicated that the system prepared from vitamin K-deficient rats would be expected to incorporate less H^14CO_3^- than the system prepared from normal rats.
normal rat livers incorporated less bicarbonate than that prepared from vitamin K-deficient rats.

Although the administration of Warfarin in vivo blocks prothrombin synthesis, the in vitro synthesis of prothrombin in liver microsomes is not significantly inhibited by Warfarin (11). However, as indicated in Table II, B, mitochondrial supernatants derived from Warfarin-treated rats form less prothrombin in vitro than do supernates from vitamin K-deficient rats. A further correlation between prothrombin synthesis and carboxylation is provided by the observation (Table II, B) that H$^{14}$CO$_3^{-}$ incorporation as well as prothrombin synthesis is reduced in systems derived from Warfarin-treated rats when compared to systems derived from vitamin K-deficient rats. These data (Table II, B) also indicate that prothrombin synthesis under these conditions may have been inhibited more than H$^{14}$CO$_3^{-}$ incorporation into the Triton extract, and suggest that there may be enhanced carboxylation of some protein other than prothrombin in the presence of Warfarin. The amount of radioactivity which was incorporated in the cytosol was investigated as well as that incorporated into the microsomal pellet. Approximately the same amount of radioactivity was incorporated into the cytosol proteins as into the microsomal extract from the vitamin K-treated system. The amount of radioactivity in this fraction was, however, not dependent on the presence or absence of the vitamin in the incubation medium.

Identification of Prothrombin as Radioactive Protein—The degree of correlation between the radioactive proteins of the BaSO$_4$ eluate and the vitamin K-dependent clotting proteins was examined by ion exchange chromatography (Fig. 1). Most of the protein in the eluate eluted before any of the radioactivity, but a small amount of protein eluted at the same position as prothrombin which was detected by both two-stage activity and venom activation. Prothrombin activity and radioactivity appeared to co-chromatograph except for a reproducible shoulder of radioactivity on the trailing edge of the prothrombin peak. This fraction of the radioactivity elutes in the position expected for rat factor X, but bioassay failed to detect any factor X activity. The similar chromatographic behavior of the prothrombin activity and the radioactivity, and the small amount of protein in this region of the chromatogram suggests that much of the radioactivity has been incorporated into prothrombin.

The properties of radioactive protein which eluted from the QAE (quaternary aminoethyl) column with prothrombin were studied further by sodium dodecyl sulfate gel electrophoresis. In this electrophoretic system, rat prothrombin has an apparent molecular weight of 85,000. When the chromatographically purified radioactive protein was subjected to electrophoresis under these conditions, most of the radioactivity was associated with the gel slice corresponding to this molecular weight (Fig. 2). Activation of prothrombin with factor Xa leads to formation of thrombin and two large activation peptides (fragment 1, $M_r = 23,000$ and fragment 2, $M_r = 13,000$) (15, 20). All of the $\gamma$-carboxyglutamic acid residues of prothrombin are reported to reside in the amino-terminal activation peptide, fragment 1 (10). Therefore, if the H$^{14}$CO$_3^{-}$ was specific-

![Fig. 1 Chromatography of the proteins of the BaSO$_4$ eluate on QAE (quaternary aminoethyl)-Sephadex Q-50. Radio-labeled proteins were prepared from an incubation of 80 ml of postmitochondrial supernatant as described under "Materials and Methods". The BaSO$_4$ cluate was dialyzed against 0.05 M NH$_4$Cl, 0.02 M Na$_2$ citrate, 0.001 M benzamidine-HCl, 0.05 M imidazole-HCl, pH 7.8, and then chromatographed on a QAE-Sephadex Q-50 column (0.9 x 28 cm) equilibrated in this same buffer. The column was developed at room temperature with a linear gradient (80 ml total) of NH$_4$Cl (0.05 to 0.35 M). Samples of the column fractions (0.1 ml) were added to 10 ml of Aqueus, and the radioactivity determined with a counting efficiency of 80%. O--O, absorbance 280 nm; A—A, prothrombin activity by two-stage assay; o—o, thrombin activity following activation with Echis carinatus venom; o—o, radioactivity (counts/min/0.1 ml).]

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**Table II**

| Treatment                  | Triton X-100 microsomal extract | BaSO$_4$ Eluate |
|----------------------------|-------------------------------|----------------|
|                            | Radio-activity: total dpm     | Radio-activity: total dpm |
|                            | Prothrombin: total units      | Prothrombin: total units       |
| A                          |                                |                                |                |
| Control                    | 1,500                          | 4                             | 333            | 0.8           |
| + Vitamin K                | 16,240                         | 78                            | 9,371          | 28.8          |
| + Vitamin K – cytosol      | 3,760                          | 6                             | <50            | 0.8           |
| B                          |                                |                                |                |
| Control                    | 3,960                          | 22                            | 167            | 4.5           |
| + Vitamin K                | 21,480                         | 74                            | 3,770          | 28.9          |
| Normal rats + vitamin K    | 8,540                          | 48                            | 866            | 22.9          |
| Warfarin-treated rats +    | 16,400                         | 48                            | 2,636          | 19.5          |
| vitamin K                  |                                |                                |                |
| Warfarin-treated rats –    | 2,400                          | 20                            | 26             | <1            |

* All values are corrected to 20 ml (equivalent to 8 g of liver).

*Postmitochondrial supernatant sampled from the same pooled preparation contained 43 units of prothrombin when treated as above but without incubation or addition of vitamin K.

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**Fig. 1 Chromatography of the proteins of the BaSO$_4$ eluate on QAE (quaternary aminoethyl)-Sephadex Q-50. Radio-labeled proteins were prepared from an incubation of 80 ml of postmitochondrial supernatant as described under "Materials and Methods". The BaSO$_4$ cluate was dialyzed against 0.05 M NH$_4$Cl, 0.02 M Na$_2$ citrate, 0.001 M benzamidine-HCl, 0.05 M imidazole-HCl, pH 7.8, and then chromatographed on a QAE-Sephadex Q-50 column (0.9 x 28 cm) equilibrated in this same buffer. The column was developed at room temperature with a linear gradient (80 ml total) of NH$_4$Cl (0.05 to 0.35 M). Samples of the column fractions (0.1 ml) were added to 10 ml of Aqueus, and the radioactivity determined with a counting efficiency of 80%. O--O, absorbance 280 nm; A—A, prothrombin activity by two-stage assay; o—o, thrombin activity following activation with Echis carinatus venom; o—o, radioactivity (counts/min/0.1 ml).**
cally incorporated into prothrombin, activation should generate a new band of radioactivity corresponding to the molecular weight of fragment 1 (23,000). After activation with factor Xa (Fig. 2B), a new radioactive band does appear which electrophoreses in the fragment 1 region. Thrombin cleaves prothrombin to form fragment 1 and a M, = 50,000 activation intermediate, intermediate 1 (15, 20). Thrombin proteolysis of the radioactive protein again resulted in the movement of the radioactivity to a portion of the gel which corresponds to the mobility of fragment 1. These data suggest that H14CO3− was specifically incorporated into the fragment 1 region of prothrombin.

Identification of γ-Carboxyglutamic Acid as the Labeled Amino Acid—It has been shown (8, 9) that acid hydrolysis of a peptide containing γ-carboxyglutamate residues results in decarboxylation of this residue to yield glutamic acid. If the H14CO3− incorporation observed was into one of the two carboxyl groups of γ-carboxyglutamic acid, this residue would be subjected to random decarboxylation by acid hydrolysis, and a 50% loss of the label should be observed. The data in Table III demonstrate that about 53% of the radioactivity associated with the prothrombin isolated from the in vitro system was lost following hydrolysis in 6 M HCl. Further, ion exchange chromatography of this hydrolysate revealed the presence of only one 14C peak in the eluate from the amino acid analyzer (Fig. 3), which corresponded to the elution position of glutamic acid, the decarboxylation product of γ-carboxyglutamic acid. Addition of [3H]glutamic acid and [14C]-norleucine to the sample before hydrolysis resulted in a co-elution of 3H and 14C in the glutamic acid position of the chromatogram which was verified by its position on the chromatogram relative to the standard norleucine.

### DISCUSSION

The data presented above are consistent with the hypothesis that the vitamin K-dependent step in prothrombin synthesis involves the carboxylation of glutamyl residues in a liver prothrombin precursor protein. There was a rapid, vitamin K-dependent incorporation of H14CO3− into protein, even when de novo protein synthesis was blocked. A significant amount of this radioactivity was associated with the vitamin K-dependent clotting factors, primarily prothrombin, and the radioactivity incorporated into prothrombin was located exclusively in the NH2-terminal activation fragment (fragment 1) of prothrombin. Acid hydrolysis of the in vitro labeled prothrombin resulted in a loss of 50% of the radioactivity, and the remaining radioactivity was associated with glutamic acid residues, which would be consistent with the presence of radioactivity in the carboxyl groups of γ-carboxyglutamyl residues.

### TABLE III

| Experiment | % 14C lost |
|------------|-----------|
| A          | 52.4      |
| B          | 52.5      |
| C          | 54.7      |

**Fig. 2.** Sodium dodecyl sulfate electrophoresis of the chromatographed prothrombin preparation. Fractions 32 and 33 (Fig. 1) were pooled and dialyzed against 0.05 M NaCl, 0.05 M imidazole-HCl, pH 7.8. Samples were removed and treated as follows: A, no treatment; B, activation with factor Xa, 0.2 μg/mL; factor V, 2.5 μg/mL; phospholipid, 20 μg/mL; Ca2+, 10 mM, for 5 min at 37°C; C, proteolysis with thrombin (7 μg/mL) for 1 hour at 37°C. The reactions were stopped by adding sodium dodecyl sulfate to a final concentration of 1%. Electrophoresis was performed by the method of Laemmli (19). The distribution of radioactivity was determined following combustion of dried gel slices. The mobility of rat fragment 1 and rat prothrombin as indicated by the arrows was determined with purified proteins run on separate gels.

**Fig. 3.** Chromatography of [14C]prothrombin hydrolysate. Purified 14C-labeled prothrombin (Fig. 1) was hydrolyzed in 6 N HCl and the hydrolysate chromatographed (see “Materials and Methods”). [3H]-Glutamic acid and [14C]-norleucine were added as standards. Recovery of radioactivity applied to the column from the 14C-labeled protein hydrolysate was 99%, from the [3H]glutamic acid was 99%, and from the [14C]norleucine was 97%. O—O, 14C activity; O—O, 3H activity.
These data would appear to offer final evidence that the vitamin K-dependent step in prothrombin synthesis is not at the level of de novo synthesis. Although arguments have been made (21, 22) that much of the previous data supporting a precursor theory of vitamin action (1, 20, 24) could also fit a de novo synthesis theory, these data cannot. The specificity of the reaction, the large amount of prothrombin synthesized in this cycloheximide-blocked system, and the failure to observe carboxylation when the incubated micromoles were obtained from normal rats and did not contain precursor are all inconsistent with the de novo synthesis theory.

Girardot et al. (25) have recently reported the in vivo incorporation of $^{14}$ CO$_3^-$ into rat prothrombin as proof of glutamyl carboxylation. Although such incorporation must occur, the data they have presented do not support the conclusions. The specific radioactivity of the isolated prothrombin was only 3 to 7 times that of the total plasma proteins, and it was claimed that this radioactivity was associated with an acidic amino acid which could be obtained by enzymatic digestion. It was further claimed that all of the radioactivity was lost following acid hydrolysis. Other workers (7, 10, 26, 27) have reported extreme difficulty in obtaining complete enzymatic hydrolysis of peptides containing γ carboxyglutamic acid, and have found (8, 9) as shown here, that decarboxylation by strong acid will result in formation of boxyglutamic acid, and have found (8, 9) as shown here, that decarboxylation by strong acid will result in formation of glutamic acid which would contain approximately 50% of the radioactivity. It would also be expected that in the absence of an inhibitor of protein biosynthesis there would be a substantial labeling of arginine, glutamic acid, and aspartic acid following the administration of $^{14}$ CO$_3^-$ (28). We have been able to demonstrate a vitamin K-dependent incorporation of $^{14}$ CO$_3^-$ into BaSO$_4$-absorbable proteins in vivo, but the extent of labeling is at least an order of magnitude lower than that reported by Girardot et al. (25). There is a strong possibility that what was observed was largely a nonspecific incorporation of $^{14}$ CO$_3^-$ into plasma proteins, rather than the specific carboxylation reaction.

These data do not provide any information regarding the molecular action of vitamin K in this carboxylation. Neither do they establish that this vitamin K-dependent reaction is the only modification of the precursor molecule. They do, however, establish this system as a new physiologically important carboxylation reaction, and provide a tool to study the molecular action of the vitamin.

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