β-Hydroxyaspartic acid in vitamin K-dependent plasma proteins from scorbutic and warfarin-treated guinea pigs

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β-Hydroxyaspartic acid is a rare amino acid, present in all vitamin K-dependent plasma proteins except prothrombin, and is formed by a post-translational hydroxylation of aspartic acid. We have now investigated whether this hydroxylation, like that of proline in collagen, is vitamin C-dependent. The vitamin K-dependent plasma proteins were isolated from normal and scorbutic guinea pig plasma by barium citrate adsorption and the β-hydroxyaspartic acid content was determined. Compared with normal animals, scorbutic animals showed no significant reduction of β-hydroxyaspartic acid content. In warfarin-treated animals there was a decreased content of both β-hydroxyaspartic acid and γ-carboxyglutamic acid in the barium citrate adsorbed fraction. It was concluded that the post-translational hydroxylation of aspartic acid is unlikely to be vitamin C-dependent.

Blood coagulation *γ-Carboxyglutamic acid* *Hydroxylation* *Post-translational modification*
Scurvy *Vitamin C* *Vitamin K* *Warfarin*

1. INTRODUCTION

β-Hydroxyaspartic acid is a rare amino acid recently shown to be present in several of the vitamin K-dependent plasma proteins [1–5]. Bovine protein C [1,3,4], bovine and human factor X [2,4,5], bovine and human factor IX [3,4,5], bovine factor VII [3] and bovine protein Z [4] all contain approx. 1 mol β-hydroxyaspartic acid per mol protein, and protein S contains 2–3 mol β-hydroxyaspartic acid per mol protein [4]. Prothrombin, however, the most abundant vitamin K-dependent plasma protein, lacks β-hydroxyaspartic acid both in the cow and the human [3,4]. The γ-carboxyglutamic acid-containing bone protein, which is vitamin K-dependent, also lacks β-hydroxyaspartic acid [4]. Analysis of a number of common non-vitamin K-dependent proteins suggests that β-hydroxyaspartic acid is confined to γ-carboxyglutamic acid-containing proteins [4].

Bovine protein C has β-hydroxyaspartic acid in position 71 of its light chain [1]. According to sequence homology, β-hydroxyaspartic acid occupies a corresponding position in human [2] and bovine factor X [5], and in human [3] and bovine factor IX [3,5].

The nucleotide sequences coding for human factor IX [6,7] and bovine factor IX [8] have been determined. They both have a triplet coding for aspartic acid at the position for the β-hydroxyaspartic acid residue, proving that β-hydroxyaspartic acid is formed by a post-translational hydroxylation of aspartic acid. Post-translational hydroxylation of proteins, e.g., formation of hydroxyproline and hydroxylysine in collagen, has long been known to depend on vitamin C. Lack of vitamin C causes scurvy with decreased formation of secreted collagen, which is thought to be due to an inadequate hydroxylation and consequent increased intracellular degradation of the newly synthesized procollagen polypeptide chains [9]. The resulting scarcity of extracellular collagen is regarded as a major cause of fragility in the connective tissue, and of the bleeding and impaired wound healing in scorbutic individuals.

Bleeding is one of the most prominent signs in

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the adult scorbutic patient [10], and several attempts have been made to demonstrate a defect in the hemostatic system [11], but with questionable success [12]. The finding of a post-translationally hydroxylated amino acid in several of the coagulation factors, and the possibility that the hydroxylation of this amino acid is vitamin C-dependent, suggest an attractive hypothesis for the explanation of the bleeding in scurvy: i.e., inadequate formation of β-hydroxyaspartic acid in the coagulation factors results in a deficient hemostatic function.

To test this hypothesis, proteins containing γ-carboxyglutamic acid were isolated by barium citrate adsorption from the plasma of normal and scorbutic guinea pigs, and the β-hydroxyaspartic acid content in the isolated fraction was determined. Here we report the results of these experiments and the finding of normal amounts of β-hydroxyaspartic acid in the barium citrate adsorbed fraction from scorbutic animals. In a control experiment, guinea pigs treated with warfarin showed an expected decrease of the γ-carboxyglutamic acid content with a parallel decrease of β-hydroxyaspartic acid.

2. MATERIALS AND METHODS

Reid-Briggs guinea pig diet without ascorbic acid was obtained from USB (Cleveland, OH), ascorbic acid from Merck (Darmstadt), and sodium warfarin (Waran®) from Nyegaard & Co. (Oslo). β-Carboxyglutamic acid was prepared as in [13]. Bovine prothrombin fragment 1 was isolated from bovine prothrombin as in [14]. Immediately after ^{125}I labeling by the lactoperoxidase method [15], it was subjected to gel chromatography on a column (1.5 × 5 cm) of Sephadex G-25 (PD-10 column, Pharmacia, Uppsala) at 20°C in 50 mM Tris–HCl buffer (pH 7.4). Before use 1 ml of a solution of bovine serum albumin in the Tris buffer (1 g/l) was passed through the column. The specific activity of the labeled fragment 1 was approx. 9.5 μCi/μg. It was kept in the chromatography buffer at −20°C and used within 34 days after labeling.

2.1. *Experimental scurvy*

Guinea pigs weighing 380–600 g were randomly divided into two groups, both of which were fed only Reid-Briggs ascorbic acid-deficient diet and supplied with tap water ad libitum. For one group (control) the water contained ascorbic acid (1 g/l), while for the second group (experimental) no additions were made to the water. The animals were weighed daily. At the end of the experiment, when scurvy had developed in the experimental group, blood was collected from both control and experimental animals by cardiac puncture. The blood was drawn into Vacutainer® test tubes (Becton Dickinson & Co., Orangeburg, NY), containing 1/10 vol. of 0.13 M sodium citrate. The animals were then killed and dissected to reveal any hemorrhage. The blood was centrifuged within an hour of collection and the plasma stored at −70°C.

2.2. *Warfarin treatment*

Guinea pigs weighing 400–600 g and kept on ordinary guinea pig diet were each injected intraperitoneally with sodium warfarin (1 mg/kg body wt) dissolved in 1 ml saline, control animals receiving saline only. Blood was collected 24 h later and plasma separated as described in section 2.1.

2.3. *Isolation of proteins containing γ-carboxyglutamic acid by barium citrate adsorption*

To 1.0 ml of citrated plasma was added 10 μl of a solution of ^{125}I-prothrombin fragment 1 (approx. 0.1 μCi), and barium citrate was then precipitated in the sample by the addition of 80 μl of 1 M $BaCl_2$. After standing for 1 h at 20°C the precipitate was sedimented by centrifugation at 5000 × *g* for 5 min, and the supernatant discarded. The precipitate was washed twice by suspension in 200 μl of 0.15 M NaCl, followed by centrifugation as above.

The washed sediment was dissolved in 400 μl of 0.2 M sodium EDTA (pH 7.4) and then dialyzed against 0.1 M NH_4HCO_3 at 20°C for 24 h using Spectrapore membrane tubing no.3 (Spectrum Medical Industries Inc., Terminal Annex, Los Angeles, CA). After dialysis, recovery was estimated from the radioactivity remaining in the dialyzed sample. Radioactivity was measured in a Nuclear Electronics NE1600 gamma spectrometer.

2.4. *Amino acid analysis*

Each dialyzed sample was divided into two portions: 4/5 of the sample was subjected to acid hydrolysis to measure β-hydroxyaspartic acid and ordinary amino acids; 1/5 of the sample was subjected to alkaline hydrolysis to determine the γ-carboxyglutamic acid content. Prior to hydrolysis the samples were lyophilized in the hydrolysis tube. Acid hydrolysis, ordinary amino acid analysis and measurement of β-hydroxyaspartic acid, were performed as in [4] for purified proteins.

Alkaline hydrolysis was performed in 100 μl of 4 M LiOH at 110°C for 24 h in evacuated alkali-resistant glass tubes, with 10.4 nmol β-carboxyglutamic acid added as an internal standard [13]. A standard containing 5.0 nmol γ-carboxyglutamic acid and 10.4 nmol β-carboxyglutamic acid was treated in the same way as the samples. The alkaline hydrolysates were neutralized and Li^+ precipitated by adding 20 μl of 6.67 M H_3PO_4. After centrifugation the supernatants were analyzed using the same chromatographic system as for β-hydroxyaspartic acid [4].

2.5. *Activated partial thromboplastin time (APT-time)*

Commercial reagents were used (APTT, General Diagnostics Automated APTT), and the assay performed according to the manufacturers' instructions.

2.6. *Statistical analysis*

Unpaired Student's *t*-test was used to compare means for groups of animals.

3. RESULTS

Guinea pigs kept on a vitamin C-deficient diet and without ascorbic acid supplementation of their drinking water all developed symptoms of scurvy after 2–3 weeks, whereas control animals kept on the same diet but with ascorbic acid added to their drinking water showed no evidence of scurvy. The symptoms included loss of body weight (fig.1), loss of hair, joint swelling and tenderness, petechial hemorrhage, and inability or unwillingness to stand up. Autopsy showed that all animals in the ascorbic acid-deficient group had periarticular hemorrhages whereas there were no pathological findings in the control group. Plasma coagulation, as determined by APT-time, did not differ significantly between the two groups ($p > 0.2$).

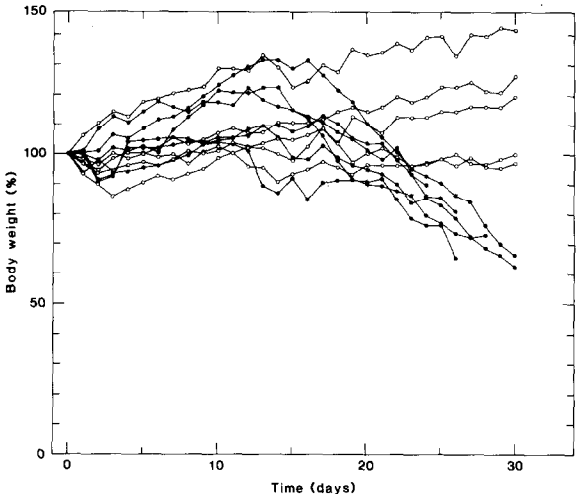

Fig.1. Body weight curves for guinea pigs on a vitamin C-deficient diet (●—●), and for guinea pigs on the same diet but with vitamin C added to their drinking water (○—○). The body weights are expressed as per cent of the weight at the start of the experiment.

Plasma proteins containing γ-carboxyglutamic acid were isolated by barium citrate adsorption and the β-hydroxyaspartic acid content was determined after acid hydrolysis. Typical chromatograms for the analysis of β-hydroxyaspartic acid are shown in fig.2. As noted with purified proteins [1,4], acid hydrolysis causes a partial racemization of β-hydroxyaspartic acid, which explains the appearance of some *threo*-β-hydroxyaspartic acid eluting at 60 min in the analyzer chromatogram (fig.2). There was no significant difference ($p > 0.2$) in β-hydroxyaspartic acid content between the scorbutic and the control group (table 1). γ-Carboxyglutamic acid in the isolated proteins was measured after alkaline hydrolysis with β-carboxyglutamic acid as an internal standard. On alkaline hydrolysis, the β-carboxyglutamic acid gives rise to two diastereomeric forms (fig.3). As with the β-hydroxyaspartic acid content and that of other amino acids (only that of leucine is shown in table 1), the yield of γ-carboxyglutamic acid did not differ significantly between the two groups (table 1).

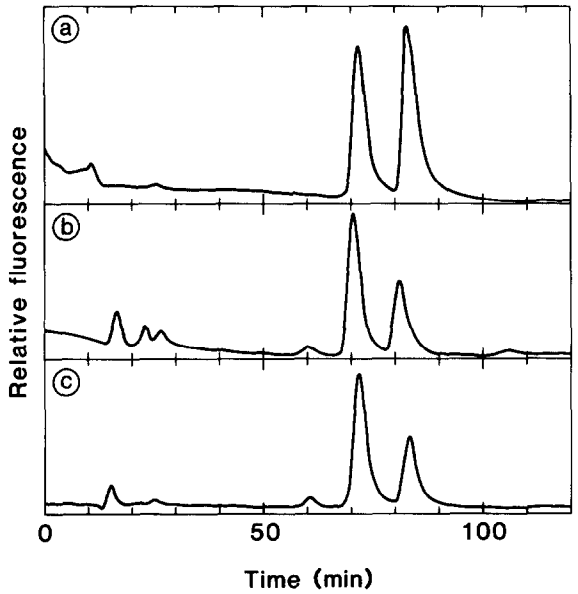

Fig.2. Analysis of β-hydroxyaspartic acid. (a) Standard mixture with 2.0 nmol each of γ-carboxyglutamic acid (elution time 72 min) and *erythro*-β-hydroxyaspartic acid (elution time 83 min). (b) Acid hydrolysate of barium citrate adsorbed proteins from 1.0 ml of plasma from guinea pigs on vitamin C-deficient diet but with ascorbic acid in their drinking water (control animals). (c) As b, but from guinea pigs without ascorbic acid in their drinking water (scorbutic animals). The peak at 60 min in b and c is *threo*-β-hydroxyaspartic acid. γ-Carboxyglutamic acid was added as an internal standard to the samples after hydrolysis [4].

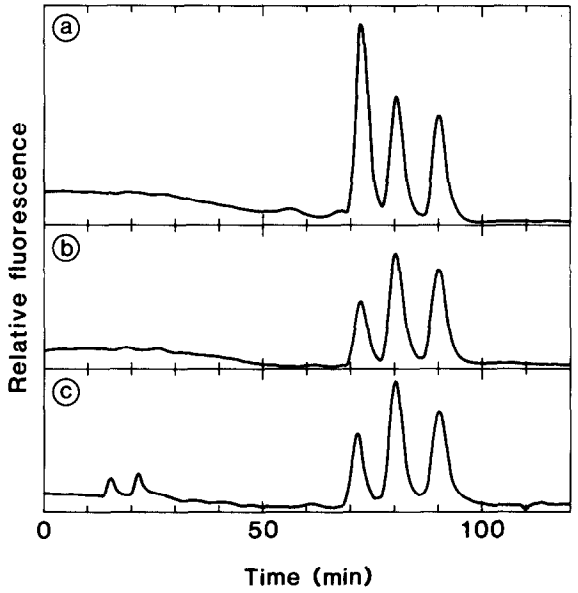

Fig.3. Analysis of γ-carboxyglutamic acid. (a) Alkaline hydrolysate of a standard mixture of 5.0 nmol γ-carboxyglutamic acid and 10.4 nmol β-carboxyglutamic acid. (b) Alkaline hydrolysate of barium citrate adsorbed plasma proteins from non-scorbutic animals. (c) As b, but from scorbutic animals. In b and c, 10.4 nmol β-carboxyglutamic acid was added as an internal standard to the sample before hydrolysis. γ-Carboxyglutamic acid elutes at 73 min and one of the two diastereomeric forms of β-carboxyglutamic acid at 80 min, the other at 90 min.

Table 1

Yields of amino acids from barium citrate-adsorbed plasma proteins in control and scorbutic guinea pigs

	Control animals	Scorbutic animals
No. of animals	5	6
Yield of amino acids (nmol/ml plasma)		
β-Hydroxyaspartic acid	2.2 ± 0.22	2.2 ± 0.12
γ-Carboxyglutamic acid	30 ± 1.2	33 ± 2.9
Leucine	400 ± 24	420 ± 46

The values, which are the arithmetic means ± SE, are expressed as nmol amino acid obtained from 1 ml plasma with correction for losses according to the recovery of ^{125}I-labeled bovine prothrombin fragment 1. β-Hydroxyaspartic acid is the sum of the *threo* and *erythro* forms

The recoveries of amino acids given in table 1 were corrected for procedural losses based on the recovery of ^{125}I-labeled prothrombin fragment 1, a correction open to question since, during the isolation, fragment 1 does not necessarily behave like other plasma proteins that contain γ-carboxyglutamic acid. However, the yield of fragment 1 did not vary much between samples (range 45–72%), nor did a comparison of the uncorrected yields show any significant difference between the control and scorbutic groups (not shown).

To evaluate the method used for measuring β-hydroxyaspartic acid and γ-carboxyglutamic acid, untreated guinea pigs were compared with animals given one large dose of the vitamin K-antagonist, warfarin, 24 h previously. In this experiment both the β-hydroxyaspartic acid and γ-carboxyglutamic acid yields were significantly reduced in the warfarin-treated animals ($p < 0.0001$) (table 2).

Table 2

Yields of amino acids from barium citrate-adsorbed plasma proteins in control and warfarin-treated guinea pigs

	Control animals	Warfarin-treated animals
No. of animals	5	5
Yield of amino acids (nmol/ml plasma)		
β-Hydroxyaspartic acid	2.0± 0.12	1.0± 0.02
γ-Carboxyglutamic acid	35 ± 1.2	15 ± 0.7
Leucine	370 ±13	340 ±24

The values are expressed as in table 1

4. DISCUSSION

Since β-hydroxyaspartic acid seems only to be present in the vitamin K-dependent plasma proteins, except prothrombin, and at most amounts to but a few residues in these proteins [4], its concentration in plasma is low. A sensitive method based on acid hydrolysis followed by cation-exchange chromatography at pH 2.0 with fluorimetric detection has been designed for measuring β-hydroxyaspartic acid in purified proteins [4]. As shown here, this method can be used for analysing the crude protein fraction obtained by barium citrate adsorption of plasma (fig.2). After base hydrolysis of the samples the same chromatographic system could be used for measuring γ-carboxyglutamic acid (fig.3).

With a sensitive method available for measuring β-hydroxyaspartic acid in proteins, a possible vitamin C-dependency of the post-translational hydroxylation of aspartic acid and a hypothesis for the explanation of the bleeding in scurvy could be tested. If the formation of β-hydroxyaspartic acid, like that of hydroxyproline in collagen, is dependent on vitamin C, then the bleeding might be caused by incompletely hydroxylated, defective coagulation factors. The results presented here, however, do not support this hypothesis. Despite the fact that the guinea pigs in the experimental group were all severely scorbutic and had bleeding, they all had a normal β-hydroxyaspartic acid content in the vitamin K-dependent proteins. The normal APT-time in the scorbutic animals also argues against the hypothesis that defective humoral coagulation factors are a major cause of the bleeding in scurvy.

That the analytical methods used in the scurvy experiment were capable of detecting changes in the content of protein-bound β-hydroxyaspartic acid was demonstrated by treating guinea pigs with the vitamin K-antagonist, warfarin, and subsequently isolating the γ-carboxyglutamic acid-containing proteins. As expected, the yield of γ-carboxyglutamic acid decreased, and that of β-hydroxyaspartic acid decreased correspondingly. With decreased carboxylation, the amount of vitamin K-dependent proteins adsorbed to the barium citrate is reduced. Whether the hydroxylation of aspartic acid in vitamin K-dependent proteins is in some way related to the carboxylation reaction is not yet known.

In conclusion, this study has shown that β-hydroxyaspartic acid content can be determined in the barium citrate adsorbed plasma protein fraction, and that this amino acid is not reduced in severely scorbutic guinea pigs. Thus the post-translational hydroxylation of aspartic acid is unlikely to be vitamin C-dependent.

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