erythro-β-Hydroxyaspartic acid in bovine factor IX and factor X

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To localize β-hydroxyaspartic acid in factor IX and factor X the two proteins were cleaved with cyanogen bromide and trypsin, respectively. Peptides containing β-hydroxyaspartic acid were isolated and subjected to Edman degradation. The phenylthiohydantoin derivative of β-hydroxyaspartic acid was identified by HPLC in position 3 in the factor IX fragment and in position 1 in the factor X fragment. This corresponds to position 64 in factor IX and position 63 in the light chain of factor X. The assignments were confirmed by subtractive Edman degradation and with the dansyl method.

β-Hydroxyaspartic acid *Posttranslational modification* *Factor IX* *Factor X* *Vitamin K-dependent protein* *Blood coagulation*

1. INTRODUCTION

The vitamin K-dependent plasma proteins contain 10–12 γ-carboxyglutamic acid residues, which are formed by posstranslational carboxylation of glutamic acid residues in a vitamin K-dependent reaction [1–3]. The γ-carboxyglutamic acid residues are prerequisites for Ca^{2+} binding and biological activity. Recently, another posttranslationally modified amino acid, *erythro*-β-hydroxyaspartic acid, was found in bovine protein C [4] and in human factor X [5]. β-Hydroxyaspartic acid has thus far been identified in all the vitamin K-dependent plasma proteins except prothrombin and in none of a number of other common proteins studied [6,7]. As judged from amino acid analysis of acid hydrolysates factor X, protein C and protein Z all contain about 1 mol β-hydroxyaspartic acid per mol protein whereas factor IX seems to contain less than 1 mol per mol protein [6]. Protein S on the other hand contains 2–3 mol β-hydroxyaspartic acid per mol protein [6]. Although β-hydroxyaspartic acid had not been found in proteins prior to its identification in the vitamin K-dependent ones it had been demonstrated in urine [8] and in microorganisms [9]. Specific analysis has demonstrated its absence in a number of common non-vitamin K-dependent proteins [6]. The function of β-hydroxyaspartic acid in the vitamin K-dependent proteins is not known.

In protein C the β-hydroxyaspartic acid residue is in position 71 of the light chain [10]. In this region protein C, factor IX and factor X show a pronounced sequence homology [10]. Recently, authors in [11] published the sequence of a cDNA for bovine factor IX mRNA. In the position corresponding to no. 71 in bovine C the cDNA coded for aspartic acid instead of threonine as has been reported in the protein sequence [12]. The rest of the sequence of the cDNA was in complete agreement with the published protein sequence. This suggested that the amino acid in bovine factor IX corresponding to position 71 in the light chain of bovine protein C is β-hydroxyaspartic acid and furthermore that it is formed by posttranslational hydroxylation of an aspartic acid residue. We show here that the β-hydroxyaspartic acid residue is in position 64 in bovine factor IX and in position 63 in the light chain of bovine factor X.

2. MATERIALS AND METHODS

DL-*threo*-β-Hydroxyaspartic acid was obtained from Fluka. DL-*erythro*-β-Hydroxyaspartic acid

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was obtained by synthesis of racemic β-hydroxyaspartic acid and separation of the DL-*threo* and DL-*erythro* forms by ion exchange chromatography [13]. TPCK-trypsin was obtained from Worthington and purified by HPLC [14]. Bovine factor IX and factor X_1 were purified by standard procedures [15,16]. They were reduced and carboxymethylated with iodo[2-^{14}C]acetic acid as in [16]. The light and heavy chains of factor X_1 were separated by gel filtration as described for protein C [16].

HPLC was performed on a Waters liquid chromatograph equipped with a model 600 solvent programmer. A 22-cm Aquapore RP 300 column (Brownlee Laboratories) was eluted with a linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid (for details see fig.2 legend). This system was used for peptide isolation and for an improved analysis of the phenylthiohydantoin (PTH) derivatives of β-hydroxyaspartic acid and aspartic acid. Amino acid analysis was performed after acid hydrolysis in 6 M HCl at 110°C for 24 h in vacuo. A Beckman 119 CL single-column automatic analyzer with a conventional ninhydrin detection system was used. For quantitation of β-hydroxyaspartic acid a Jeol 5AH amino acid analyzer was used after modification for increased resolution of acidic amino acids and fluorimetric detection with the *o*-phthalaldehyde reagent [4,6]. Automated amino acid sequence analysis was performed as in [10] and the PTH-derivatives were identified by HPLC [17]. Manual Edman degradations were performed as in [18] with identification of the PTH-derivatives by back hydrolysis and with acid hydrolysis of the remaining peptide for quantitation of β-hydroxyaspartic acid. Dansyl derivatives of amino acids and peptides were prepared with standard methods and identified by two-dimensional chromatography as in [19].

The reduced and *S*-carboxymethylated light chain of bovine factor X_1 was digested with trypsin for 4 h at 37°C in 0.1 M NH_4HCO_3 at an enzyme: substrate ratio of 1:50 (w/w). After lyophilization the digest was chromatographed on a column (1.6 × 194 cm) of Sephadex G-50 equilibrated with 10% formic acid and eluted at a rate of 9 ml/h. As judged from amino acid hydrolysates of the pooled fractions the β-hydroxyaspartic acid-containing peptide was eluted earlier than and well separated from the other peptides (not shown). The peptide was lyophilized, extracted with 5% ethanol in 0.1% trifluoroacetic acid and dried by rotary evaporation. It was then subjected to a second digestion with trypsin in 0.1% NH_4HCO_3, this time at an enzyme: substrate ratio of 1:5 (w/w) for 6 h at 37°C. The resulting peptides were separated by HPLC.

Reduced and *S*-carboxymethylated factor IX, which has 3 methionine residues, was cleaved with CNBr (500-fold molar excess relative to methionine) in 70% formic acid [20]. After lyophilization the fragments were separated by chromatography on a column (1.6 × 94 cm) of Sephacryl S-200 in 50 mM Tris–HCl (pH 7.5) containing 6 M guanidine hydrochloride and eluted at 15 ml/h. The largest fragment, residues 62–349, suspected to have β-hydroxyaspartic acid in position 3 was eluted in the first major peak. It was dialyzed against 0.1 M NH_4HCO_3 and lyophilized.

3. RESULTS AND DISCUSSION

3.1. *Identification of* erythro-*β-hydroxyaspartic acid in bovine factor X*

The β-hydroxyaspartic acid-containing peptide in factor X_1 was isolated by sequential tryptic digestion. The first tryptic peptide (T0) isolated by gel filtration had an amino acid composition indicating that it contained residues 44–79 (fig.1). After a second tryptic digestion of this peptide at

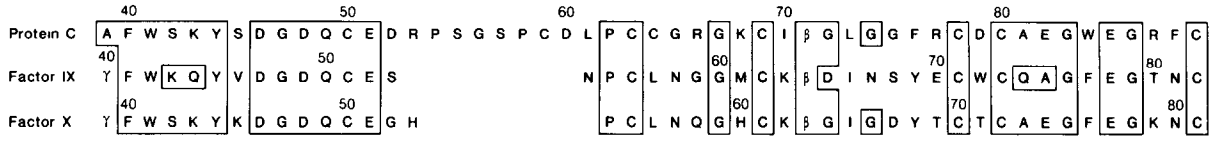

Fig.1. Comparison of sequences surrounding the β-hydroxyaspartic acid residues in bovine protein C, bovine factor IX and bovine factor X. The blocks enclose residues identical with the residue in protein C. β and γ refer to β-hydroxyaspartic acid and γ-carboxyglutamic acid, respectively. The sequence of protein C light chain was taken from [10] of factor IX from [12] of factor X light chain from [21].

high enzyme: substrate ratio 3 peptides (T1–T3) could be isolated by HPLC (fig.2). Amino acid analysis of T1 and T2 showed that they occupied positions 46–62 and 44–62 in the light chain, respectively. T3 contained β-hydroxyaspartic acid and corresponded to residues 63–79 as judged from amino acid analysis and 14 steps of automated Edman degradation. The amino acid composition of the peptide T3 was CM-Cys 2.0, Asp 1.2, Thr 1.8, Glu 2.2, Gly 4.0, Ala 1.0, Ile 1.0, Tyr 0.9, Phe 1.0, Lys 1.4. The β-hydroxyaspartic acid content of the light chain was 0.73 mol/mol, the peptide T0 (residues 44–79) contained 1.23, and the peptide T3 (residues 63–79) contained 0.90 mol/mol peptide. The peptide T3 was isolated in an overall yield of 31% based on the recovery of β-hydroxyaspartic acid. In this context it is noteworthy that in the sequence determination of the light chain of bovine factor X authors in [21] obtained peptide T3 in high recovery from tryptic digests of the light chain at an enzyme:substrate ratio of 1:50 (w/w) whereas we obtained virtually no cleavage at lysine 62 unless very high enzyme: substrate ratios were employed. A possible explanation for this difference is that they used vinylpyridine for cysteine modification and thus introduced a positive charge on the cysteine residue in position 61. We used iodoacetic acid which caused a negatively charged carboxymethylcysteine residue in position 61 and presumably contributed to the relative resistance of the adjacent lysyl-β-hydroxyaspartyl bond to tryptic digestion.

The amino composition of peptide T3 (residues

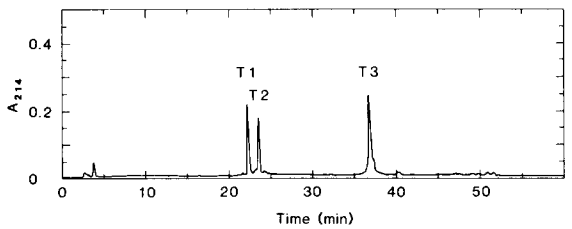

Fig.2. Chromatography of a tryptic digest of a peptide containing residues 44–79 from the light chain of factor X. About 540 nmol was digested and applied to an Aquapore RP 300 column in 0.1% (v/v) trifluoroacetic acid and 5% (v/v) acetonitrile. A linear gradient to 70% acetonitrile was run in 60 min. The absorbance was monitored at 214 nm and the individual peaks were pooled.

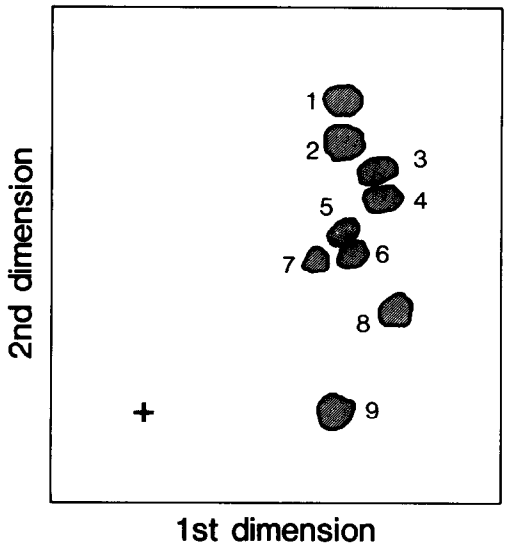

Fig.3. Polyamide thin layer chromatography of the dansyl derivative of *erythro*-β-hydroxyaspartic acid (spot no. 8). The chromatogram was developed with formic acid/water (1:15, v/v) in the first dimension and with formic acid/methanol/ethyl acetate (1:1:14, by vol.) in the second dimension. The compounds are (1) dansylamine, (2) glycine, (3) threonine, (4) serine, (5) glutamic acid, (6) aspartic acid, (7) carboxymethylcysteine, (8) *erythro*-β-hydroxyaspartic acid, (9) dansylhydroxide.

63–79) was in complete agreement with the published sequence [21] except that one aspartic acid was missing. NH_2-terminal acid analysis with the dansyl method gave an amino acid that migrated exactly as β-hydroxyaspartic acid on two-dimensional polyamide thin-layer chromatography (fig.3). Manual Edman degradation of peptide T3 in the first cycle gave a PTH-derivative which on HPLC eluted in the same position as the co-eluting PTH-aspartic acid and PTH-β-hydroxyaspartic acid. There was no PTH-asparagine [21]. In a second HPLC analysis, which used an acid eluting system allowing separation of PTH-aspartic acid and PTH-β-hydroxyaspartic acid, two doublet peaks were obtained (fig.4). The two first eluting peaks had mobilities identical with the two peaks obtained with synthetic PTH-β-hydroxyaspartic acid and presumably corresponded to the *erythro* and *threo* forms of β-hydroxyaspartic acid. The last peak in the second doublet (fig.4) had the same retention time as PTH-aspartic acid whereas the

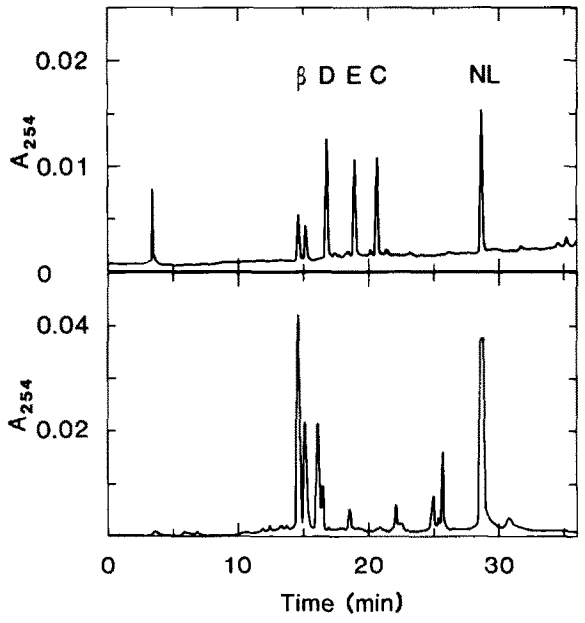

Fig.4. HPLC with an acid eluting system of PTH-derivates obtained from the first step of manual Edman degradation of peptide T3 from the light chain of factor X. Top, standard mixture with components denoted by the standard one letter code. β denotes the doublet obtained with β-hydroxyaspartic acid. NL is norleucine. Bottom, first step of manual Edman degradation. For experimental details, see text and legend to fig.2.

first peak in this doublet did not correspond to any of the 20 common PTH amino acids. Acid hydrolysis of the material obtained in the first cycle of Edman degradation of peptide T3 gave a small amount of aspartic acid on amino acid analysis but no β-hydroxyaspartic acid. An identical treatment of synthetic PTH-β-hydroxyaspartic acid led to destruction of the amino acid and recovery of some aspartic acid. It should be pointed out that PTH-serine and PTH-threonine are also destroyed by acid hydrolysis [18]. HPLC analysis of the material obtained by automated Edman degradation of peptide T3 gave the same doublet corresponding to PTH-β-hydroxyaspartic acid as with manual degradation but with a lower recovery (15%), presumably due to less efficient extraction from the sequencer cup. β-Hydroxyaspartic acid was also determined by acid hydrolysis in the remaining peptide during the manual Edman degradation. As seen in table 1, β-hydroxyaspartic acid was almost completely removed from T3 with

Table 1

β-Hydroxyaspartic acid recovery during subtractive Edman degradation

Stage	Factor X T3 (mol/mol peptide)	Factor IX CB1 (mol/mol peptide)
Peptide	0.90	0.94
1	0.06	0.91
2	<0.05	1.07
3	ND*	0.32
4	ND	0.31

* not determined

the first step of Edman degradation confirming the NH_2-terminal position of β-hydroxyaspartic acid in the peptide and thus in position 63 in the light chain of factor X. With subtractive Edman analysis only a little racemization of β-hydroxyaspartic acid takes place during acid hydrolysis of the peptide [4]. Therefore, as the two diasteromeric forms of β-hydroxyaspartic acid are completely separated on the amino acid analyzer [4], it was possible to determine that the diastereomeric form present in the peptide is the *erythro* form. In contrast, the PTH-derivative of β-hydroxyaspartic acid seems to be almost completely racemized and therefore is not helpful in this respect. Neither can polyamide layer chromatography of the dansyl derivative of β-hydroxyaspartic acid be used since the two diasteromeric forms do not separate completely in the thin-layer chromatographic system used.

3.2. *Identification of* erythro-*β-hydroxyaspartic acid in bovine factor IX*

The CNBr fragment (CB1) of factor IX containing β-hydroxyaspartic acid was isolated by gel filtration. Automated amino acid sequence determination gave CM-Cys-Lys-X-Asp-Ile-Asn-Ser-Tyr, indicating that the fragment was residues 62-349 in factor IX [12]. The sequence analysis is also indicated that the fragment was contaminated with 29% of a fragment with a sequence identical to the NH_2-terminal sequence of intact factor IX, presumably caused by incomplete cleavage at methionine 61. In the third cycle the HPLC gave a PTH-derivative which eluted as a doublet in exactly the same position as the doublet obtained with synthetic PTH-β-hydroxyaspartic acid. The re-

covery was about 8%. PTH-threonine was not found [12]. Subtractive Edman degradation of CB1 (table 1) with β-hydroxyaspartic acid determination in the acid hydrolysate of the remaining peptide clearly indicated an *erythro*-β-hydroxyaspartic acid residue in position 3 of CB1 corresponding to position 64 of bovine factor IX. However, about 0.3 mol/mol peptide of the amino acid was found even beyond position 3. This was caused by the contamination of CB1 with a larger CNBr fragment due to incomplete cleavage at methionine 61.

3.3. *β-Hydroxyaspartic acid in vitamin K-dependent plasma proteins*

This work has unambigously shown that β-hydroxyaspartic acid occupies position 64 in bovine factor IX and position 63 in the light chain of bovine factor X. These findings are in agreement with those in [5,7]. Until now β-hydroxyaspartic acid has been demonstrated in the following positions in proteins: position 71 in the light chain of bovine protein C, position 64 in bovine factor IX, position 63 in the light chain of bovine factor X, and in position 63 in the light chain of human factor X. In all these proteins the β-hydroxyaspartic acid residue occupies the corresponding position according to the sequence homology of the surrounding parts of the polypeptide chains. This pronounced conservation points to an important role of this unusual amino acid in vitamin K-dependent proteins.

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