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THE OCCURRENCE OF β-HYDROXYASPARTIC ACID IN THE VITAMIN K-DEPENDENT BLOOD COAGULATION ZYMOGENS

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SUMMARY: Previous work has shown that two vitamin K-dependent plasma zymogens, factor X and protein C, each contain one residue of erythro-β-hydroxyaspartic acid. In the present study, prothrombin, factor VII and factor IX were subjected to amino acid analyses for β-hydroxyaspartic acid. Factor IX and factor VII each contain one residue of erythro-β-hydroxyaspartic acid. Edman sequence analyses revealed that this residue occurs at position 64 in human and bovine factor IX. Inasmuch as the nucleotide sequence codes for aspartic acid at this position, it appears highly likely that β-hydroxyaspartic acid is formed in these proteins by a post-translational hydroxylation of aspartic acid. In contrast, neither human nor bovine prothrombin contain β-hydroxyaspartic acid.

Five plasma glycoproteins including prothrombin, factor IX, factor VII, factor X and protein C exist in plasma as precursors to serine proteases and require vitamin K for their biosynthesis (1). Vitamin K is required for the formation of γ-carboxyglutamic acid (Gla) residues in these proteins. These residues bind calcium ions which facilitates the interaction of these proteins with acidic phospholipid membranes and greatly augments their biological activity. Recently, an unusual amino acid, erythro-β-hydroxyaspartic acid was discovered independently in bovine protein C (2,3) and in human and bovine factor X (3). The biological function of this residue is presently unknown. As part of our studies to determine the functional significance of β-hydroxyaspartic acid in these proteins, we initially focused on the occurrence of this residue in the other vitamin K-dependent zymogens present in plasma. We report here that, by amino acid composition and sequence analyses, human and bovine factor IX and bovine factor VII each contain one

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Abbreviations: Bha, β-hydroxyaspartic acid; Gla, γ-carboxyglutamic acid; Pth, phenylthiohydantoin; HPLC, high-performance liquid chromatography.

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residue of β-hydroxyaspartic acid. In contrast, β-hydroxyaspartic acid was not detected in either human or bovine prothrombin by amino acid analyses.

MATERIALS AND METHODS

Cyanogen bromide, polybrene and 4-vinyl pyridine were obtained from Pierce. Sephadex G-15, G-50(SF) and G-75 were Pharmacia products. Chymotrypsin was obtained from Worthington and purified further on benzamidine-agarose (4). Iodoacetic acid was obtained from Sigma. The columns used in reverse-phase HPLC (μBondapak C_{18} and Ultrapore RPSC) were purchased from Waters and Altex, respectively. Authentic L-threo-β-hydroxyaspartic acid was obtained from CalBiochem. Authentic L-erythro-β-hydroxyaspartic acid was kindly provided by Drs. N. Izumiya and T. Kato, Faculty of Biochemistry, Kyushu University, Fukuoka, Japan.

Bovine and human prothrombin were prepared according to Mann (5) and Kisiel and Hanahan (6), respectively. Bovine factor VII was purified according to Kisiel and Davie (7). Bovine and human factor IX were isolated by procedures described by Fujikawa et al. (8) and Amphlett et al. (9), respectively. All proteins were found to be homogeneous when examined by SDS-polyacrylamide gel electrophoresis in the presence or absence of 2-mercaptoethanol (10).

Amino acid analyses were performed on a Dionex Model D-500 amino acid analyzer according to standard procedure (11,12). Automated Edman degradations were performed with a Beckman sequencer (Model 890C) according to a slight modification of the program described by Brauer et al. (13). Polybrene (3 mg) was added to the peptide samples to reduce washout in the cup (14). Manual Edman degradation was performed according to Tarr (15) and phenylthiohydantoin (Pth) derivatives were identified by HPLC as described (3).

Peptides containing β-hydroxyaspartic acid were isolated from human and bovine factor IX as follows: 50 mg of human or bovine factor IX were initially reduced and alkylated with either 4-vinyl pyridine (16) or iodoacetic acid (17). The reduced and alkylated proteins were treated with hydroxylamine as described (18), and the reaction mixture desalted in Sephadex G-15 equilibrated with 0.1 M NH_4HCO_3. The void-volume fraction was lyophilized and subsequently digested in 0.1 M NH_4HCO_3 with chymotrypsin at 37°C for 18 h at an enzyme-to-substrate weight ratio of 1:100. The digest was fractionated by gel filtration in a Sephadex G-50(SF) column (1.5 x 95 cm) equilibrated with 0.1 M NH_4HCO_3. Those fractions containing β-hydroxyaspartic acid by amino acid analysis were further fractionated by HPLC in a Waters liquid chromatograph equipped with a Model 660 solvent programmer and a μBondapak C_{18} column. In the separation of peptides by HPLC, the mobile phase consisted of 0.1% trifluoroacetic acid, and the mobile-phase modifier was acetonitrile containing 0.08% trifluoroacetic acid. The concentration of acetonitrile was increased linearly (1-2%/min) during 35 min at a flow rate of 1.5 ml/min. The HPLC-fractionated peptide containing β-hydroxyaspartic acid was then subjected to automated sequence analysis. In the isolation of the β-hydroxyaspartate-containing peptide from bovine factor VII, approximately 15 mg of factor VII was reduced, S-pyridylethylated and digested with cyanogen bromide as previously described (19). The cyanogen bromide digest was fractionated in a Sephadex G-75 column (1.6 x 95 cm) equilibrated with 2.3 M HCOOH/3 M urea. Those fractions containing β-hydroxyaspartic acid were then pooled and digested with chymotrypsin as described above. The peptide containing β-hydroxyaspartic acid was then purified by a combination of gel filtration and HPLC as described above for factor IX with the exception that an Ultrapore RPSC column was employed in the HPLC fractionation.

RESULTS AND DISCUSSION

Previous work from our laboratory revealed that human and bovine factor X, as well as bovine protein C, contained one residue of erythro-β-hydroxy-aspartic acid (3). The presence of β-hydroxyaspartic acid (Bha) in bovine protein C has also been reported independently by Drakenberg et al. (2). Bha is highly acidic, and elutes between cysteic acid and carboxymethyl-cysteine on the amino acid analyzer. Thus, provided the protein is relatively small (M_r <30,000), one can detect a single residue of this amino acid in a protein sample by standard amino acid analysis. For larger proteins, however, one residue of Bha generates a relatively small peak on the chromatogram that may easily be interpreted as a spurious background peak. Examination of amino acid chromatograms of human factor IX and bovine factor IX and factor VII, revealed a small, but distinct, peak at the Bha elution position. In contrast, no such peak was observed in this region in amino acid chromatograms of human and bovine prothrombin. Given the limitation of detecting one residue of Bha in a large protein, we digested both human and bovine prothrombin with chymotrypsin, gel filtered each digest and subjected fractions of the effluent to amino acid analysis in an attempt to enrich the Bha content in a chymotryptic fragment. As observed with the intact molecule, no chymotryptic fragment was found that produce a peak on the amino acid chromatogram corresponding to Bha. From this data, we conclude that Bha is not present in prothrombin.

In order to both quantitate and define the position(s) of Bha in factor IX and factor VII, carboxymethylated or pyridylethylated derivatives of these proteins were subjected to chemical and enzymatic digestions, and the peptides arising from these digests fractionated by a combination of gel filtration and HPLC. Those peptides containing Bha by amino acid analysis were then subjected to automated Edman degradations. In the case of human factor IX, a single Bha-containing peptide was isolated that yielded a sequence of Gly-Gly-Ser-Cys-Lys-?-Asp-Ile-Asn-Ser-Tyr. The Pth-derivative at cycle 6 exhibited an extremely low yield (~1%) of a double peak that

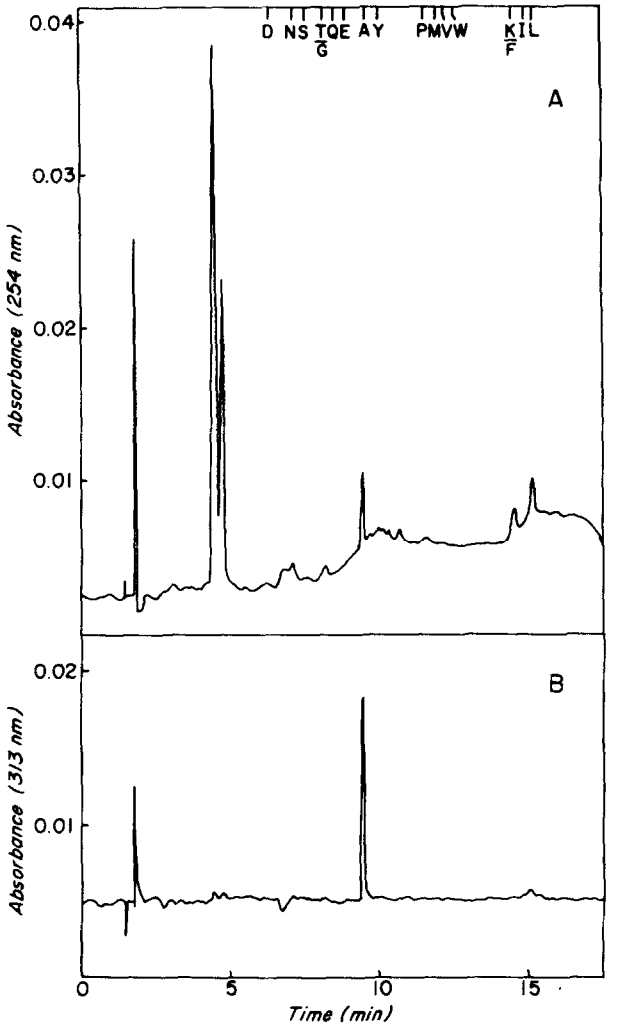

Figure 1. HPLC of Pth-Bha obtained from cycle 6 during manual Edman degradation of a peptide derived from human factor IX containing residues 59-69. (A) Effluent monitored at 254 nm. The sharp peak at 2 min represents the solvent front. The elution positions of standard Pth-amino acids, in one letter code (3), are inserted at the top. (B) Effluent monitored at 313 nm.

migrated ahead of Pth-Asp by HPLC. By comparison to the published sequence for human factor IX (20,21), this peptide corresponded to residues 59-69 where the nucleotide sequence codes for aspartic acid at position 64. In view of our earlier experience with factor X regarding the poor extraction of Pth-Bha from the sequencer cup (3), we subjected this peptide to manual Edman degradation which resulted in good yield of Pth-Bha at cycle 6 (Figure 1A). By HPLC analysis, the Pth-derivative of this cycle appeared to corre-

Human Factor IX	Gly	Gly	Ser	Cys	Lys	Bha	Asp	Ile	Asn	Ser	Tyr
Bovine Factor IX	Gly	Gly	Met	Cys	Lys	Bha	Asp	Ile	Asn	Ser	Tyr
Bovine Factor VII	Gly	Gly	Ser	Cys	Glu	Bha	Gln	Leu	Arg	Ser	Tyr
Human Factor X	Gln	Gly	Lys	Cys	Lys	Bha	Gly	Leu	Gly	Glu	Tyr
Bovine Factor X	Gln	Gly	His	Cys	Lys	Bha	Gly	Ile	Gly	Asp	Tyr
Bovine Protein C	Arg	Gly	Lys	Cys	Ile	Bha	Gly	Leu	Gly	Gly	Phe

Figure 2. Amino acid sequences surrounding the β-hydroxyaspartic acid (Bha) in factor IX, factor VII, factor X and protein C. Amino acids that are identical in all peptides are shown in solid blocks; amino acids that are conservatively replaced are shown in dashed blocks. Residues underlined are tentative assignments. Sequence data for factor X and protein C are from McMullen et al. (3).

spond to roughly a 75:25 erythro:threo mixture of L-Bha. This is consistent with earlier observations regarding the interconvertibility of the two stereoisomers under the conditions of the assay (3). As noted earlier with factor X and protein C (3), when the HPLC effluent of cycle 6 was monitored at 313 nm, a significant peak was observed migrating slightly ahead of Pth-Ala (Figure 1B). It appears likely that this species represents an unsaturated dehydration product of Bha.

Like the human molecule, chemical and enzymatic digestion of carboxymethylated bovine factor IX and subsequent HPLC yielded a single Bha-containing peptide with a sequence of Gly-Gly-Met-Cys-Lys-Bha-Asp-Ile-Asn-Ser-Tyr. This sequence corresponds to residues 59-69 of bovine factor IX (22), where again the nucleotide sequence codes for Asp at position 64 (23). In the case of bovine factor VII, a single Bha-containing peptide was isolated that yielded a sequence of Gly-Gly-Ser-Cys-Glu-Bha-Gln-Leu-Arg-Ser-Tyr. Although the precise location of the Bha in factor VII is unknown, it is likely that it occurs in a region of the molecule corresponding to that found in factor IX, factor X and protein C.

The apparent homology of the sequence surrounding the Bha residue in factor IX, factor VII, factor X and protein C is presented in Figure 2. Of interest is the strict conservation of glycine and cysteine preceding the Bha. Two features of this comparative sequence are noteworthy. First, our finding of Bha at position 64 in factor IX by Edman degradation, coupled

with the nucleotide sequence data on these proteins (20,21,23), substantiates that the formation of Bha most probably occurs via a post-translational hydroxylation of aspartic acid. Secondly, in contrast to that observed in factor X and protein C, glycine was not observed in factor IX and factor VII on the carboxyl side of the Bha residue. Thus, unlike the hydroxylation of prolyl and lysyl residues in collagen and elastin (24), a glycine residue on the carboxyl side of the susceptible aspartic acid does not appear to be an absolute substrate requirement in the enzymatic post-translation hydroxylation of aspartic acid in these proteins.

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REFERENCES

1. Davie, E.W., Fujikawa, K., Kurachi, K., and Kisiel, W. (1979) Adv. Enzymol. 48, 277-318.
2. Drakenberg, T., Fernlund, P., Roepstorff, P., and Stenflo, J. (1983) Proc. Natl. Acad. Sci. USA 80, 1802-1806.
3. McMullen, B.A., Fujikawa, K., Kisiel, W., Sasagawa, T., Howald, W.M., Kwa, E.Y., and Weinstein, B. (1983) Biochemistry 22, 2875-2884.
4. Fujikawa, K., and McMullen, B.A. (1983) J. Biol. Chem., in press.
5. Mann, K.G. (1976) Methods Enzymol. 45, 123-156.
6. Kisiel, W., and Hanahan, D.J. (1973) Biochim. Biophys. Acta 329, 221-232.
7. Kisiel, W., and Davie, E.W. (1975) Biochemistry 14, 4928-4934.
8. Fujikawa, K., Thompson, A.R., Legaz, M.E., Meyer, R.G., and Davie, E.W. (1973) Biochemistry 12, 4938-4945.
9. Amphlett, G.W., Kisiel, W., and Castellino, F.J. (1981) Arch. Biochem. Biophys. 208, 576-585.
10. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
11. Moore, S., and Stein, W.H. (1963) Methods Enzymol. 6, 819-831.
12. Hugli, T.E., and Moore, S. (1972) J. Biol. Chem. 247, 2828-2834.
13. Brauer, A.W., Margolies, M.N., and Haber, E. (1975) Biochemistry 14, 3029-3035.
14. Tarr, G.E., Beecher, J.F., Bell, M., and McKea, D.J. (1978) Anal. Biochem. 84, 622-627.
15. Tarr, G.E. (1982) in Methods in Protein Sequence Analysis (Elzinga, M., Ed.) pp. 223-232, Humana Press, Clifton, NJ.
16. Friedman, M., Krull, L.H., and Cavins, J.F. (1970) J. Biol. Chem. 245, 3868-3881.
17. Crestfield, A.M., Moore, S., and Stein, W.H. (1963) J. Biol. Chem. 238, 622-627.
18. Steinman, H.M., Vishweshwar, R.N., Abernethy, J.L., and Hill, R. L. (1974) J. Biol. Chem. 249, 7326-7338.
19. Kisiel, W., Fujikawa, K., and Davie, E. W. (1977) Biochemistry 16, 4189-4194.
20. Kurachi, K., and Davie, E.W. (1982) Proc. Natl. Acad. Sci. USA 79, 6461-6464.

21. Jaye, M., LaSalle, H., Schamber, F., Balland, A., Kohli, V., Findeli, A., Tolstoshev, P., and Lecocq, J.P. (1983) Nucl. Acid Res. 11, 2325-2335.
22. Katayama, K., Ericsson, L.H., Enfield, D.L., Walsh, K.A., Neurath, H., Davie, E.W., and Titani, K. (1979) Proc. Natl. Acad. Sci. USA 76, 4990-4994.
23. Choo, K.H., Gould, K.G., Rees, D.J.G., and Brownlee, G.G. (1982) Nature (London) 299, 178-180.
24. Kivirikko, K.I., and Myllyla, R. (1980) in The Enzymology of Post-Translational Modification of Proteins (Freedman, R.B., and Hawkins, H.C., Eds.) pp. 53-104, Academic Press, New York.