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Biochemistry

Identification of the molecular defect in factor $IX_{Chapel\ Hill}$: Substitution of histidine for arginine at position 145

(blood coagulation/protein primary structure/amino acid substitution)

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ABSTRACT **Hemophilia $B_{Chapel\ Hill}$ is a mild hereditary hemorrhagic disorder in which the factor IX antigen is present in normal amounts but factor IX biological activity is markedly reduced. Previous studies have demonstrated that purified factor $IX_{Chapel\ Hill}$ has 8% of the activity of normal human factor IX and that the activation of factor $IX_{Chapel\ Hill}$ is defective in that only one of the two peptide bonds hydrolyzed during activation of normal factor IX is cleaved. The tryptic peptides from normal human factor IX and factor $IX_{Chapel\ Hill}$ were subjected to analysis by high-performance liquid chromatography. Comparison of the elution profile of the peptides obtained from factor $IX_{Chapel\ Hill}$ and normal factor IX demonstrated that the tripeptide Leu-Thr-Arg, which is derived from the normal molecule (positions 143–145) immediately amino-terminal from the Arg-Ala peptide bond at 145–146 that is cleaved during the activation of factor IX with factor XIa, was absent in the digest obtained from factor $IX_{Chapel\ Hill}$. The elongated "activation peptide" from factor $IX_{Chapel\ Hill}$ was obtained by further high-performance liquid chromatographic fractionation and subjected to primary structure analysis. The following sequence, corresponding to positions 143–147, was obtained: Leu-Thr-His-Ala-Glu. Thus, the primary molecular defect in factor $IX_{Chapel\ Hill}$ is the substitution of histidine for arginine at position 145. This substitution precludes cleavage by factor XIa at this peptide bond, and the activation peptide region remains associated with the light chain of factor $IXa_{Chapel\ Hill}$.**

Blood coagulation factor IX is a glycoprotein of M_r 57,000 that participates during the middle phase of intrinsic blood coagulation (1–3). Factor IX circulates as an inactive precursor or zymogen form; upon activation with either factor XIa and calcium ions or tissue factor/factor VIIa and calcium ions, two peptide bonds (Arg-Ala at position 145–146 and Arg-Val at position 180–181) are cleaved with the release of an activation peptide and the formation of factor IXa (4–7). Factor IXa is a serine protease composed of two polypeptide chains, a "light chain" consisting of the amino-terminal 145 residues of factor IX and containing the 12 γ-carboxyglutamic acid residues connected by a disulfide bridge to a "heavy chain" consisting of the carboxyl-terminal 236 residues of factor IX. The "heavy chain" contains the residues actually involved in the process of catalysis and demonstrates a high degree of homology with the heavy chains of the other vitamin K-dependent coagulation factors and the chymotrypsin family of proteases (8, 9). Factor IXa in turn activates factor X in a reaction requiring the presence of calcium ions, phospholipid, and factor VIII (2, 3).

Individuals with Christmas disease (hemophilia B; deficiency of factor IX activity) can be divided into several classes: cross-reacting material-positive (CRM^+) individuals who possess normal levels of factor IX protein as assessed by immunological assay but decreased-to-undetectable levels of factor IX coagulant activity; CRM^- individuals with greatly reduced or undetectable levels of both factor IX activity and factor IX antigen; and CRM^r individuals with variably reduced levels of both factor IX activity and factor IX antigen (1, 10, 11).

Our laboratory has been interested in the study of CRM^+ variants with the goal of elucidating the nature of the molecular defect(s) and using this information to establish relationships between chemical structure and biological function in factor IX. Recent work has focused on one of these CRM^+ variants, factor $IX_{Chapel\ Hill}$. This abnormal protein has been purified by techniques developed for the purification of normal human factor IX and has been characterized partially (12). More recent studies (13) have shown that factor $IX_{Chapel\ Hill}$ is activated by factor XIa in the presence of calcium ions but, in contrast to normal factor IX, only the Arg-Val bond at position 180–181 is cleaved. The present study demonstrates that there is the substitution of a histidine for the arginine at position 145, thus explaining the lack of cleavage at this position in factor $IX_{Chapel\ Hill}$.

MATERIALS AND METHODS

Normal human factor IX and factor $IX_{Chapel\ Hill}$ were purified to homogeneity by published procedures (12, 14). Trypsin (L-1-tosylamide-2-phenylmethyl chloromethyl ketone-treated trypsin, 222 units/mg; 92% protein) was obtained from Worthington and was allowed to stand at ambient temperature (24°C) in 0.001 M HCl for 2 hr prior to use (15). Urea was recrystallized from water after treatment with a mixed-bed ion-exchange resin (Bio-Rad AG501-X8) prior to use (16).

Proteins were taken into 10% acetic acid (vol/vol) by dialysis, lyophilized, and then reduced and carboxymethylated as described by Canfield and Anfinsen (17). Tryptic hydrolysis was performed with a 1:20 (wt/wt) ratio of enzyme to substrate protein in 0.1 M NH_4HCO_3 for 16 hr at 37°C. The hydrolysis reaction was terminated by the addition of glacial acetic acid to pH 4.0, and the reaction mixture was then lyophilized. Because small amounts of proteins were used (2–4 nmol), all of the above manipulations were performed in the same reaction vessel (18).

The tryptic hydrolysates were analyzed by high-performance liquid chromatography with a Beckman–Altex 324 liquid chromatographic system. The initial analysis (19) was performed by using a 4.6 × 250 mm Ultrasphere-ODS column (Beckman) at 25°C with a flow rate of 1.0 ml/min. The initial solvent was 0.05 M sodium phosphate (pH 2.87). Elution was accomplished with a linear gradient to 28% acetonitrile in 114 min and then to 62% acetonitrile in 30 min. Rechromatography of isolated peptides prior to primary structure analysis utilized a 4.6 × 250 mm Vydac TP C_8 5-μm column (The Separations Group, Hesperia, CA). The initial solvent was 0.1% trifluoroacetic acid.

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Abbreviation: CRM, crossreacting material.

Elution in a linear gradient to 60% 2-propanol was accomplished in 120 min at 45°C with a flow rate of 1.0 ml/min. Absorbance was monitored at 210 and 280 nm.

The isolated peptides were subjected to automated Edman degradation (20) in a Beckman 890C protein/peptide sequencer. The 0.1 M Quadrol program of Brauer *et al.* (21) was used with modification of the cleavage cycle to minimize blowing of heptafluorobutyric acid vapor through the effluent valve. Polybrene (Aldrich) was used as a carrier (22, 23); 3 mg was dried from aqueous solution in the sequencer cup and run through one complete cycle before the sample was loaded. We identified the phenylthiohydantoin derivatives of amino acids produced by the Edman degradation by HPLC using the 4.6 × 250 mm Ultrasphere-ODS column with detection at 254 and 323 nm and using gradient elution at 55°C with an acetate/acetonitrile solvent system (24).

RESULTS

The HPLC analysis of the peptides derived from the tryptic hydrolysis of reduced and carboxymethylated normal human factor IX is shown in Fig. 1*B*. This pattern is reproducible and has been obtained for several different preparations of factor IX. The HPLC analysis of the peptides derived from the tryptic hydrolysis of reduced and carboxymethylated factor $IX_{Chapel\ Hill}$ is shown in Fig. 1*A*. As can be observed, there is a single peptide missing from the early portion of the chromatogram as indicated by the arrow. We previously had determined that this

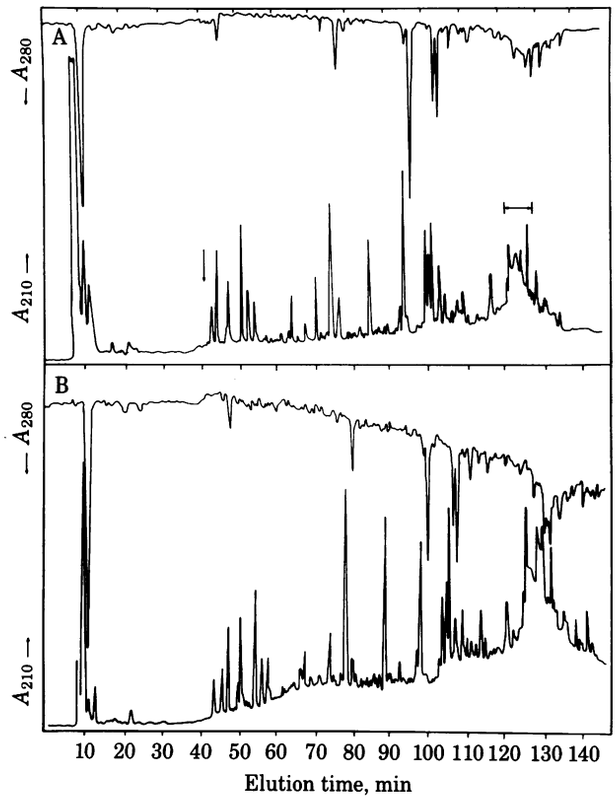

FIG. 1. Tryptic peptides of factor $IX_{Chapel\ Hill}$ and normal human factor IX. The separation of the tryptic peptides from reduced and carboxymethylated factor $IX_{Chapel\ Hill}$ (*A*) and normal factor IX (*B*) was accomplished by reverse-phase chromatography on Ultrasphere-ODS in a phosphate buffer/acetonitrile solvent gradient as described. The vertical arrow indicates the elution position of the peptide Leu-Thr-Arg obtained from the tryptic hydrolysis of normal factor IX. The eluent from the region within the bars in *A* was collected and subjected to a second chromatographic fractionation as shown in Fig. 2.

Table 1. Primary structure surrounding peptide bonds in factor IX cleaved by factor XIa*

		140 145
Arg-Ala	Human	Thr-Ser-Lys-Leu-Thr-Arg-Ala-Glu-Ala
		146
	Bovine	Ser-Lys-Lys-Leu-Thr-Arg-Ala-Glu-Thr
		175 180
Arg-Val	Human	Phe-Asn-Asp-Phe-Thr-Arg-Val-Val-Gly-Gly
		181
	Bovine	Phe-Asp-Glu-Phe-Ser-Arg-Val-Val-Gly-Gly

* Data for the bovine protein is from ref. 8. The data for the human protein was obtained from analysis of cDNA (25).

was a tripeptide, Leu-Thr-Arg, derived from the portion of the factor IX molecule immediately amino-terminal from the Arg-Ala peptide bond at 145–146, which is cleaved by factor XIa during the formation of factor IXa as shown in Table 1. The majority of the other peptides had been identified previously by primary structure analysis (unpublished data) and are in agreement with the primary structure determined from nucleotide sequence analysis of cDNA for human factor IX (26). The material within the bars of Fig. 1*A* was subjected to a second HPLC analysis as shown in Fig. 2. The material within the bars was collected and subjected to primary structure analysis as shown in Table 2. The amino acid sequence derived from the analysis of this peptide is compared to that of normal human factor IX. From this analysis it is clear that the defect in factor $IX_{Chapel\ Hill}$ is a result of the substitution of a histidyl residue for an arginyl residue at position 145. This substitution precludes cleavage by either trypsin or factor XIa under the present reaction conditions.

The activation peptide of normal human factor IX contains no internal lysine or arginine and is, therefore, a single 35-residue tryptic peptide. Thus, the variant peptide in the tryptic digest of factor $IX_{Chapel\ Hill}$ is a 38-residue peptide consisting of the last three residues of the light chain plus the activation peptide.

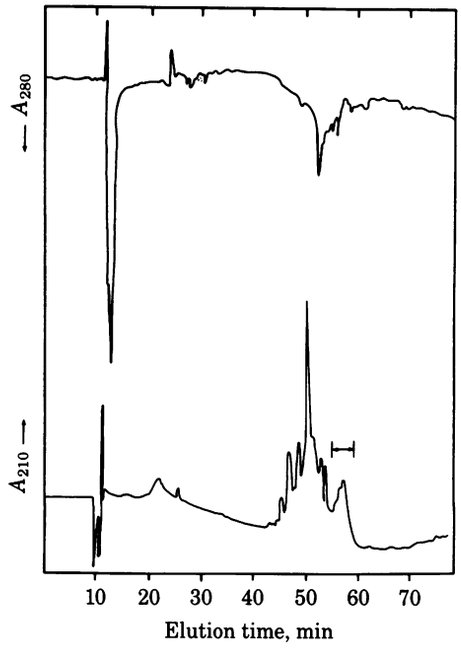

FIG. 2. Rechromatography of the eluent containing the variant peptide. The portion of the eluent indicated in Fig. 1*A* was concentrated to about 1 ml and rerun on a Vydac TP C_8 column in a 0.1% trifluoroacetic acid/2-propanol solvent gradient as described. The material indicated was subjected to primary structure analysis.

Table 2. Amino-terminal sequence of variant peptide from factor $IX_{Chapel\ Hill}$

Cycle	Variant peptide: Found	Variant peptide: Amount, nmol	Normal human factor IX*
1	Leu	0.26	Leu
2	Thr	NQ†	Thr
3	His	0.11	Arg
4	Ala	0.22	Ala
5	Glu	0.12	Glu
6	Ala	0.19	Ala
7	Val	0.22	Val
8	Phe	0.18	Phe
9	Pro	0.05	Pro
10	Asp	0.14	Asp
11	Val	0.16	Val
12	Asp	0.14	Asp
13	Tyr	0.13	Tyr
14	Val	0.11	Val

About 50% of the peak indicated in Fig. 3 was subjected to Edman degradation.
*From ref. 25.
†NQ, not quantitated.

DISCUSSION

The above results suggest that the physiological defect in hemophilia $B_{Chapel\ Hill}$ is due to the failure of the Arg-Ala peptide bond at position 145–146 to be cleaved during the process of coagulation because of the substitution of histidine for arginine at position 145. The substitution of a histidine for an arginine residue can be the result of a point mutation resulting from a single nucleotide change in the codon responsible for position 145 in the factor IX polypeptide chain. In the case of factor $IX_{Chapel\ Hill}$, the change is likely from C-G-T to C-A-T. As previously demonstrated by work in this laboratory (13), the activation of factor $IX_{Chapel\ Hill}$ results in a two-chain molecule with a structure similar to that reported for bovine factor IXaα, the activation product formed from the activation of factor IX with the coagulant protein from Russell's Viper Venom (6). Lindquist *et al.* reported that bovine factor IXaα has equally reduced esterase activity N^{α}-tosyl-L-arginine methyl ester and coagulant activity as compared to bovine factor IXaβ (6). Byrne *et al.* (26) subsequently reported that, in the presence of calcium ions, there is essentially no difference in the hydrolysis of N^{α}-benzoyl-L-arginine ethyl ester by bovine factor IXaα and factor IXaβ. Further work from Castellino's laboratory (F. J. Castellino, personal communication) has shown that both bovine factor IXaα and factor IXaβ effectively hydrolyze the thioester substrates developed by Powers and co-workers (27). With the bovine proteins there is a clear difference in their effectiveness in the activation of factor X in the presence of cofactors, with factor IXaα being much less effective than factor IXaβ (F. J. Castellino, personal communication). In studies with the human protein, DiScipio *et al.* (7) presented evidence that there is no difference in the activity of factor IXaα and factor IXaβ in the catalysis of reactions in the intrinsic coagulation pathway. It would seem reasonable that factor $IXa_{Chapel\ Hill}$ should have enzymatic properties similar to human factor IXaα. It is clear that a more rigorous comparison of the catalytic properties of human factor IXaα and factor IXaβ is required before a meaningful relationship can be established between normal human factor IXaα and factor $IXa_{Chapel\ Hill}$.

Although we have not observed cleavage of the His-Ala bond at position 145–146 either in our present studies with trypsin or in previous studies on the interaction of factor XIa with factor $IX_{Chapel\ Hill}$ (13), it is possible that cleavage of the bond could be accomplished with higher amounts of factor XIa or with factor VIIa/tissue factor. In this regard, it is of interest to note that Shafer's laboratory (28) has reported the substitution of histidine for arginine at residue 16 in the A (α) chain of fibrinogen Petoskey, forming a peptide bond that is slowly cleaved by thrombin.

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