THE HETEROGENEITY OF THE LIGHT CHAINS OF FACTOR X

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1. Introduction

It has been shown in many laboratories [1–3] that Factor X is resolved into two protein peaks on chromatography on DEAE-Sephadex. So far no chemical differences in the chemical composition of these two forms of the protein have been described which might account for this difference in behaviour on chromatography. Although Jackson [4] has shown small differences in the sialic acid and hexose content of the two forms of Factor X these differences have not been observed by others [2,5].

On the other hand Fujikawa et al. [2] and Esnouf et al. [3] have shown that there is a difference in the specific activity of Factor X_1 and Factor X_2, Factor X_1 having 86% [2] and 70% [3] the activity of Factor X_2.

The simplest interpretation of the difference in the chromatographic behaviour of the two forms of Factor X is that Factor X_2, which is eluted after Factor X_1, has a higher negative charge than Factor X_1 and is retained on the column longer. Against this idea is the observation that Factor X_1 and X_2 are not resolved by electrophoresis on polyacrylamide gels [2,3,6], but this objection could be invalid because normal and abnormal prothrombin are also not resolved by this technique [7]. Normal prothrombin has the more negative charges than abnormal prothrombin [8,9], which arise from the carboxylation of the γ carbon of ten glutamic acid residues in the N-terminal portion of normal prothrombin. Recently this 1,3,3′-tricarboxy amino propane (Gla) residue has been indirectly identified in the N-terminal portion of the light chain of Factor X [10]. In view of these observations we have analysed alkaline hydrolysates of the light chains of Factor X_1 and X_2 to test whether the difference in the chromatographic behaviour of these two forms of the protein could be accounted for by a difference in their Gla content.

2. Materials and methods

2.1. *Preparation of Factors X_1 and X_2*

Factor X was prepared as described by Esnouf et al. [3], except that all buffers contained 1 mM benzamidine and X_1 and X_2 were further separated by rechromatography on DEAE-Sephadex A-50 (Pharmacia Fine Chemicals Ltd., London) with 200–400 mM gradient of sodium citrate–citric acid buffer, pH 6.8, containing 1 mM benzamidine.

The two cows treated with vitamin K received 10 mg/day intramuscularly for ten days prior to the collection of the blood.

2.2. *Preparation of light chains*

The light chains were obtained by two different procedures (a) The separated Factor X_1 and X_2 were reduced, carboxy-methylated and the light chains recovered by gel filtration. (b) Factors X_1 and X_2 were pooled, reduced and the mixed light chains, obtained by gel filtration, were separated by ion exchange chromatography.

2.2.1. Route A

The pools containing Factor X_1 (40 mg) and X_2 (20 mg) were separately concentrated to about 5 mg protein/ml and dialysed overnight into 6 M guanidine hydrochloride buffered with 0.2 M Tris-HCl buffer, pH 8.0. The solution was then made 5% with respect to 2-mercaptoethanol and incubated at 37°C for 1 h and the reduced protein carboxy-methylated after

adding iodoacetic acid to a final concentration of 0.1 M; the pH was maintained at 8.0 by the addition of 1.0 M Tris. After 15 min the excess iodoacetic was eliminated by the further addition of 2-mercapto-ethanol. The carboxy-methylated heavy and light chains were separated by gel filtration on a column of LKB (LKB Ltd., Croydon, Surrey) Ultrogel AcA 44 (2.5 × 80 cm) eluted with 0.1 M NH_4HCO_3. The fractions containing the light chains of X_1 and X_2 were pooled and taken to dryness on a rotary evaporator.

2.2.2. Route B

A pool containing both Factor X_1 and X_2 was concentrated to approximately 60 mg protein/ml and made 4 M with respect to guanidine hydrochloride. After incubation at 37°C for 30 min with a 3-fold molar excess of dithiothreitol, the mixed (X_1 + X_2) heavy and light chains were separated on a column of LKB Ultrogel AcA 44 (2.5 × 100 cm) eluted with 0.1 M sodium citrate buffer pH 6.9 containing 1 mM dithiothreitol. The mixed (X_1 + X_2) light chains were concentrated and separated from each other by chromatography on DEAE-Sephadex A-50 (1.5 × 20 cm) eluted with a citrate gradient containing 1 mM dithiothreitol, from 0.2–0.6 M sodium citrate, pH 6.9. Samples of X_1 and X_2 light chains were taken for alkaline hydrolysis.

3. Other methods

Electrophoresis was performed by the method of Weber and Osborn [11]. Factor X was assayed as described by Prowse et al. [12]. Amino acid analyses were carried out using Aminex A5 and A4 resins (Bio-Rad Laboratories, Bromley, Kent) by the procedure of Moore and Stein [13]. Samples were either hydrolysed in 6 M HCl in sealed glass tubes or 15% NaOH in polypropylene tubes, at 110°C, for 18 h after flushing with nitrogen. Norleucine was added to each sample as an internal standard. After hydrolysis HCl was removed by rotary evaporation and the NaOH by ion exchange on a Dowex 50 W × 8 column in 1 M NH_4OH, and the sample dried by rotary evaporation. Alkaline hydrolysis gave a 90% recovery of GLA from prothrombin. γ-Carboxyglutamic acid was synthesised by the method of Fernlund et al. [9].

4. Results

SDS gel electrophoresis of the light chains obtained by either route A or B showed that the separation from the heavy chain had been complete. Two routes for the preparation of the light chains were employed, firstly to check that none of the techniques employed resulted in the decarboxylation of Gla residues and secondly to test whether the light chains of X_1 and X_2 could be resolved by ion exchange chromatography. Taking the last point first, fig.1 shows that the light chains are readily separated by chromatography on DEAE-Sephadex and that the separation achieved is substantially better than the separation of Factor X_1 from Factor X_2, which would support the hypothesis that the difference in the chromatographic behaviour of the two forms of Factor X is caused by the heterogeneity of the light chains. Alkaline hydrolysis showed that pool 1 (fig.1) was the light chain of Factor X_1.

Amino acid analysis of the light chains after acid hydrolysis shows no differences in their composition

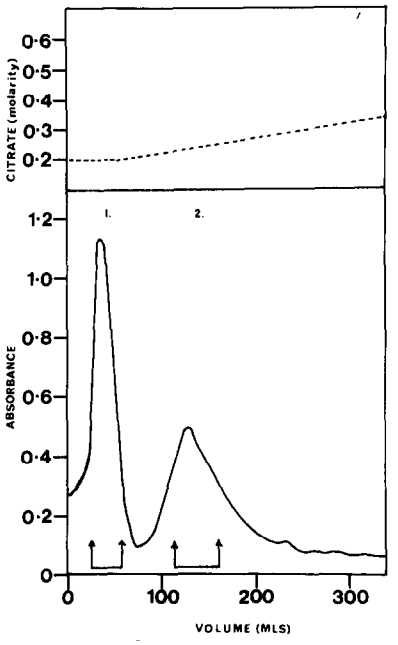

Fig.1. The separation of the light chains of Factor X_1 and Factor X_2 on a column of DEAE-Sephadex A-50 (1.5 × 20 cm). The protein was eluted at a flow rate of 40 ml/h with a linear gradient of 0.2–0.6 M sodium citrate pH 6.9 containing 1 mM dithiothreitol. The protein was pooled as indicated.

Table 1
Amino acid composition of the light chains of Factor X_1 and X_2 (moles per 17 000 g)

	Acid hydrolysates		
	X_1	X_2	X_1^a
Lys	6.8	6.6	7
His	2.9	2.8	3
Arg	7.1	7.0	8
Asp	13.4	13.2	14
Thr	5.8	6.2	6
Ser	8.6	8.6	11
Glu	23.6	23.7	27
Gly	13.6	14.0	14
Ala	6.3	6.3	6
Val	4.4	6.1	5
Ile	1.7	2.9	2
Leu	6.5	7.3	7
Tyr	1.9	2.7	3
Phe	6.3	5.4	8

	Alkaline hydrolysate			
	X_1		X_2	
	Route A	Route B	Route A	Route B
Glu	15.8	14.9	10.9	10.9
Gla	6.8	8.1	12.8	13.3

[a] Values taken from Enfield et al. [14].

(table 1) a result which confirms the findings of others [2,4]. However analysis of the amino acids released by alkaline hydrolysis revealed a considerable difference in the Glu and Gla content. Gla is not seen in acid hydrolysates because it is readily decarboxylated to Glu during the hydrolysis. Gla was identified in the alkaline hydrolysates by co-chromatography with an authentic sample of the amino acid, and was quantitated taking the ninhydrin colour value for glutamic acid. This was necessary because the colour value for the Gla standard decreased on storage, probably due to spontaneous cyclization to pyro γ-carboxyglutamic acid.

The results show (table 1) that while the sum of Gla and Glu residues obtained by alkaline hydrolysis is in good agreement with the number of Glu residues seen in acid hydrolysates; the Gla content of X_1 light chain is only 62% of X_2 light chain which implies X_2 has 5 more negative charges than X_1, an observation that is consistent with the difference in the chromatographic behaviour of the two chains.

It might be supposed that the reduced carboxylation of X_1 could be the result of a partial vitamin K deficiency in cattle. To test this two cows were given intramuscular injections of 10 mg vitamin K per day for 10 days prior to the collection of the blood. In both cases the Factor X was recovered in two separate peaks, as in the untreated animals, also like the untreated cattle the specific activity of X_1 was 64% that of X_2.

5. Discussion

At present it is difficult to understand why these two forms of Factor X circulate in the plasma, and when assayed using Russell's viper venom have a different specific activity. The possibility that the two forms of Factor X arise as an artefact of the initial purification process cannot be eliminated entirely. But the finding that the Glu:Gla ratio of X_1 and X_2 and the amounts of X_1 and X_2 are not altered by re-chromatography on DEAE-Sephadex would suggest that if these changes do take place during the preparation of Factor X they can only be associated with the blood collection or the adsorption on insoluble barium salts. Since this last step is used for the separation of Gla containing peptides from enzymic digests of prothrombin [8,9] it would seem to be unlikely that the adventitious decarboxylation of Factor X_1 occurs at this step. Since it is unlikely that Factor X_1 is decarboxylated during blood collection, it must be that X_1 and X_2 coexist in plasma.

Hitherto the only other indication that Factor X_1 and X_2 differed comes from measurements of the specific activity using Russell's viper venom. In both instances [2,3] the less carboxylated Factor X_1 has a lower specific activity than Factor X_2. Recently incomplete carboxylation of prothrombin [12] has been implicated in causing a reduction in the specific activity of that protein isolated from the blood of cattle during Warfarin treatment, as calcium ions are required both for the activation of Factor X and for prothrombin it would not be unreasonable to suppose that reduced specific activity of the Factor X_1, like abnormal prothrombin, arises from a slower rate of activation.

The thirteen γ-carboxyglutamic acid residues found for X_2 are in close agreement with the twelve modified

glutamic acid residues suggested by Enfield et al. [14] for X_1 and X_2. However these authors have not identified the γ-carboxyglutamic acid residues as such, but have based their interpretations on the basis of poor yields at certain cycles of the sequenator, and on sequence homology with prothrombin. Howard and Nelsestuen [10] have also concluded that the N-terminal portion of the light chain, prepared from a pool of X_1 and X_2 has fourteen γ-carboxyglutamic acid residues.

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