N-TERMINAL AMINO ACID SEQUENCE OF INTACT HUMAN FIBRINOGEN

Eckart IRION and Birger BLOMBÄCK
Department of Blood Coagulation Research, Karolinska Institutet. Stockholm, Sweden

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N-Terminal amino acid sequence analysis by Edman's phenylisothiocyanate method on intact human fibrinogen revealed the first seven amino acids of the γ-chain: H–Tyr–Val–Ala–Thr–Arg–Asp–Asn–.

1. Introduction

The fibrogen molecule is most likely composed of two identical subunits, each having a molecular weight of about 170,000 and 3 different peptide chains named α(A), β(B) and γ [1, 2]. By the action of thrombin, fibrinopeptide A is released from the α(A)-chain and fibrinopeptide B from the β(B)-chain. The γ-chain is not susceptible to hydrolysis by thrombin. The fibrinopeptides are located at the *N*-terminal end of the fibrinogen molecule [3, 4]. The amino acid sequences of the fibrinopeptides are known for several species [5]. Since the *N*-terminal amino acid of the human fibrinopeptide B is a pyroglutamyl residue [6] and therefore not degradable by the phenylisothiocyanate method [7], sequence analysis of the whole human fibrinogen should yield only two amino acids for each step, one belonging to the α(A)-chain and the other to the γ-chain. Since the *N*-terminal sequence of the α(A)-chain is known, the sequence of the γ-chain should be deducible by stepwise degradation of whole fibrinogen.

In the present study, radioactive phenylisothiocyanate-^{35}S was used for stepwise degradation of milligram quantities of human fibrinogen. This allowed a quantitative estimation of the PTH-amino acids and facilitated their identification on the thin-layer chromatograms. The results have been referred to in a previous publication [1].

Abbreviations:
PTH– = phenylthiohydantoin–
PTC– = phenylthiocarbamyl–

2. Materials and methods

Fibrinogen. A 1.82% solution of purified, dialyzed human fibrinogen (Fraction I-4) in 0.3 M NaCl was used [8]. Coagulability was around 98%.

Phenylisothiocyanate-^{35}S was obtained from the Radiochemical Centre, Amersham, England. (Cat. No SJ 95). Specific activity was 25–30 μCi/μmole. Before use freshly distilled carrier phenylisothiocyanate was added.

Thin-layer chromatography was performed in silicagel (0.5 mm) containing 0.5% starch, 0.1% EDTA and fluorescence indicator. The plates were 20 cm × 20 cm. The silica was Camag Type DF 5. The two-dimensional scanner of Berthold, Wildbad, Germany was used for localization and quantitation of radioactivity in the spots. The instrument is provided with equipment for automatic registration of radioactivity (counts per min) as well integration of the peaks.

Sequence analysis. The three-stage phenylisothiocyanate method of Edman [7, 9, 10] was used. The details of the procedure have been described elsewhere [4, 11]. To duplicate samples of 0.75 ml (13.7 mg) of fibrinogen solution was added 1 ml of pyridine and 0.5 N NaOH to adjust to the pH to 8.5–9. After flushing the mixture with nitrogen 25 μl of phenylisothiocyanate-^{35}S (specific activity: 2–3 μCi/μmole) was added. The mixture was incubated for 2 hr at room temperature. If necessary the pH was adjusted to 9 with NaOH. Washing of the PTC-protein with benzene (2–3 ml), precipitation with acetone (5 ml) and washing with aqueous acetone (3 ml) was a previously des-

cribed [11]. The lyophilized PTC-protein was subjected to cleavage in trifluoroacetic acid (0.1 ml) at 40° for 20 min and the residual protein precipitated with ethylenechloride (3 ml) [4]. The thiazolinones contained in the ethylenechloride phase were subsequently converted to PTH-derivatives in N HCl (0.3 ml) at 80°. The PTH-amino acids were extracted from the aqueous solution with ethylenechloride [4]. The solvent was evaporated and the PTH-amino acids dissolved in 90% acetic acid (25 μl) for thin-layer chromatography. PTH-derivatives left in the water-phase were identified by electrophoresis. The protein residue left after extraction with ethylenechloride was lyophilized and used for the next degradation cycle.

In the second and following degradation cycles dimethyl-allylamine (DMAA) buffer, pH 9.5, containing pyridine was used for the coupling reaction [4]. The protein was suspended in 1 ml of buffer. It was rather insoluble in the solvent but could be suspended evenly by crushing and vigorous "whirl-mixing". After coupling with phenylisothiocyanate-^{35}S the samples were washed 5 times with benzene (about 1.5 ml). The water-phase was lyophilized and the residues extracted with ethyl-acetate (0.5 ml, 3 times) [6]. Next the residual PTC-protein was cleaved with trifluoroacetic acid. This and the following procedure were the same as described above for the first degradation cycle.

PTH-^{35}S-tyrosine standard. A PTH-^{35}S-tyrosine standard was prepared from 0.5 μmoles of tyrosine using the same radioactive phenylisothiocyanate-^{35}S and essentially the same conditions as described above for the sequence analysis in the second degradation cycle. However, because of the solubility properties of PTC-tyrosine washing with ethyl-acetate was omitted. After thorough washing with benzene the water-phase was lyophilized. The PTC-derivative was converted to the PTH-derivative in HCl as described above.

Thin-layer chromatography and electrophoresis. Aliquots (1–3 μl) of the samples, PTH-^{35}S-tyrosine standard and reference PTH-amino acids were chromatographed on thin-layers of silica gel. Solvents D and E of Edman and Sjöquist [12] and II and III of Brenner et al. [13] were generally used. To distinguish PTH-valine from PTH-phenylalanine system V of Jeppsson and Sjöquist [14] was used.

After drying the plates were mounted on the scanner table. The radioactivity (counts per min) was registered and integrated. The yields of the unknown PTH-^{35}S-amino acid samples were calculated from the PTH-^{35}S-tyrosine standard curve.

After scanning, the plates were checked under UV-light, sprayed with the iodine azide reagent [15] and photographed. By comparison with the reference PTH-amino acids the unknown PTH-amino acids could be unequivocally identified.

The aqueous phases were examined by means of paper electrophoresis [16] for demonstration of PTH-derivatives of histidine and arginine. No quantitation of those amino acids was attempted.

3. Results

Intact human fibrinogen, degraded stepwise by the Edman method through seven cycles, yielded as expected two major amino acids for each step. Since one set corresponded exactly to the known *N*-terminal sequence of the fibrinopeptide A, the *N*-terminal sequence of the γ-chain could be easily deduced as:

H–Tyr–Val–Ala–Thr–Arg–Asp–Asn– (table 1).
1 2 3 4 5 6 7

Traces of other PTH-amino acids were also observed in each cycle. These possibly derived from the chain variant α(AY) [6]. However, only the residues of the α(A)- and α(AP)-chain have been quantitatively determined. Other unidentifiable derivatives were occasionally observed. The latter, with two exceptions, did not influence the quantitative determination, since they could be chromatographically separated from the main PTH-amino acids in at least one of the solvent systems. In step 3, multiple radioactive spots due to decomposition products of serine were found, thus rendering quantitative determination of serine in the α(A)-chain impossible. Quantitation of PTH-alanine from the γ-chain however, seemed not to be influenced by the degradation products (table 1). In step 4, about 2/3 of the threonine was present as PTH-dehydrothreonine. This could not be determined accurately since it was not in any of the solvents completely separated from other side reaction products. The PTH-derivative of arginine was found in the aqueous phase of step 5. The electrophoretic mobility and the positive Sakaguchi reaction indicated arginine. The yield of the PTH-glycine in the sixth degradation cycle seems to be somewhat high. It is likely that monophenylthiourea which has about the same R_f-value as glycine is inter-

Table 1
Stepwise degradation of intact human fibrinogen.

Step	α(A)-Chain			γ-Chain		
	PTH-amino acid	Total[a] nmoles	μmoles/ 170 mg	PTH-amino acid	Total[a] nmoles	μmoles/ 170 mg
1	Ala	36.0 (4)	0.45	Tyr	66.0 (4)	0.82
2	Asp	23.9 (6)	0.30	Val	60.7 (17)	0.75
3	Ser	b	–	Ala	41.1 (12)	0.51
4	Gly	27.5 (6)	0.34	Thr[c]	14.0 (6)	0.17
5	Glu	18.8 (6)	0.23	Arg	present[d]	–
6	Gly	42.1 (5)	0.52	Asp	28.5 (5)	0.35
7	Asp	27.3 (8)	0.34	Asn	19.1 (8)	0.24

[a] mean values of 2 samples, numbers of scannings in parenthesis.
[b] quant. determination not possible.
[c] PTH-dehydrothreonine not determined.
[d] not quantitatively determined.

fering in this step. Beginning at step 4, some overlapping between successive steps was observed. The quantitative estimation, however, allowed a clear distinction of the main PTH-amino acids.

4. Discussion

The existing evidence [1, 3, 4, 17] that fibrinopeptide A is an *N*-terminal fragment of fibrinogen is further substantiated by the present findings. Stepwise degradation of the intact fibrinogen molecule resulted in residues corresponding to the first seven amino acids of the fibrinopeptide A. Furthermore, the first seven amino acids of the γ-chain could be deduced. This information made it possible to identify the *N*-terminal γ-chain fragment in fibrinogen degraded with cyanogen bromide [1, 18].

The *N*-terminal portions of fibrinopeptides A and B of various mammals show a high variability in sequence [5]. The γ-chain may have a more constant structure in the *N*-terminal region. *N*-terminal tyrosine is found in fibrinogen of at least seven mammalian species [11]. Pirkle et al. [19] have found in the γ-chain of bovine fibrinogen the same sequence for seven residues as in human fibrinogen. In the corresponding portion of the γ-chain of pig fibrinogen there was only one substitution, glutamic acid substituted for aspartic acid at position 6.

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