Isolation of thermostable structure from the fibrinogen D fragment

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1. INTRODUCTION

According to the current concept, fibrinogen consists of 3 large structural blocks regarded as domains [1]. Judging by electron microscopy studies these domains are attached by rod-like connectors [2,3] which were supposed to have a coiled-coil structure [4]. The regions with a specific sequence for coiled-coils were indeed revealed in the fibrinogen polypeptide chains between the two 'disulfide rings' at each half of the molecule [5,6]. Our denaturation studies showed that the D fragment of fibrinogen, corresponding to both its terminal parts, consists of 2 structures with a drastically different stability [7]. It was tempting to find whether one of these structures, namely the thermostable one, represents the hypothetical coiled-coil connector of fibrinogen domains. We isolated this thermostable structure of the D fragment and showed that it includes a 'disulfide ring' and has a high α-helix content; i.e., it is probably the connector which was expected to exist.

2. MATERIALS AND METHODS

Bovine fibrinogen was prepared from oxolate plasma by salting out with sodium sulphate [8]. The early D_H fragment (M_r 95 000) was isolated according to [9] by ion-exchange chromatography and subsequent gel filtration on Sephadex G-200 from a 4 h tryptic hydrolyzate of fibrinogen. Hydrolysis was performed in 0.1 M sodium phosphate (pH 7.0) in the presence of 4.4×10^{-4} M $CaCl_2$; the ionic strength was adjusted to 0.2 by the addition of NaCl. Protein was 15 mg/ml and enzyme/substrate was 1:2500 (w/w).

The M_r of the studied fragment was determined by high-speed equilibrium ultracentrifugation according to [10] and by SDS–polyacrylamide gel electrophoresis according to [11].

The concentration of the fragment in the solution was determined by the nitrogen content measured in the stock solution on a Perkin-Elmer 240 B elemental analyser.

Spectrophotometric measurements were done on a Beckman M25 spectrophotometer. The CD spectrum was determined on the Jasco J-41 A spectropolarimeter. The α-helicity was calculated according to [12].

Calorimetric studies were carried out on a scanning microcalorimeter DASM-1M [13] at a heating rate of 1 K/min and a solution concentration of 1–2 mg/ml. The partial molar heat capacity and enthalpy of thermal transition of the fragment were determined as in [14].

3. RESULTS AND DISCUSSION

Fig.1 presents the calorimetrically obtained heat capacity function of the D_H fragment illustrating that the intensive heat absorption connected with its melting proceeds in 2 separate temperature regions. It follows from the observed melting profile that the D_H fragment must consist of 2 parts which drastically differ in thermostability. This in its turn indicates a pathway for the isolation of the assumed parts of the fragment. As evident, the part with a lower thermostability can be easily eliminated if the fragment is treated with the proteolytic enzyme under conditions in which this part is un-

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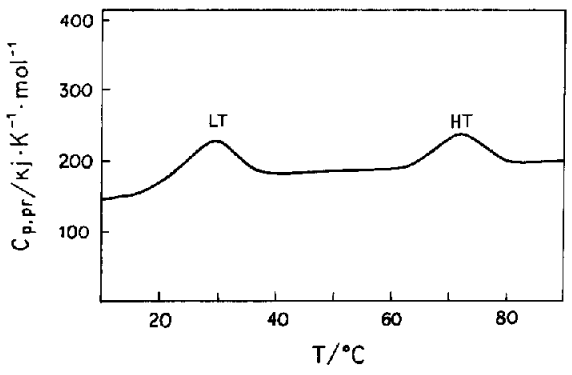

Fig.1. Temperature dependence of the partial molar heat capacity of the D_H fragment in 0.05 M glycine (pH 2.8): LT and HT – low and high temperature transitions.

folded while the thermostable one is compact; i.e., at >20°C and pH <2.8. Since this condition is optimal for pepsin activity, the following procedure has been suggested: the D_H fragment in 0.05 M glycine (pH 2.5) was incubated with pepsin at 25°C (fragment conc. 7 mg/ml, enzyme/substrate 1:200 (w/w), pepsin act. 2580 unit/mg). Hydrolysis was terminated by addition of 1.0 M glycine (pH 9.0) up to a final pH 8.0. The time course of hydrolysis under these conditions is shown in fig.2a. The 2.5 h hydrolysate was fractionated on a 1.5 × 65 cm column of Sephadex G-75 Superfine equilibrated by 0.05 M KH_2PO_4 (pH 7.0), 0.1 M NaCl. The first fraction corresponding to the first band on the SDS electrophoretic pattern (fig.2a) was isolated and studied by various physicochemical methods.

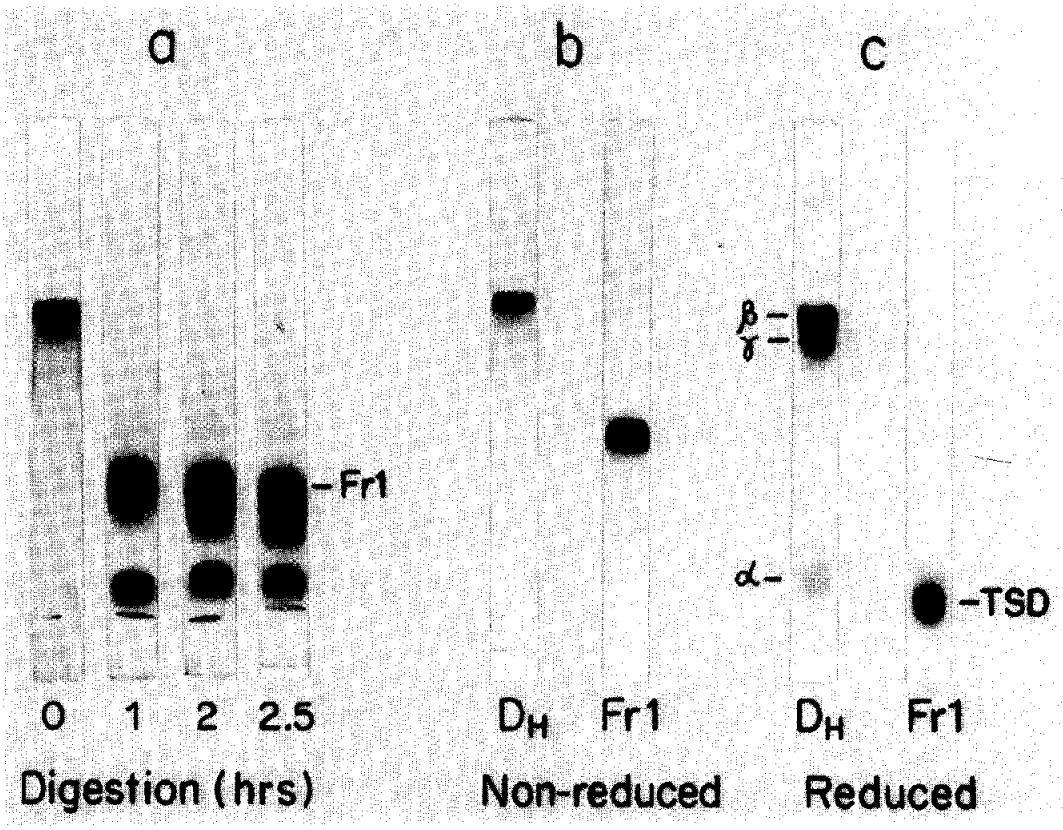

Fig.2. SDS–polyacrylamide gel electrophoretic patterns of timed peptic digests of the D_H fragment (a) and patterns of D_H and fraction I in non-reduced (b) and reduced (c) conditions. β, γ, α the bands of the corresponding polypeptide chains of the D_H fragment.

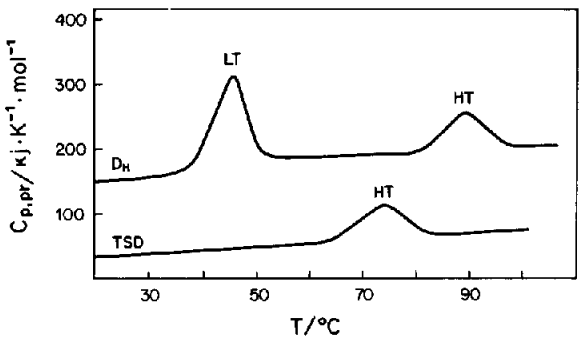

Fig.3. Temperature dependence of the partial molar heat capacity of the D_H and fraction I (TSD) fragments in 0.05 M glycine (pH 3.5).

A calorimetric study showed that fraction I undergoes a reversible co-operative transition at heating with a significant heat absorption and heat capacity change (fig.3); in the solution with pH 3.5 the following values have been obtained: T_m = 74.0°C, $\Delta_m H$ = 350 kJ . mol^{-1} and $\Delta_m C_p$ = 7.1 kJ . K^{-1} . mol^{-1}. These melting temperatures and enthalpy values are somewhat lower than those found in the same conditions for the high temperature process of the D_H fragment: T_m = 89.2°C; $\Delta_m H$ = kJ . mol^{-1}. But the important point is that the extrapolation of the enthalpy value for the isolated fragment I to 89.2°C, by the equation:

$$\Delta H(T) = H(T_m) + \int_{T_m}^{T} \Delta_m C_p dT$$

gives 460 kJ . mol^{-1}, which is close to the value for the high temperature process in the D_H fragment, we can conclude that the isolated fragment corresponds almost completely to a structure which melts in the D_H fragment at higher temperatures; i.e., to its thermostable part. Therefore, we have denoted the isolated thermostable portion of the D_H fragment as TSD fragment.

Each of the D_H and TSD fragments show a single band on the SDS electrophoretic pattern corresponding to M_r 95 000 and 28 000, respectively (fig.2b). The same M_r has been obtained by equilibrium ultracentrifugation.

It is known that the D_H fragment consists of 3

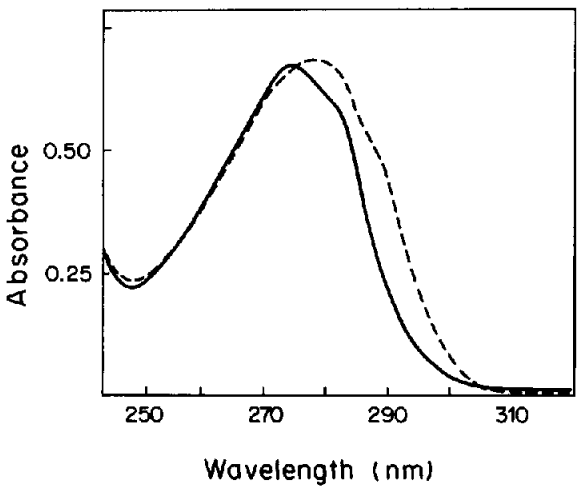

Fig.4. Absorption spectra of the D_H (---) and TSD (——) fragments in 0.05 M KH_2PO_4, 0.1 M NaCl (pH 7.0).

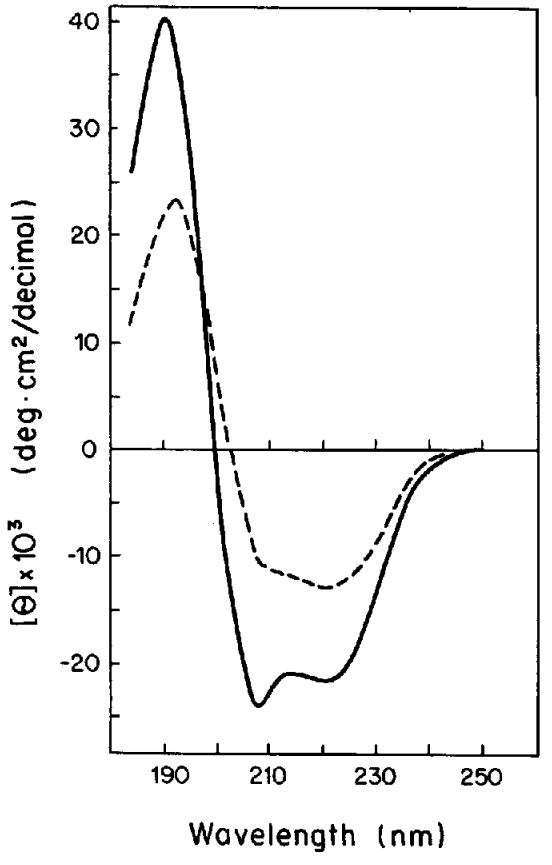

Fig.5. Circular dichroism spectra of the D_H (---) and TSD (——) fragments in 0.05 M KH_2PO_4 (pH 7.0), 0.1 M NaCl.

unequal polypeptide chains α, β and γ, held together by a 'disulfide ring' [5,15,16]. Correspondingly, the SDS electrophoretogram of the D_H fragment with the reduced S–S bonds obtained in the presence of 0.2% β-mercaptoethanol shows 3 bands (fig.2c). The SDS electrophoretogram of the TSD fragment in reducing condition gives only one band but it corresponds to an M_r thrice smaller than that of the TSD fragment without β-mercaptoethanol. It follows that the TSD fragment consists of 3 polypeptide chains of equal size held together by disulfide crosslinks; i.e., this fragment includes a 'disulfide ring'. It is noteworthy also that the chains in the TSD fragment are about the same size as the α-chain in the D_H fragment. Therefore, preparation of the TSD fragment from D_H is connected with the reduction mainly of β- and γ-chains to the size of the α-chains.

Spectrophotometric studies of the TSD fragment have shown that its light absorption spectrum is of the tyrosine type with the maximum at 275 nm and extinction $E^{1\%}_{275} = 8.0$ (fig.4). According to the fibrinogen chemical structure [5,17,18] only the N-terminal part of the D fragment with the 'disulfide ring' does not include a tryptophan residue. Therefore it is likely that the TSD fragment originates from this part of the D_H fragment.

The α-helicity determined from the CD spectra (fig.5) was found to be 65% for the TSD fragment and only 44% for the D_H fragment. Thus, the α-helical conformation seems to be concentrated mostly in this N-terminal part of the D_H fragment. This experimental fact confirms the model, suggested [5] on the basis of a theoretical analysis of the fibrinogen sequence, according to which this part of the molecule, enclosed between two 'disulfide rings' has an α-superhelical conformation. From this model the M_r of the superhelical part of the D fragment should be 15 000–20 000; i.e., this part composes 50–70% of the whole TSD fragment. The close correspondence of this estimate of the size of the superhelical part of the TSD fragment with its experimentally found helicity leaves little doubt that the isolated TSD fragment serves as a part of superhelical stretch connecting the central and terminal regions in the intact fibrinogen molecule.

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