

# Neutron scattering study of the ( $\gamma$ -B) catalytic domains of complement proteases C1r and C1s

Giuseppe Zaccari<sup>1</sup>, Catherine A. Aude<sup>2</sup>, Nicole M. Thielens<sup>2</sup> and Gérard J. Arlaud<sup>2</sup>

<sup>1</sup>CNRS URA 1333, Institut Laue Langevin, 156X, 38042 Grenoble Cedex, France and <sup>2</sup>Unité INSERM 238, Département de Recherche Fondamentale, Laboratoire d'Immunochimie, Centre d'Etudes Nucléaires de Grenoble, 85X, 38041 Grenoble Cedex, France

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The catalytic domains of C1r and C1s, comprising the C-terminal region of the A chain ( $\gamma$ ), disulphide-linked to the B chain, were obtained by limited proteolysis of the native proteases with chymotrypsin and plasmin, respectively, and studied by small angle neutron scattering. For C1s ( $\gamma$ -B), a molar mass of  $45\,000 \pm 5\,000$  g/mol, and a relatively large radius of gyration ( $R_g$ ) of  $28 \pm 1$  Å were determined, excluding a single globular domain. The corresponding values for C1r ( $\gamma$ -B)<sub>2</sub> (90,000 g/mol,  $R_g = 34 \pm 1$  Å) are consistent with a dimer involving the loose packing of two ( $\gamma$ -B) subunits. Various models of the dimer are discussed in the light of neutron scattering and other data.

Complement system; Serine protease; C1r; C1s; Protein domain; Neutron scattering

## 1. INTRODUCTION

The catalytic subunit of human C1, the first component of the classical pathway of complement, is a  $\text{Ca}^{2+}$ -dependent tetrameric association (C1s-C1r-C1r-C1s) of two serine proteases, C1r and C1s, which are sequentially activated upon binding of C1 to activators [1–3]. Activation occurs in both cases through cleavage of a single Arg-Ile bond [4,5], that converts single chain proenzymes into active enzymes (C1r, C1s) comprising two disulphide-linked chains, A (N-terminal) and B (C-terminal). Each monomeric protease is thought to be organized in at least two functional domains or regions: (i) an interaction region ( $\alpha$ ) corresponding to the N-terminal half of the A chain, responsible for  $\text{Ca}^{2+}$ -dependent C1r-C1s interaction; (ii) a catalytic domain, comprising the intact B chain and the C-terminal  $\gamma$  region of the A chain, connected to each other through a single disulphide bridge (Fig. 1). The B chains are homologous to trypsin and chymotrypsin, whereas regions  $\gamma$  comprise two structural motifs (IV and V). The catalytic regions have initially been considered as domains on the basis of their resistance to proteolysis and their globular appearance in electron microscopy. Thus, treatment of C1s with plasmin yields a ( $\gamma$ -B) fragment that is visualized as a monomeric globular structure [6], whereas limited pro-

teolysis of the native C1r-C1r dimer [6–8] ends in a truncated ( $\gamma$ -B)<sub>2</sub> molecule visualized as twin domains. However, recent studies by differential scanning calorimetry [9] indicate that the ( $\gamma$ -B) fragment of C1s comprises three independently folded domains, two in the  $\gamma$  region (motifs IV and V) and one in the catalytic B chain.

With a view to obtain further information about their shape and structural organization, the isolated C1s ( $\gamma$ -B) and C1r ( $\gamma$ -B)<sub>2</sub> fragments were studied by small angle neutron scattering. The neutron scattering curve at small angles from a protein solution provides *simultaneous* and *independent* measurements of the molar mass of the particle formed by the protein in the particular solvent, and of its radius of gyration, and therefore an indication of the shape.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Human plasmin was obtained from Kabi Vitrum, Stockholm, Sweden, and bovine  $\alpha$ -chymotrypsin (type IV) was from Sigma. Activated C1r and C1s were isolated from human plasma as described previously [10].

### 2.2. Isolation of the fragments by limited proteolysis

C1r was first blocked with iPr<sub>2</sub>-P-F as described in [8], and iPr<sub>2</sub>-P-C1r (0.5 mg/ml) in 145 mM NaCl/50 mM triethanolamine hydrochloride (pH 7.4) was submitted to limited proteolysis with chymotrypsin (4%, w/w) for one hour at 30°C. The reaction was stopped by addition of iPr<sub>2</sub>-P-F to a final concentration of 1 mM, and fragment ( $\gamma$ -B)<sub>2</sub> was purified by high-pressure gel permeation as described [8]. C1s (1.0 mg/ml) in 145 mM NaCl/50 mM triethanolamine hydrochloride (pH 7.4) was cleaved with plasmin (5%, w/w) for 90 min at 37°C, and the C1s ( $\gamma$ -B) fragment was purified by high-pressure hydrophobic interaction chromatography as described [11]. Based on identification by N-terminal sequence analysis [8,11] of the cleavage sites of chymotrypsin and plasmin

*Correspondence address:* G.J. Arlaud, Département de Recherches Fondamentales, Laboratoire d'Immunochimie, Unité INSERM 238, CEN-Grenoble 85X, 38041 Grenoble Cedex, France

*Abbreviations:* The nomenclature of complement components is that recommended by the World Health Organization; activated components are indicated by a superscript bar, e.g. C1r. iPr<sub>2</sub>-P-F, diisopropylphosphorofluoridate

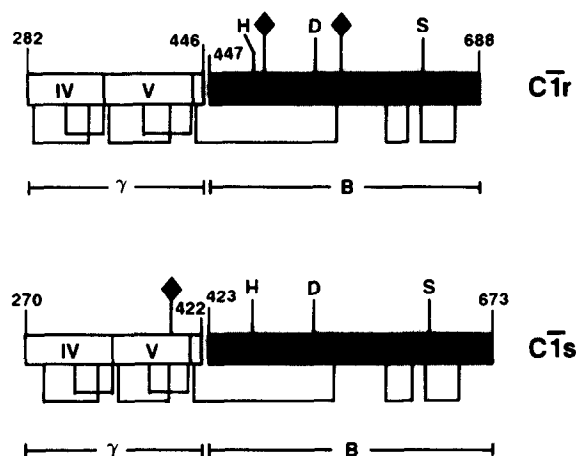


Fig. 1. Schematic representation of the linear structures of the  $\gamma$ -B fragments obtained by limited proteolysis with chymotrypsin (C1r) and plasmin (C1s). The disulphide bridge patterns, and the positions of the N-terminal and C-terminal residues of the B chains and  $\gamma$  fragments are indicated. (H, D, S), histidine, aspartic acid and serine residues of the active site. (♦) Asn-linked carbohydrates.

(Fig. 1), the  $M_r$  of the ( $\gamma$ -B) fragments of C1r and C1s were calculated from the amino acid sequences [12–18], plus 2000 for each carbohydrate moiety, and estimated as 50300 and 46600, respectively. Protein concentrations in the samples were measured using values of  $E_{280,1\%}^{1\text{cm}} = 14.5$  for C1r ( $\gamma$ -B)<sub>2</sub> and 18.3 for C1s ( $\gamma$ -B), which were determined experimentally by amino acid analysis of protein solutions of known absorbance at 280 nm.

### 2.3. Neutron scattering

Experiments were done on the D11 camera at the Institut Laue Langevin [19]. The wavelength ( $\lambda$ ) was set at 10 Å with a  $\Delta\lambda/\lambda$  of 8%, and the sample detector distance was 2.80 m. This gave an observable  $Q$  range of  $0.013 \leq Q \leq 0.076 \text{ Å}^{-1}$ , where the scattering parameter  $Q = (4\pi \sin \theta)/\lambda$ , and  $\theta$  is one-half the scattering angle. Samples were placed in quartz cuvettes of optical pathlength 0.100 cm and the temperature was controlled at 6°C. Times of sample exposure to the beam varied between 15 min and a few hours depending on the signal-to-noise ratio.

The scattered intensity,  $I(Q)$ , obtained after subtraction of the background due to the solvent alone in a similar cell, and calibration by the scattering of 0.100 cm of H<sub>2</sub>O [20], was interpreted in the Guinier approximation [21]:

$$\ln I(Q) = \ln I(0) - (1/3)R_g^2 Q^2 \quad (1)$$

from which the two independent parameters  $I(0)$  and  $R_g^2$  were calculated. In the case of a monodisperse solution, in which there is no correlation between the positions or the orientations of the particles, the two parameters have the following meanings:

$$I(0) = (M_2 c_2 / N_A) (b_2 - \rho^0 \bar{v}_2)^2 \quad (2)$$

and

$$R_g^2 = [\sum_i (b_i - \rho^0 v_i) / \sum_i (b_i - \rho^0 v_i)]^2 \quad (3)$$

where  $M_2$  is the molar mass of the particle in solution (g/mol);  $c_2$  is the concentration of macromolecule in g/cm<sup>3</sup>;  $N_A$  is Avogadro's number (mol<sup>-1</sup>);  $b_2$  (cm/g) is the scattering length per gram of macromolecule;  $\bar{v}_2$  is the partial specific volume of the macromolecule (cm<sup>3</sup>/g);  $b_i$  (cm),  $v_i$  (cm<sup>3</sup>) are the scattering length and volume, respectively, of the small volume  $i$  in the particle;  $r_i$  (cm) is the distance of this volume to the center of mass of the  $(b_i - \rho^0 v_i)$  distribution (contrast);  $V$  is the total volume of the particle;  $\rho^0$  (cm<sup>-2</sup>) is the scattering length density of the solvent; the summation should be over the entire particle [22]. The values of  $(b_2 - \rho^0 \bar{v}_2)$  required for

Table I

Neutron scattering data and chemical parameters of the proteins

Protein	$I(0)/c^a$ (cm <sup>2</sup> /g)	$R_g^a$ (Å)	$(b_2 - \rho^0 \bar{v}_2)^b$ (10 <sup>9</sup> cm/g)
C1r ( $\gamma$ -B) <sub>2</sub>	$0.050 \pm 0.005$	$34 \pm 1$	17.8
C1s ( $\gamma$ -B)	$0.025 \pm 0.002$	$28 \pm 1$	18.2

<sup>a</sup> Values of  $I(0)/c$  (on an absolute scale) and  $R_g$  from the experimental data in the 0.15 M NaCl buffer

<sup>b</sup>  $(b_2 - \rho^0 \bar{v}_2)$  values calculated from the chemical compositions of the proteins and solvent (see text). The partial specific volumes  $\bar{v}_2$  estimated from the amino acid and sugar compositions were 0.758 and 0.748 cm<sup>3</sup>/g for the protein moieties of C1r ( $\gamma$ -B)<sub>2</sub> and C1s ( $\gamma$ -B), respectively, and 0.60 cm<sup>3</sup>/g for the sugars. The value of  $\rho^0 = 5.49 \times 10^9 \text{ cm}^{-2}$  was calculated for the 0.15 M NaCl buffer. Estimated uncertainties in  $I(0)/c$  and  $R_g$  include systematic errors in the concentration determination and background subtraction

the analysis were calculated from the chemical compositions of the proteins and of the solvent (Table I). The radius of gyration of excess scattering amplitude obeys the same laws as its mechanical counterpart. The parallel axes theorem applies. It states that the  $R_g^2$  of a body about any axis  $A$  is equal to  $R_{\text{com}}^2 + D^2$ , where  $R_{\text{com}}$  is its radius of gyration about an axis parallel to  $A$  through its center of mass and  $D$  is the distance between  $A$  and the center of mass.

### 2.4. Solvent conditions

The choice of solvent will affect the value of  $\rho^0$  (and therefore of the contrast) and the background due to incoherent scattering which dominates the solvent contribution to the scattering. Replacing H<sub>2</sub>O by D<sub>2</sub>O in the protein solvent increases the contrast enormously and also reduces the background. However, D<sub>2</sub>O may not be a suitable solvent because it is known to strengthen hydrophobic effects thus favouring protein aggregation [23].

## 3. RESULTS AND DISCUSSION

### 3.1. Solvent conditions

Initial neutron scattering studies of the C1r ( $\gamma$ -B)<sub>2</sub> fragment were performed in H<sub>2</sub>O and D<sub>2</sub>O buffers (145 mM NaCl/50 mM triethanolamine hydrochloride, pH 7.4). The data collected in both cases were not satisfactory due to the low solubility of the protein at neutral pH (0.4–0.5 mg/ml) and to its marked tendency to form aggregates in D<sub>2</sub>O. Further comparative studies on C1r ( $\gamma$ -B)<sub>2</sub> and C1s ( $\gamma$ -B) were conducted in H<sub>2</sub>O buffer at alkaline pH (0.15 M NaCl/0.1 M Tris, pH 10.0). Both fragments showed increased solubility in this solvent (1.5–4.5 mg/ml) and were expected to retain a functional conformation, as judged from the fact that the ability of proenzyme C1r ( $\gamma$ -B)<sub>2</sub> to self-activate is not modified at pH 10.0 [24].

### 3.2. Neutron scattering

The Guinier plots of the proteins are shown in Fig. 2. The  $I(0)/c$  values interpreted by using the parameters in Table I and Eqn. 2 yielded molar masses of  $45000 \pm 5000$  and  $90000 \pm 8000$  g/mol for C1s ( $\gamma$ -B) and C1r ( $\gamma$ -B)<sub>2</sub>, respectively, in good agreement with the calculated values of the monomer and dimer, respec-

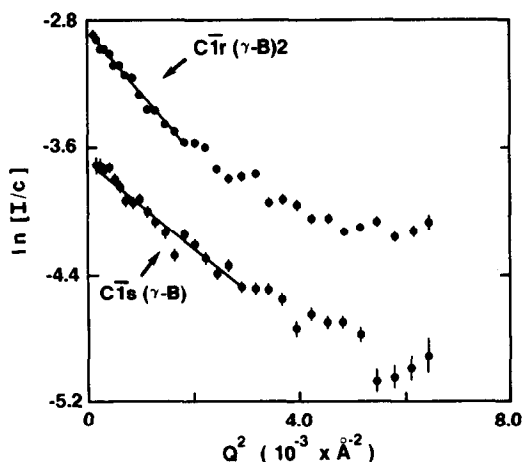


Fig. 2. Neutron scattering data plotted in the Guinier approximation for CTr (γ-B)<sub>2</sub> and CTr (γ-B). See text for experimental conditions.

tively. This confirmed that the solutions were monodisperse in these conditions. The radii of gyration found were  $28 \pm 1$  Å and  $34 \pm 1$  Å for CTr (γ-B) and CTr (γ-B)<sub>2</sub>, respectively. These are much larger than the values expected for monodomain globular proteins of the same mass (Fig. 3). Data collected on CTr (γ-B)<sub>2</sub> in 2 M NaCl (same buffer as above, pH 10.0) showed no significant difference in either  $R_g$  or  $I(0)/c$ . Thus, these high salt concentration conditions, which inhibit activation of the proenzyme form of CTr (γ-B)<sub>2</sub> [24], do not dissociate the dimer or cause gross conformational changes.

### 3.3. Models

The proteins were modelled on the basis of the following information: (a) the mass, radius of gyration values and the parallel axes theorem; (b) the assumption that the (γ-B) subunits have the same  $R_g$  in CTr and CTr; (c) the B chains have a structure similar to that of chymotrypsin ( $R_g = 17$  Å,  $M_r = 25000$ ) [25,26]; (d) the

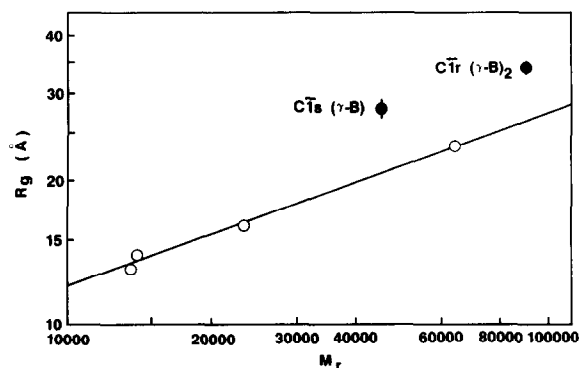


Fig. 3. Dependence of the radii of gyration of proteins on their molecular weights. The line ( $R_g^2 = \text{constant} \times M_r$ ) in logarithmic coordinates is for globular proteins such as lysozyme ( $R_g = 13 \pm 0.5$  Å), ribonuclease ( $14 \pm 0.5$  Å), papain ( $16 \pm 0.5$  Å), and hemoglobin ( $23.5 \pm 0.5$  Å) (neutron data collected by us and reference [27]). Data points are shown for CTr (γ-B) and CTr (γ-B)<sub>2</sub>.

γ regions comprise two domains, IV and V, of respective  $M_r$  about 8000 and 11000.

For a particle made up of two components of mass  $m_1$ ,  $m_2$  and radius of gyration  $R_1$ ,  $R_2$ , the parallel axes theorem states that:

$$mR_g^2 = m_1R_1^2 + m_2R_2^2 + m_1m_2D_{12}^2$$

where  $m$  is the total mass,  $R_g$  is the radius of gyration of the particle and  $D_{12}$  is the distance between the centres of mass of the components. In the case of scattering data, the  $m$  values are equivalent to  $(\sum_i b_i - \rho^0 V)$  values. The equation was applied to CTr (γ-B) using values in Table I and with  $R_g = 28$  Å,  $R_1 = 17$  Å (B), and  $R_2 =$  between 15.5 Å and 20 Å (γ) (this broad range was taken to allow for different arrangements of the two components of γ).  $D_{12}$  was calculated to be 46 Å for  $R_2 = 15.5$  Å (i.e. an approximately globular γ component) and 43 Å for  $R_2 = 20$  Å (a very elongated γ component). The equation was then applied to CTr (γ-B)<sub>2</sub> with  $R_g = 34$  Å,  $R_1 = R_2 = 28$  Å and  $m_1 = m_2 =$  half the mass of (γ-B)<sub>2</sub>, to yield a distance of 46 Å between (γ-B) protomers.

The models tested are shown in Fig. 4. The measured  $R_g$  (28 Å) excludes a monodomain globular shape for CTr (γ-B) (model A) which would have a radius of gyration of approximately 20 Å (for  $M_r = 45000$ ; Fig. 3). The data are in agreement with models (B) and (C). Both these models have a larger  $R_g$  than the globular protein arising from a separation between the centres of mass of the γ and B domains. A separation of 43–46 Å yields an  $R_g$  value in agreement with the measured one. Model (C) is consistent with the report [9] indicating that γ is made up of two independently folded domains. For CTr (γ-B)<sub>2</sub>, the neutron data exclude models (D) and (E) in which the monomers interact end-to-end, which have calculated  $R_g$  values of 49 Å and 57 Å, respectively. The neutron results are compatible with models (F) and (G). Both these models represent a loose packing of the (γ-B) subunits with 46 Å between their

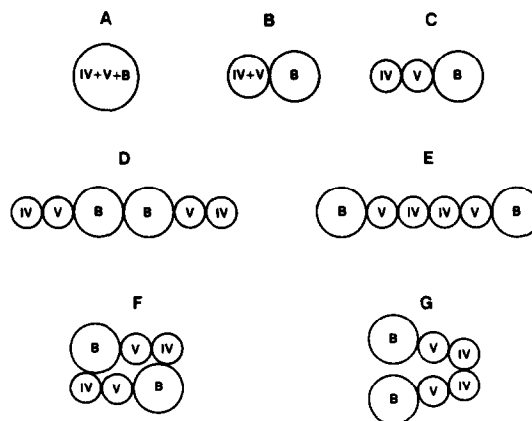


Fig. 4. Models for CTr (γ-B) (A, B, C) and CTr (γ-B)<sub>2</sub> (D, E, F, G). The data are only compatible with (B) and (C) for (γ-B), and (F) and (G) for (γ-B)<sub>2</sub>. See text for discussion.

centres of mass, a value similar to the distance between the centres of mass of  $\gamma$  and B in the monomer. Model (F) is consistent with the 'head-to-tail' structure proposed on the basis of chemical cross-linking [8]. Model (G) is consistent with electron micrographs of the protein obtained by Weiss et al. [7,28]. Further studies are necessary in order to discriminate between the two alternative models.

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