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Neutron scattering study of the (γ-B) catalytic domains of complement proteases C$\bar{1}$r and C$\bar{1}$s

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The catalytic domains of C$\bar{1}$r and C$\bar{1}$s, comprising the C-terminal region of the A chain (γ), 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 C$\bar{1}$s (γ-B), a molar mass of 45 000 ± 5000 g/mol, and a relatively large radius of gyration (R_g) of 28 ± 1 Å were determined, excluding a single globular domain. The corresponding values for C$\bar{1}$r (γ-B)$_2$ (90,000 g/mol, $R_g = 34 \pm 1$ Å) are consistent with a dimer involving the loose packing of two (γ-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 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 (C$\bar{1}$r, C$\bar{1}$s) 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 (α) corresponding to the N-terminal half of the A chain, responsible for Ca^{2+}-dependent C1r-C1s interaction; (ii) a catalytic domain, comprising the intact B chain and the C-terminal γ 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 γ 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 C$\bar{1}$s with plasmin yields a (γ-B) fragment that is visualized as a monomeric globular structure [6], whereas limited proteolysis of the native C$\bar{1}$r-C$\bar{1}$r dimer [6–8] ends in a truncated (γ-B)$_2$ molecule visualized as twin domains. However, recent studies by differential scanning calorimetry [9] indicate that the (γ-B) fragment of C$\bar{1}$s comprises three independently folded domains, two in the γ 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 C$\bar{1}$s (γ-B) and C$\bar{1}$r (γ-B)$_2$ 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.

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Abbreviations: The nomenclature of complement components is that recommended by the World Health Organization; activated components are indicated by a superscript bar, e.g. C$\bar{1}$r. iPr$_2$P-F, diisopropylphosphorofluoridate

2. MATERIALS AND METHODS

2.1. *Materials*

Human plasmin was obtained from Kabi Vitrum, Stockholm, Sweden, and bovine α-chymotrypsin (type IV) was from Sigma. Activated C$\bar{1}$r and C$\bar{1}$s were isolated from human plasma as described previously [10].

2.2. *Isolation of the fragments by limited proteolysis*

C$\bar{1}$r was first blocked with iPr$_2$-P-F as described in [8], and iPr$_2$-P-C$\bar{1}$r (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$_2$-P-F to a final concentration of 1 mM, and fragment (γ-B)$_2$ was purified by high-pressure gel permeation as described [8]. C$\bar{1}$s (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 C$\bar{1}$s (γ-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

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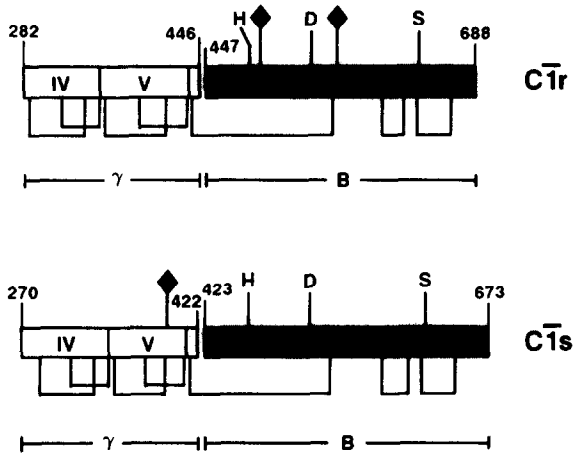

Fig. 1. Schematic representation of the linear structures of the γ-B fragments obtained by limited proteolysis with chymotrypsin (C1r) and plasmin (C1s). The disulphide bridge patterns, and the positions of the N-terminal and C-terminal residues of the B chains and γ fragments are indicated. (H, D, S),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

(Fig. 1), the M_r of the (γ-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 $E_{280,1\%}$ = 14.5 for C1r $(\gamma\text{-B})_2$ and 18.3 for C1s (γ-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 (λ) 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 $0.013 \leq Q \leq 0.076$ Å^{-1}, where the scattering parameter $Q = (4\pi \sin\theta)/\lambda$, and θ 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_2O [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:

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

and

$$R_g^2 = [\Sigma_i(b_i - \varrho^\circ v_i]/\Sigma(b_i - \varrho^\circ V) \quad (3)$$

where M_2 is the molar mass of the particle in solution (g/mol); c_2 is the concentration of macromolecule in g/cm^3; N_A is Avogadro's number (mol^{-1}); b_2 (cm/g) is the scattering length per gram of macromolecule; $\bar{v}_s$ is the partial specific volume of the macromolecule (cm^3/g); b_i (cm), v_i (cm^3) 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 $(b_i - \varrho^\circ v_i)$ distribution (contrast); V is the total volume of the particle; ϱ° (cm^{-2}) is the scattering length density of the solvent; the summation should be over the entire particle [22]. The values of $(b_2 - \varrho^\circ \bar{v}_2)$ required for 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 $R_{cofm}^2 + D^2$, where R_{cofm} 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.

Table I

Neutron scattering data and chemical parameters of the proteins

Protein	$I(0)/c$[a] (cm^2/g)	R_g[a] (Å)	$(b_2 - \varrho^\circ \bar{v}_2)$[b] ($10^9$ cm/g)
C1r $(\gamma\text{-B})_2$	0.050 ± 0.005	34 ± 1	17.8
C1s (γ-B)	0.025 ± 0.002	28 ± 1	18.2

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

[b] $(b_2 - \varrho^\circ \bar{v}_2)$ 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^3/g for the protein moieties of C1r $(\gamma\text{-B})_2$ and C1s (γ-B), respectively, and 0.60 cm^3/g for the sugars. The value of ϱ° = 5.49 × 10^9 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

2.4. *Solvent conditions*

The choice of solvent will affect the value of ϱ° (and therefore of the contrast) and the background due to incoherent scattering which dominates the solvent contribution to the scattering. Replacing H_2O by D_2O in the protein solvent increases the contrast enormously and also reduces the background. However, D_2O 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 $(\gamma\text{-B})_2$ fragment were performed in H_2O and D_2O 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_2O. Further comparative studies on C1r $(\gamma\text{-B})_2$ and C1s (γ-B) were conducted in H_2O 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 $(\gamma\text{-B})_2$ 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 ± 5000 and 90000 ± 8000 g/mol for C1s (γ-B) and C1r $(\gamma\text{-B})_2$, respectively, in good agreement with the calculated values of the monomer and dimer, respec-

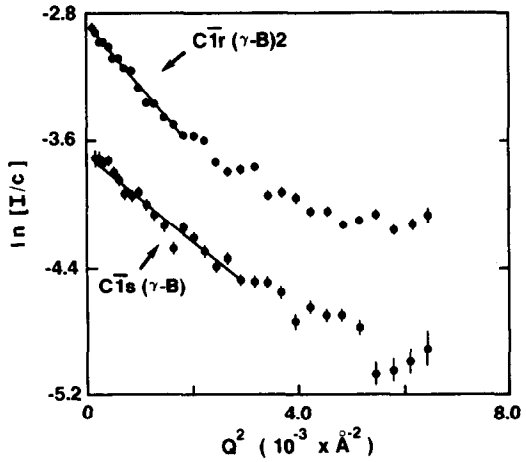

Fig. 2. Neutron scattering data plotted in the Guinier approximation for C1r (γ-B)$_2$ and C1s (γ-B). See text for experimental conditions.

tively. This confirmed that the solutions were monodisperse in these conditions. The radii of gyration found were 28 ± 1 Å and 34 ± 1 Å for C1s (γ-B) and C1r (γ-B)$_2$, respectively. These are much larger than the values expected for monodomain globular proteins of the same mass (Fig. 3). Data collected on C1r (γ-B)$_2$ 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 C1r (γ-B)$_2$ [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 C1r and C1s; (c) the B chains have a structure similar to that of chymotrypsin (R_g = 17 Å, M_r = 25000) [25,26]; (d) the 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 $(\Sigma_i\, b_i - \varrho^\circ V)$ values. The equation was applied to C1s (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 C1r (γ-B)$_2$ with R_g = 34 Å, $R_1 = R_2$ = 28 Å and $m_1 = m_2$ = half the mass of (γ-B)$_2$, 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 C1s (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 C1r (γ-B)$_2$, 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

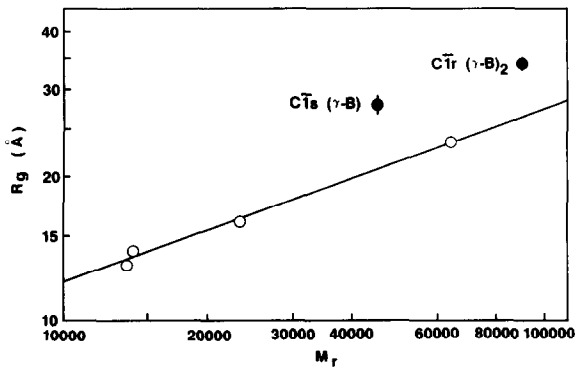

Fig. 3. Dependence of the radii of gyration of proteins on their molecular weights. The line (R_g^3 = constant × M_r) in logarithmic coordinates is for globular proteins such as lysozyme (R_g = 13 ± 0.5 Å), ribonuclease (14 ± 0.5 Å), papain (16 ± 0.5 Å), and hemoglobin (23.5 ± 0.5 Å) (neutron data collected by us and reference [27]). Data points are shown for C1s (γ-B) and C1r (γ-B)$_2$.

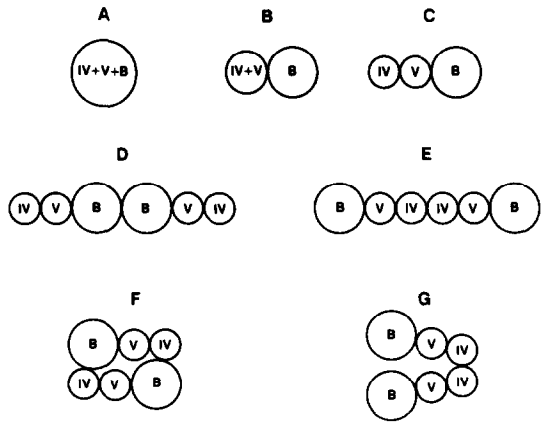

Fig. 4. Models for C1s (γ-B) (A, B, C) and C1r (γ-B)$_2$ (D, E, F, G). The data are only compatible with (B) and (C) for (γ-B), and (F) and (G) for (γ-B)$_2$. See text for discussion.

centres of mass, a value similar to the distance between the centres of mass of γ 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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