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METASTABLE STATE OF THE Fc FRAGMENT

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Abstract

The metastable state appearing at neutral pH in the Fc fragment after its brief incubation at pH 2.5 was studied using scanning microcalorimetry, high-flow sedimentation and fluorescence polarization. It was shown that the metastable state of the Fc fragment is characterised by smaller melting enthalpy of C_H2 domains as compared with that of the stable state which is explained by reduced interaction of C_H2 and C_H3 domains. As seen from the decrease in the sedimentation coefficient, this leads to diminution of compactness of the Fc fragment molecule. The intramolecular motility of C_H2 domains increases judging by the fluorescence polarization data.

Keywords: Fc fragment, metastable state

Introduction

An IgG molecule consists of two identical light and two identical heavy chains. Every light chain is folded in two homologous domains and every heavy chain is folded in four homologous domains [1–3]. These twelve domains form three compact structures: two Fab subunits and one Fc subunit [4] joined by a flexible hinge region. As a rule, within each subunit there are intense interactions between domains pertaining either to one polypeptide chain or to different chains. It was shown earlier [5] that after brief incubation of intact immunoglobulin at pH 2.5 and the following reverse transition to pH 7.0, the Fc subunit has the state that can be described as a metastable one. With such a state at pH 7.0 it is possible to carry out limited proteolysis with plasmin between C_H2 and C_H3 domains at the Lys345-Asp346 connection. As a result, a Facb fragment is formed preserving several functions of an intact molecule including the ability to interact with the complement system. Analogous hydrolysis can be made using trypsin at the Fc fragment after a brief acid treatment of the latter, which would induce its transition into a metastable state. With such a selective cleavage of the polypeptide chain, C_H2 and C_H3 domains are formed [6,7]. To investigate the metastable state of the Fc fragment, several physical methods including scanning microcalorimetry were used.

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Materials and methods

Materials

IgG1 myeloma protein Ser was isolated from the serum of patients with myelomatosis according to [8]. Fc fragments of IgG1 Ser were generated by papain digestion, according to standard procedures. Briefly, at papain hydrolysis the solution of immunoglobulins (100 mM phosphate buffer, pH 7.5) contained 100 mM cysteine and 2 mM EDTA. Papain was added at a ratio of 1:100 and the mixture was incubated at 25°C. The time of hydrolysis was 60 min. The reaction was stopped with an addition of 10 mM iodoacetamide. Intact molecules were separated from the fragments by gel-filtration on an ultragel ACA-44 column. The Fab- and Fc-fragments were obtained using ion-exchange DEAE chromatography equilibrated by 10 mM Tris-HCl buffer, pH 8.0. As a result of the hydrolysis, Fc-fragments were obtained that did not dissociate into individual polypeptide chains at pH 2.2 if interchain disulfide bonds remained intact. Protein purity was controlled by SDS-PAGE on 10 or 15% cross-linked gels, under reducing or non-reducing conditions according to the method of Weber and Osborn [9]. It was established that the heavy chains within the Fc fragments were covalently linked. 1-dimethylaminonaphthalene-5-sulphonyl (DNS) conjugate of human Fc fragment was prepared according [10]. An analogous procedure was used to obtain DNC-C_H2-domains. Fc fragments labeled by C_H2 domains were obtained using antibodies to DNC-C_H2-domains. Antisera to C_H2-domains and to Fc and pFc′ fragments were prepared by immunizing goats with purified antigens. An analogous procedure was used to prepare antiserum to the fragments containing the fluorescent label.

Experimental procedures

Scanning calorimetry experiments (DSC) were performed using a computerized version of the DASM-4A microcalorimeter [11] with a cell volume of 0.47 ml at a heating rate of 0.5, 1.0 and 2 K min^{-1}. The protein solutions for calorimetric experiments were dialysed *vs*. 1 mM phosphate buffer, pH 7.0. The calorimetric measurements were carried out using 10 mM glycine buffer (or 10 mM acetate) pH 2.2–4.2 or using a glycine (acetate) buffer plus 150 mM NaCl. Gel-filtration on an ultragel ACA-34 column equilibrated with a corresponding buffer was used prior to the calorimetric measurements of the samples. The protein concentrations in the calorimetric experiments varied from 0.5 to 5.0 mg ml^{-1}. The partial heat capacity of the protein, the calorimetric and effective enthalpies, the excess heat capacity function were calculated from the calorimetric data as described previously [12, 13]. The partial specific volume was taken as 0.73 ml g^{-1}. Sedimentation measurements were performed on a model E ultracentrifuge (Beckman, USA), using Schleiren optics. Sedimentation coefficients were estimated at protein concentrations of 2–10 mg ml^{-1} and extrapolated to zero concentration. Molecular mass were determined by equilibrium ultracentrifu-

gation (MOM, Hungary) using interference optics [14]. The time of rotational relaxation ρ_h was determined as described in [15, 16].

Results and discussion

Melting curves of the Fc fragment of human IgG1 in the acid region of pH (2.6–3.8) which reflect intramolecular processes have two heat absorption peaks (Fig.1, curve 2) [8, 17]. The first peak reflects the melting of the cooperative structure of C_H2 domains [11, 17]. The melting enthalpy of these domains decreases rapidly following the increase in pH in such a way that at pH 2.5 only one heat absorption peak corresponding to the melting of C_H3 domains is missing on the calorimetric curve (Fig. 1, curve 1). Thus, at pH 2.5 C_H2 domains are in a denatured state at 0°C.

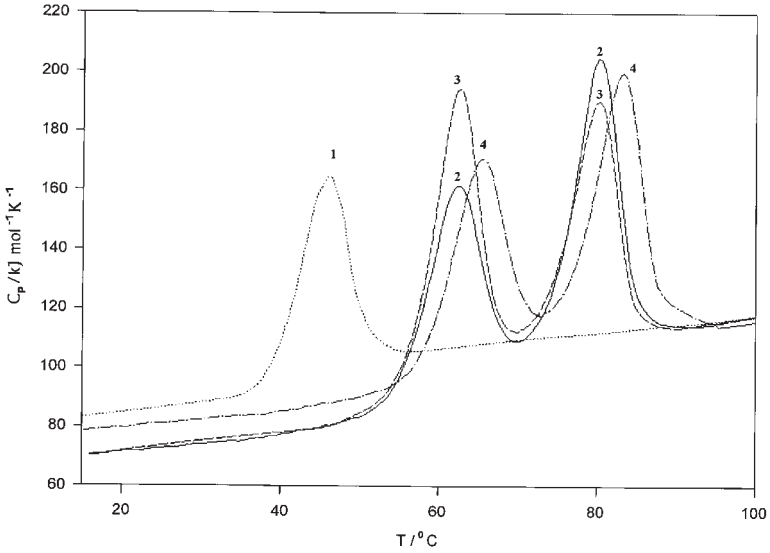

Fig. 1 Temperature dependence of the partial molar heat capacity of Fc fragment of IgG1 Ser in the stable and metastable states at different pH. The stable state at pH 2.5 is shown by a dotted line (curve 1), at pH 3.8 by a solid line (curve 2) and at pH 7.0 by a dashed line (curve 3). The metastable state at pH 7.0 is shown by a dot-and-dash line (curve 4)

Upon melting of the Fc fragment at pH>4.2, the calorimetric curve of the intramolecular structure again has two heat absorption peaks, but its shape is changed. The intensity of the first heat absorption peak increases sharply so that the enthalpy of this peak exceeds that of the second peak (Fig. 1, curve 3). Such an increase in the first heat absorption peak is explained by the fact that at pH>4.2, in addition to the melting of C_H2 domains, there occurs disruption of numerous contacts between C_H2 and C_H3 domains [11, 18] interacting less intensively at pH<4.0 [8, 17]. When the Fc fragment, that is in a metastable state according to the data of limited

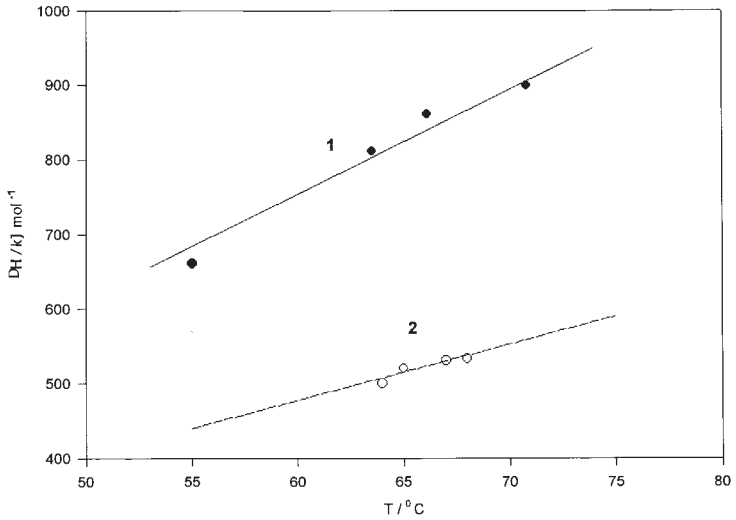

Fig. 2 Temperature dependence of the molar enthalpy of melting of C_H2 domains of the Fc fragment of IgG1 Ser in the stable (1) and metastable (2) states

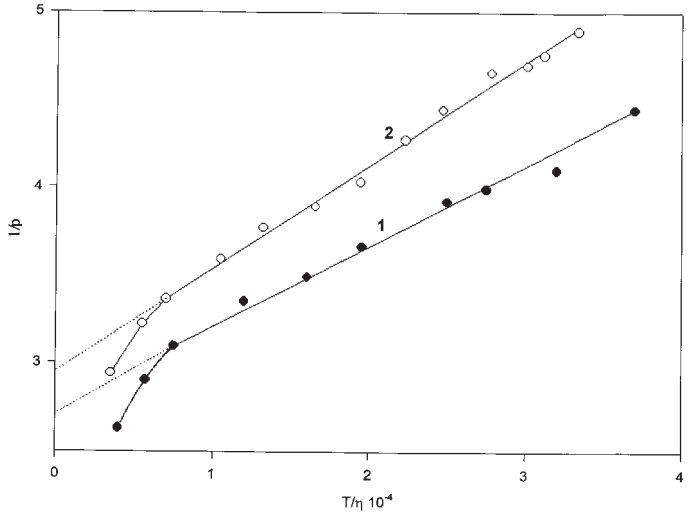

Fig. 3 Dependence of the reciprocal value of fluorescence polarization on temperature divided by viscosity for DNC-Fc fragment in the stable (1) and metastable (2) states. 100 mM Tris-HCl buffer, 280 mM NaCl, pH 8.0, 25°C, the protein concentration is 0.3 mg ml^{-1}, T/η in degree·$poise^{-1}\cdot 10^{-4}$

proteolysis, melts at pH 7.0, the first heat absorption peak is present on the calorimetric curve being characterised, however, by lower enthalpy (Fig. 2, curve 1; Table 1). The same pattern is observed also at other pH values exceeding 4.2. As shown by the

heat absorption curve and the data of limited proteolysis, after incubation for 24 h at pH 7.0, the protein relaxes to a stable state. The above arguments demonstrate that, first, scanning microcalorimetry allows to detect the metastable state per se. Second, the presence of a low-temperature peak means that C_H2 domains in a metastable state have a tertiary cooperative structure. Third, the interaction between C_H2 and C_H3 domains is weaker in this state. The diminution of interaction between the domains is connected with the number of contacts between them. The X-ray data evidence that such contact surfaces in a native Fc fragment make 1500 Å^2 per a pair of domains [19, 20]. In the metastable state, at least part of amino acid residue become accessible for the solvent at room temperature. As a consequence, the thermodynamic parameters characterising the metastable state have additional difference from those of the stable (native) state. As seen from the data given in Fig. 1 and Table 1, the initial partial heat capacity changes. The decrease in the heat capacity change (the heat capacity jump) changes also on melting of C_H2 domains. This is clearly seen on the plots showing the dependence of the melting enthalpy of C_H2 domains on the melting temperature (Fig. 3, Table 1).

Table 1 Thermodynamic parameters of melting of Fc fragment at stable and metastable state

Fragment (state)	pH	Temperature/°C		Enthalpy/ kJ mol^{-1}	
		1st peak	2nd peak	1st peak	2nd peak
Fc (stable)	2.5	–	45.3	–	479
Fc (stable)	3.8	62.4	80.1	436	723
Fc (stable)	7.0	62.6	80.3	857	638
Fc (metastable)	7.0	64.8	83.1	478	691

After acid denaturation of the C_H2 domain the sedimentation coefficient of the Fc fragment dropped to 3.1S at pH 2.5 [21]. The sedimentation coefficient of this fragment is 3.4S in a metastable state at pH 7.0 as compared to 3.7S in a stable native state at the same pH. Such an intermediate value of the sedimentation coefficient can be explained by decompactisation of the Fc fragment caused by changes in the mutual arrangement of individual domains or by their increased role in relative motility due to weakening of interdomain interaction. To clarify in what way the weakening of interaction between C_H2 and C_H3 domains affects the change in the motility of domains within the Fc fragment, the extent of fluorescence polarisation was measured and the time of rotational relaxation of the protein in the two states at pH 7.0 was estimated. To compare the results obtained with the available data, the measurements were made in conditions described earlier [15, 16]. The results are given in Fig. 3. For a Fc fragment with a non-selective fluorescence label, the time of rotational relaxation ρ_i in a stable state (curve 1) is 34 nsec which is very close to the data obtained earlier (33 nsec). For the fragment in a metastable state (curve 2) this value drops to 28 nsec. This can be the evidence of the higher lability of the Fc fragment in a metastable state and is in good agreement with both calorimetric and hydrodynamic data. More convincing data were obtained when studying Fc fragments in which only

C_H2 domains were modified selectively with a fluorescence label. Herewith the times of rotational relaxation in stable and metastable states were found to be 28 nsec and 22 nsec, respectively.

Thus, proceeding from the calorimetric data it can be stated that in a metastable state the interaction between C_H2 and C_H3 domains diminishes resulting in enhanced Brownian rotation of these domains within the fragment, as shown by the data of fluorescence depolarisation. As a result, the compactness of the Fc fragment per se decreases which is detected by the hydrodynamic method.

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