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Thermodynamic and functional characterization of a stable IgG conformer obtained by renaturation from a partially structured low pH-induced state

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Abstract At pH 2, rabbit IgG adopts a partially structured state that exhibits loss of thermal unfolding transition, tentatively assigned to the CH2 domain, whilst retaining a well-defined tertiary structure for the rest of the molecule and extensive secondary structure. Renaturation of IgG from this state yields a stable conformer that differs from native IgG by a lower degree of interaction between the CH2 and CH3 domains, and stronger interaction between the CH1 and CH2 domains, as judged by differential scanning calorimetry and probing the IgG conformation with specific ligands (C1q component of complement, protein A and monospecific antibodies to the CH2 domain and hinge region).

Key words: Immunoglobulin; IgG conformer; Differential scanning calorimetry; Conformational probe; C1q component of complement

1. Introduction

In the molecule of immunoglobulin G, the Fab fragments are constructed by the two variable antigen-binding (VH and VL) and two constant (CH1 and CL) domains, whereas the two CH2 and two CH3 domains constitute the Fc fragment of the molecule [1]. For multidomain proteins of this type, it is generally assumed that domain interactions are essential for stability and functionality [2,3]. However, relationships between well-defined changes of domain interactions and functional changes in immunoglobulins have not been reported. In the present study, we describe, using a combination of differential scanning calorimetry (DSC) and a functional approach, thermodynamic and functional properties of an IgG conformer obtained by low pH-induced structural reorganization and differing from native IgG in interactions of the CH2 domain with neighboring domains.

2. Experimental

Ferritin was isolated from human spleen essentially according to [4]. Total antiferritin IgG was purified by the caprylic acid procedure [5] from sera of immunized rabbits. The C1q component of human complement was isolated as described in [6]. Antibodies specific for the rabbit IgG hinge region and the CH2 domain were obtained by affinity chromatography of total sheep anti-rabbit IgG antibodies on $F(ab')_2$ and Fc fragments, respectively, immobilized on CNBr-activated Sepharose 4B. 'Anti-CH2 domain' antibodies thus obtained reacted selectively with the CH2 domain [7]. Proteolysis of rabbit IgG with pepsin or papain was carried out as described in [8,9]. Biotin *N*-hydroxysuccinimide ester (Sigma, USA) was used for preparing biotinylated C1q, IgG and protein A.

All binding assays were performed at room temperature in triplicate; mean values were used for calculation of binding parameters and graphic presentation. To immobilize ferritin, protein A (Sigma, USA) and anti-IgG by physical adsorption, polystyrene tubes were incubated overnight with 3 μg of the protein in 0.25 ml of sodium borate, pH 8.5, then for 1 h with 0.5% bovine serum albumin (BSA) in the same buffer and washed. To study C1q binding to IgG, immobilized ferritin was allowed to react with IgG (10 μg) for 3 h and, after washing, with 20–5,000 ng of biotinylated C1q in 0.25 ml of 0.05 M sodium borate, pH 7.8, containing 15 mM $CaCl_2$, 50 mM $MgCl_2$ and 0.5% BSA. After 2.5 h and washing, tubes were incubated with 250 ng of streptavidin–peroxidase conjugate (Sigma, USA) in 0.25 ml of the above buffer for 30 min and washed. After another 30 min in 0.1 M sodium citrate, pH 5 containing 0.02 M *o*-phenylenediamine and 0.02 M H_2O_2, peroxidase activity was determined spectrophotometrically at 492 nm. Protein A and anti-IgG binding assays were performed essentially as in [10]. Briefly, increasing amounts of IgG reacted first with immobilized protein A, and then with biotinylated protein A (500 ng). Streptavidin–peroxidase (250 ng) was used to measure bound protein A as described above. In competition anti-IgG binding assays, biotinylated rabbit IgG (100 ng) and varying amounts of IgG were incubated for 3 h with immobilized 'anti-hinge' or 'anti-CH2 domain' antibodies. Bound IgG was determined with streptavidin–peroxidase.

Acid treatment of rabbit IgG was performed by diluting stock IgG solution (15–17 mg/ml) with 0.05 M citrate-HCl, pH 2, to give a final concentration of 2 mg/ml and pH was brought to pH 2 with 0.01 M HCl. After 1.5 h at room temperature, the solution was dialyzed against 0.05 sodium phosphate buffer, pH 7, for 16–20 h at 6°C. The treatment at pH ranging from pH 2–7 was performed by 6–8-fold diluting the stock IgG solution with 0.1 M citrate-phosphate buffer at the required pH. After 1.5 h at room temperature, the pH was brought to pH 7 by dialysis as described above.

CD spectra were recorded with J-20 spectropolarimeter (JASCO, Japan) at IgG concentration 0.3–0.5 mg/ml. Calorimetric measurements were done with DASM-4 differential scanning calorimeter [11] (Biopribor, Pushchino, Russia) at a 60 $K \cdot h^{-1}$ heating rate. The protein concentration was 1.5–2.5 mg/ml as determined from the coefficient $E^{1\%}_{280} = 13.8$. Three to four scans were recorded for each sample, with variation of ΔH measurements being about 6%. Calorimetric enthalpy was calculated according to [12]. Deconvolution of melting curves was performed using TERMCALC software supplied by the DASM-4 manufacturer.

3. Results

The tertiary structure of rabbit IgG at pH 7 is characterized by an excess heat capacity function, quantitative deconvolution of which yields five two-state subtransitions delineating consecutive thermal unfolding of IgG domains (Fig. 1); a similar deconvolution pattern comprising five subtransitions has been previously obtained for mouse monoclonal IgG1 [13]. Upon acidification of the IgG solution to pH 2, the protein retains a β-stranded secondary structure (Fig. 1). Stronger negative ellipticity at 217 nm implies that some increase in ordered structural

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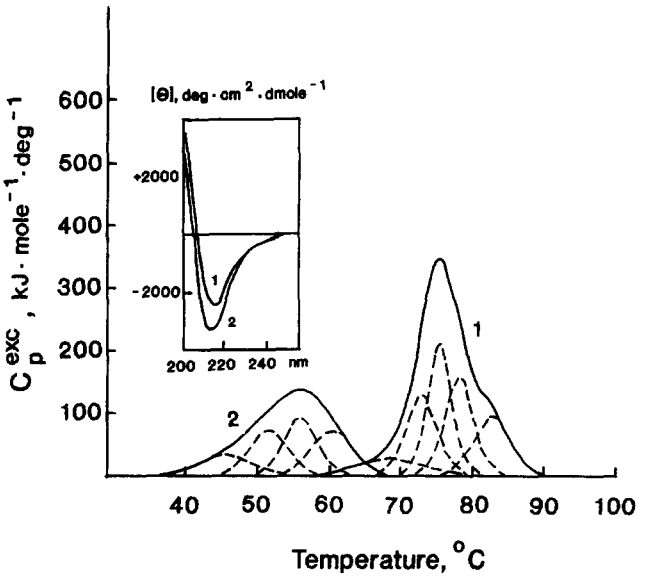

Fig. 1. Deconvolution of excess heat capacity functions of rabbit IgG at pH 7 (1) and pH 2 (2). Insert: CD spectra for IgG at pH 7 (1) and pH 2 (2).

elements might have taken place. However, the tertiary structure of IgG at pH 2 is distinct from that at pH 7 as evidenced by DSC, with major differences being associated with: (i) decrease in overall temperature of maximum heat capacity, T_M, by approx. 20°C for the state at pH 2; (ii) presence of four two-state subtransitions at pH 2 instead of five at pH 7, which indicates that IgG at pH 2 loses a well-defined tertiary structure of the domain having the lowest pH-stability; and (iii) significant decrease in overall enthalpy of thermal unfolding (from 3,350 ± 210 to 1,830 ± 110 kJ·mol^{-1}), which results from both the loss of cooperative unfolding of the least stable domain as described above and decrease in the ΔH value with lowering pH, that was found for many proteins [12].

Renaturation of IgG from the pH 2 state by dialysis against buffer with neutral pH yields a non-native (N_1) conformation different from the native (N) state as judged from the DSC data (Fig. 2). These differences are manifested as a significant (by 34%) decrease of maximal excess heat capacity and increase in half-width of the transition peak from 10.0°C to 13.2°C due to the altered contribution of five subtransitions to the resulting heat capacity curve; the overall ΔH value is by 13% lower for the N_1 conformer. These changes in cooperativity of thermal unfolding suggest changes in domain interactions in the N_1 conformation. Further study has been undertaken to elucidate the origin of differences of N and N_1 IgG conformers using probes sensitive to conformational changes in the CH2 domain and regions linking this and neighboring domains, and to investigate the reversibility of pH-dependent transitions resulting in formation of the N_1 conformer.

As evidenced by C1q and protein A binding studies, irreversible conformational changes of the IgG molecule do not occur at pH 7–3 and appear only below pH 3 (Fig. 3). After the incubation at pH 2 and subsequent renativation at neutral pH, significant increase in accessibility and/or flexibility of the protein A binding site (located in the CH2 and CH3 domain interface [1]) was observed that is suggestive of lower extent of interaction between the CH2 and CH3 domains. Concomitant increase in the C1q binding to its specific site in the CH2 domain can be seen only in the presence of antigen (human spleen ferritin), with similar C1q binding to both IgG conformers in the absence of antigen (Fig. 3). Furthermore, the N_1 conformer exhibited about 2.5-fold lower activity of ferritin binding than native IgG (not shown). These results strongly suggest that increased C1q binding affinity of the N_1 conformer originates from enhanced functional link between the CH2 domain and Fab fragment in this conformer, which may occur only when stronger interaction between the CH2 domain and the CH1 domain in the Fab fragment take place.

Monospecific 'anti-hinge' antibodies appeared the most sensitive probes for IgG conformation as they are capable of detecting conformational changes that occur in the hinge peptide not only in the pH 3–2 range, but also at pH 4–3 and pH 6–5. Antibodies recognizing epitopes of the CH2 domain do not discriminate between native IgG and the N_1 conformer. In combination with similar C1q binding to N and N_1 in the absence of antigen, these findings are indicative of unaltered conformation of the CH2 domain in the N_1 conformer of IgG. The N_1 conformer was stable for at least several months, with no conversion to native state being observed.

4. Discussion

Formation of the N_1 conformer of IgG is a process of pH-dependent conformational rearrangement in the structural block that involves the CH2 domain and regions linking this and neighboring domains. At pH 2, rabbit IgG adopts a partially structured conformation that demonstrates the lack of calorimetrically revealed tertiary structure of the least stable domain whilst retaining a well-defined tertiary structure for the rest of the molecule and extensive secondary structure (Fig. 1). The lowest stability of the CH2 domain among IgG domains has been shown previously both at neutral and at acidic pH [14,15]. Therefore, it can be safely assumed that it is the CH2 domain which is partially 'unfolded' at pH 2. Detailed analysis of pH-stability of rabbit IgG in the pH 7–2 range by DSC confirms the lack of well-defined structure of the CH2 domain at pH <3 (Martsev, Kravchuk and Vlasov, manuscript in preparation). Recently, mouse monoclonal IgG1 antibody MAK33 has been shown to adopt, at pH 2, the so-called A-state which was characterized as an alternatively folded molten globule-like

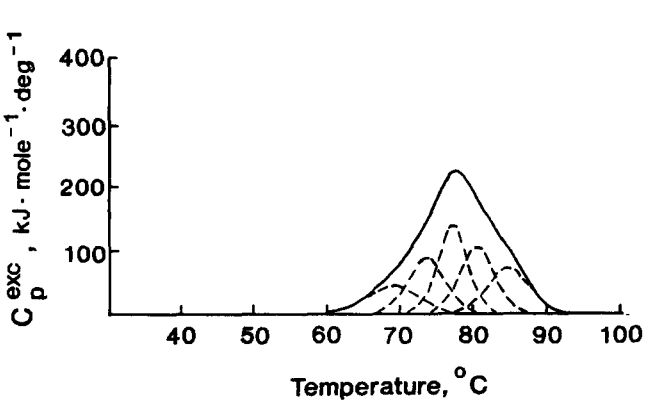

Fig. 2. Deconvolution of excess heat capacity curve of the N_1 conformer of rabbit IgG at pH 7.

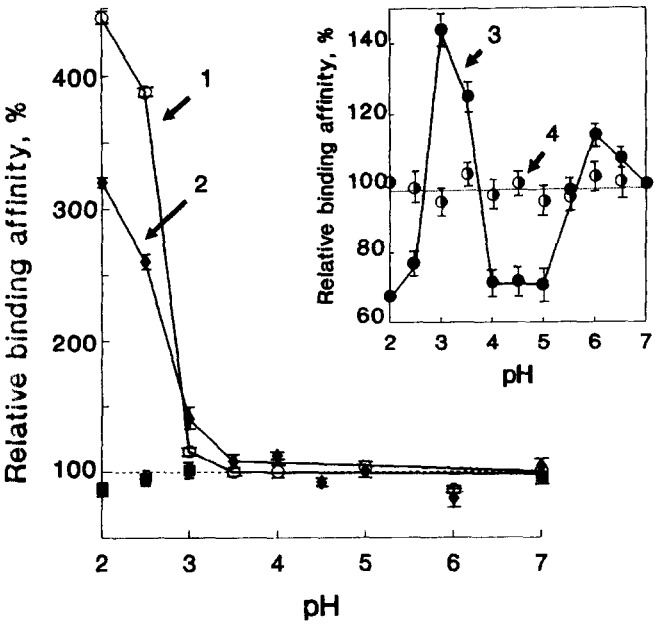

Fig. 3. Interaction of the conformational probes with IgG after incubation at varying pH and subsequent renaturation at pH 7. Curve 1, C1q; 2, protein A; 3, 'anti-hinge'; and 4, 'anti-CH2 domain' monospecific antibodies. Solid squares indicate C1q binding to the N and N_1 conformers adsorbed on the polystyrene in the absence of antigen. Relative binding affinity represents the ratio $C_{pH\ 7}/C_{pH\ x}$, where $C_{pH\ x}$ is the concentration providing half-maximal binding of the probe to IgG incubated at pH in question while $C_{pH\ 7}$ is the concentration obtained for the native IgG.

state [13]. Comparison of calorimetric data obtained in our own and previous studies [13] reveals that the A-state is clearly distinct from the partially structured (intermediate) state of rabbit IgG at pH 2 when considering overall ΔH and T_M of thermal unfolding transition, and the number of subtransitions under the resulting melting curve.

Our calorimetric and functional studies indicate that reversibility of the low pH-induced transition of rabbit IgG from native (N) to intermediate (I) state is incomplete and renaturation yields a stable native-like N_1 conformer of IgG according to the scheme: $N \rightarrow I \rightarrow N_1$. Formation of the partially structured I state occurs at pH < 3 as evidenced by the absence of irreversible changes in C1q and protein A binding sites at pH >3 (Fig. 3). Irreversible conformational transitions detectable in the pH 6–3 range using 'anti-hinge' antibodies could not be observed with other functional probes (Fig. 3) or calorimetrically (data not shown). These local conformational changes seem to be confined to the hinge region and do not involve 'meltable' structures of the IgG molecule. According to these data, the hinge peptide of IgG is a primary site of pH-induced local conformational changes occurring at pH < 6, very close to the physiological conditions. Another local site of irreversible conformational changes induced by renaturation of IgG from the I state, is the CH2 and CH3 domain interface forming the protein A binding site [1]. Conformational changes in the N_1 versus N conformer of IgG involve: (i) lower extent of interaction between the CH2 and CH3 domains as evidenced by protein A binding, (ii) increase in functional link between the Fab fragment and CH2 domain due to stronger interaction of the CH2 and CH1 domains, judging from the C1q binding, and (iii) decreased accessibility of the hinge peptide to the 'anti-hinge' antibodies, fully consistent with stronger interaction of the CH1 and CH2 domains linked through the hinge region. Taken together, these results suggest that N_1 conformer of IgG differs from the native protein in an altered balance of domain interactions involving the CH2 domain which exhibits a lower extent of interaction with the CH3 domain and a stronger interaction with the CH1 domain. This rearrangement of interdomain interactions does not change the conformation of the CH2 domain itself as evidenced by an 'anti-CH2 domain' antibody binding assay, and by the antigen-independent C1q binding. The results obtained demonstrate one of the mechanisms by which functionally significant changes in the IgG molecule can be achieved through changes in interactions of conformationally unaltered domains.

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