The Unfolding/Denaturation of Immunogammaglobulin of Isotype 2b and its F_{ab} and F_c Fragments

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ABSTRACT The unfolding and further denaturation of IgG and its F_{ab} and F_c fragments were studied both on a macroscopic and molecular level, using differential scanning calorimetry and circular dichroism spectroscopy, respectively. It was shown that the structural integrity of the F_{ab} and F_c units was retained after fragmentation of the IgG. The F_{ab} fragment denatured at ~61°C and the F_c fragment at 71°C. The structural transitions observed in the whole IgG is the sum effect of those determined for the isolated F_{ab} and F_c fragments.

INTRODUCTION

The hypothesis that proteins are made up of a number of independent, compact globular regions, called domains, has become generally accepted (Hardie and Coggins, 1986). An important class of proteins that conforms to such a common subunit structure is the class of immunoglobulins (Tischenko et al., 1982; Goto et al., 1988; Brandts et al., 1989; Miller, 1990; Martsev et al., 1994; Lilie and Buchner, 1995; Brody, 1997; Vermeer and Norde, 2000a). In general, immunoglobulins, otherwise called antibodies, consist of continuous stretches of the polypeptide chain comprising approximately 100 amino acids, that show a characteristic fold (Chothia et al., 1985; Edmundson and Ely, 1986; Padlan, 1997). For immunogammaglobulins (IgGs), these folds are grouped together in different segments, two identical F_{ab} segments connected via the hinge region to one F_c segment, thus forming a Y-shaped conformation, see Fig. 1. Data reported by Oi et al. (1984) suggested that IgG of isotype 2b exhibits considerable segmental flexibility, whereas, for example, IgG of isotype 1 is rather rigid.

It is well known that the different IgG fragments are associated with different functions of the immunoglobulin. The fragment which is best described and understood is the F_{ab}. F_{ab} fragments are involved in the binding of the immunoglobulin to the offending antigen (Mian et al., 1991). In addition, it is increasingly realized that the other part of the antibody, the F_c fragment, plays important biological roles as well. A number of recent reviews (Ravetch and Kinet, 1991; van de Winkel and Capel, 1993; Raghavan and Bjorkman, 1996) describe the binding of the F_c fragment to F_c receptors as a major step in immune defense. It enables the transport of, for example, immunoglobulins across epithelial tissues to their main sites of action (Underdown and Schiff, 1986) and maternal IgG into the bloodstream of the newborn (Burmeister et al., 1994). Above all, it is well recognized that the interactions between the different fragments might provide the intra-protein communication that is necessary to coordinate these protein functions (Harrison et al., 1994; Beaven and Metzger, 1993; van de Winkel and Capel, 1993).

In a series of papers, Vermeer and Norde (2000a,b,c) studied the unfolding and denaturation of the present IgG of isotype 2b, mainly by differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy. They showed that the folding/unfolding of this particular IgG is characterized by two main transitions that are themselves superpositions of various steps. It was shown that the bimodal distribution observed in both DSC and CD experiments did not depend on the scan rate in these experiments. The two transitions appeared to be independent, and the unfolding was irreversible. It was shown that, in addition to induction by heat, the structural perturbation of IgG in general could also be triggered by changing the pH (Vermeer and Norde, 2000a) or by interaction with a hydrophobic environment, i.e., adsorption onto Teflon surfaces or interaction with surfactants (Vermeer et al., 1998; Vermeer and Norde, 2000c,b). An important observation in relation to various manipulations in the application of this immunoglobulin was that the denaturation method did not affect the different transitions in the same way. The transition at 61°C appeared to be most sensitive to heat treatment and adsorption onto a hydrophobic surface, whereas the peak at 71°C was most sensitive to decreasing pH. It was suggested that the transitions at 61°C and 71°C represent the F_{ab} and F_c fragments, respectively. However, further evidence for this conclusion is required.

In view of the specific functions of the F_{ab} and F_c parts, it is clear that an accurate assignment of the two transitions observed in DSC and CD to the different IgG domains is most relevant for a better understanding of the adsorption and antigen-binding characteristics of this immunoglobulin under various environmental conditions.

Received for publication 13 January 2000 and in final form 22 May 2000.

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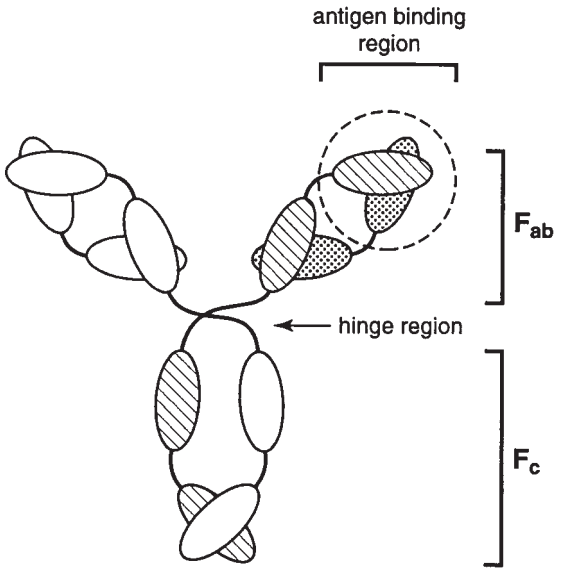

FIGURE 1 Schematic diagram of IgG, showing the F_{ab} and F_c fragments and the hinge region. An antigen binding site is indicated as well.

In the present paper we describe the enzymatic digestion of our IgG of isotype 2b and the subsequent isolation of the stable F_{ab} and F_c fragments. The secondary structure as well as the thermodynamic stability of the two single fragments are studied and compared with those of the intact immunoglobulin. Above all, it is shown that we can assign the two peaks observed for intact IgG to the F_{ab} and F_c fragments, respectively.

MATERIALS AND METHODS

All experiments were performed in a 10 mM phosphate buffer, pH 8.1, unless indicated otherwise. Chemicals were of analytical grade and were used without further purification. The water was purified by percolation through a mixed bed ion exchanger followed by an activated carbon column and a microfilter.

Immunoglobulin

The immunoglobulin G (IgG) is a monoclonal mouse anti-human antibody of isotype 2b, specific for the glycosylated N-terminal part of the β-chain of human hemoglobulin A1c. The IgG was kindly donated by Bayer AG. Further characterization of this antibody has been described previously (Vermeer and Norde, 2000a; unfortunately, the present IgG was erroneously described as mouse anti-rat in the previous paper).

F_{ab} and F_c fragments

F_{ab} and F_c fragments were isolated as major products from a papain digest. As mentioned by Parham (1986), proteolytic cleavage of IgG of isotype 2b requires a very accurate optimization, because this isotype is most sensitive to proteases and complete destruction of the IgG may result. Separation of the fragments was accomplished using a diethylaminoethyl (DEAE)-cellulose column according to Demignot et al. (1989). The fractions were identified and checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Papain digest of the IgG2b monoclonal antibody

A 5 ml solution of IgG2b at 20 mg/ml was prepared in 10 mM phosphate buffer containing 150 mM NaCl, pH 7.3. To this solution, 1 ml of 100 mM cysteine (freshly prepared) and 1 ml of 20 mM EDTA were added, together with 1 mg papain. The tube was closed, mixed, and incubated for 30 min at 37°C in a water bath. To stop the digestion, 1 ml of 150 mM iodoacetamide was added and the tube left for 30 min at room temperature to ensure complete alkylation. Digested samples were stored at −20°C.

DEAE cellulose chromatography

Digested samples (1 ml; approx. 2.5 mg on the basis of the original IgG2b sample) were dialyzed against DEAE column starting buffer, i.e., 50 mM Tris-HCl, pH 8.5, and loaded on a DEAE cellulose column (14 ml, Pharmacia Biotech (Uppsala, Sweden), C10 mm/20 cm, microgranular anion exchange DE32 diethylaminoethyl cellulose, cat. no. 4055010, Whatman, Maidstone, Kent, U.K.) at a flow rate of 0.2 ml/min. Fractions of 1.8 ml (9 min) were collected. The column was washed with starting buffer for 3 h and subsequently eluted with a linear gradient of 0 to 200 mM NaCl in 50 mM Tris-HCl, pH 8.5, during 12 h.

SDS-PAGE and immunoblotting

SDS-PAGE was performed under nonreducing conditions on a 10% acrylamide separation gel. Immunoblotting was done with goat anti-mouse IgG-F $(ab^1)_2$ and with goat anti-mouse IgG- F_c (gamma) specific antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA).

DSC

DSC was performed using a Setaram Micro-DSC III (Setaram, Caluire, France). The samples were placed in the calorimeter in a 1 ml sample cell against a 1 ml reference cell that was filled with the appropriate blank solution. The cells were stabilized for 4 h at 25°C inside the calorimeter before heating up to the final temperature at a heating rate of 0.5°C/min. The transition temperature and enthalpy were determined using the Setaram software (Setaram, Version 1.3).

CD Spectroscopy

The CD spectra were measured with a JASCO spectropolarimeter, model J-715 (JASCO International Co., Tokyo, Japan). A quartz cuvette of 0.1 cm light path length was used. Temperature regulation was carried out using a JASCO PTC-348WI (JASCO International) thermocouple. Comparison of the actual temperature in the cell with the temperature set by the Peltier element showed that the deviation from the actual temperature was <0.1°C. Temperature scans were recorded at a heating rate of 0.5°C/min using the Peltier thermocouple with a resolution of 0.2°C and a time constant of 16 s. Wavelength scans, in the far-UV region (0.2 nm resolution) were obtained by accumulation of 64 scans with a scan rate of 100 nm/min and a time constant of 0.125 s. The presented spectra are the average of these scans.

RESULTS AND DISCUSSION

Papain digest of the IgG2b monoclonal antibody

In preliminary experiments the optimal conditions for the papain digestion of the IgG2b monoclonal antibody was

established. Incubation was followed for 6 h and samples were taken at regular time intervals and analyzed by SDS-PAGE (results not shown). The optimal incubation time turned out to be 30 min.

Fractionation of IgG2b digest on DEAE cellulose and analysis of eluted fractions

In Fig. 2, the fractionation on DEAE cellulose is shown. The papain digest of the IgG2b monoclonal antibody eluted in three major peaks: peak I around fractions 32/33, peak II around 41/42, and peak III around 59/60. Peak fractions were analyzed by SDS-PAGE together with the unfractionated digest (Fig. 3). The unfractionated digest (lane 2) contained four major bands at molecular weights of 47, 42, 28, and 24 kD. It appeared that peaks I and II (peak I in lane 3; peak II not shown) both contained the bands with molecular weights of 47 and 24 kD, whereas peak III (lane 4) contained the bands with molecular weights of 42 and 28 kD. The expected molecular weight of the F_{ab} fragment is in the order of 50 kD, whereas the F_c migrates slightly slower than the F_{ab} fragment (Parham, 1986).

Immunoblotting of these peak fractions with specific anti-mouse IgG-F_{ab} and IgG-F_c revealed that the major bands of peak I and II both reacted with the anti-F_{ab} specific antibodies (peak I in lane 5; peak II not shown) and not with an anti-F_c specific antibody. The bands in peak III reacted only with the anti-F_c specific antibodies (lane 6). Pooled peak fractions (F_{ab}, fractions 29–45; F_c, fractions 57–62) were dialyzed against 100 mM phosphate buffer, pH 8.1, and further analyzed by DSC and CD.

Characterization of intact IgG

A typical result of a DSC experiment is shown in Fig. 4. The IgG concentration of the sample was 6 mg/ml and the heating rate 0.5°C/min. The curve shows two transitions: one at a denaturation temperature, T_d, of 60°C, and with an enthalpy, Δ_dH, of 12.5 J/g; and a second peak at 71°C with an enthalpy of 4.5 J/g. The overall denaturation process was irreversible and the two transitions were shown to be independent, as was indicated by reverse DSC scans and preheating the IgG at 55°C, respectively (Vermeer and Norde, 2000a). These authors also discussed the dependence of the denaturation process on the scan rate in DSC and CD

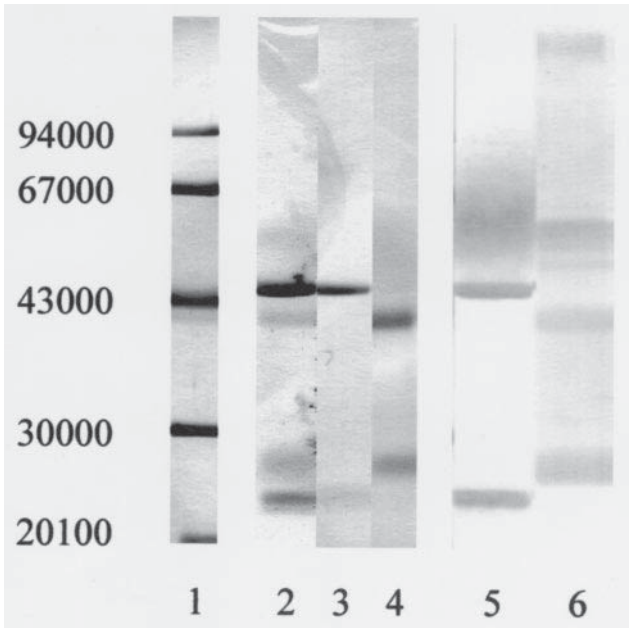

FIGURE 3 SDS-PAGE and immunoblotting analysis of peak fractions from the DEAE separation of the IgG2b papain-digest. Gels were run under nonreducing conditions. Lanes 1–4, SDS-PAGE: lane 1, Molecular weight markers; lane 2, unfractionated digest material; lane 3, DEAE fraction 32; lane 4, DEAE fraction 59. Immunoblotting, anti-F_{ab}: lane 5, DEAE fraction 32. Immunoblotting, anti-F_c: lane 6, DEAE fraction 59.

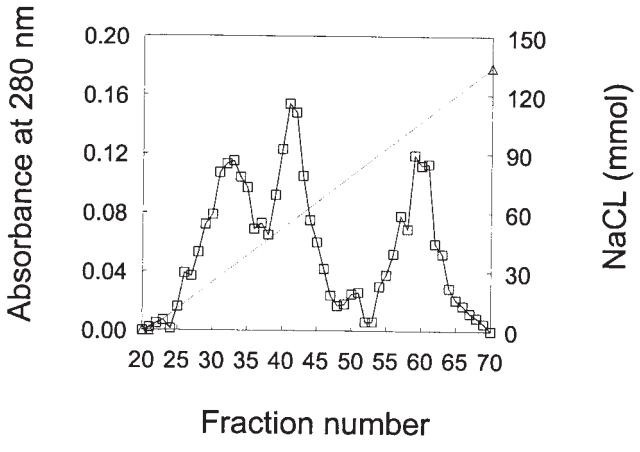

FIGURE 2 Chromatogram of the separation of the IgG2b papain-digest on DEAE anion exchange chromatography. The digest was applied in 50 mM Tris-HCl, pH 8.5 and eluted with a linear gradient of 0 to; 200 mM NaCl, pH 8.5.

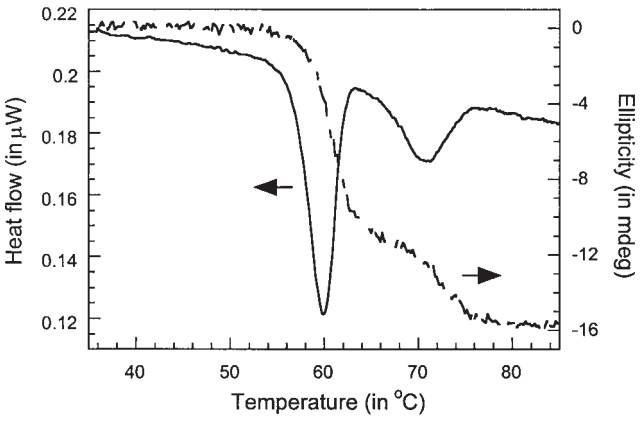

FIGURE 4 DSC thermogram of intact IgG (6 mg/ml; *left axis*) and CD (206.5 nm) temperature scan of intact IgG (0.2 mg/ml; *right axis*) in a 10 mM phosphate buffer, pH 8.1. Heating rate 0.5°C/min.

experiments and showed that the presence of these two transitions was independent of the operating conditions. Calorimetry data provide information on the protein structure stability on a macroscopic level. Complementary information, on a molecular level, may be obtained by spectroscopy. A temperature scan of a 0.2 mg/ml IgG solution, measured by circular dichroism spectroscopy at 206.5 nm, at a heating rate of 0.5°C/min, is also presented in Fig. 4. The decrease in ellipticity at 206.5 nm corresponds to a loss in β-turn and/or formation of random coil and α-helix conformations (Vermeer et al., 1998). The curve shows two steps at which the ellipticity decreases strongly. The major change occurs at 61°C, followed by a second step at about 71°C. Comparison with the DSC thermogram clearly shows that the temperature at which the changes in the secondary structure occur coincide with the transition temperatures in the DSC experiment. From these experiments it can be concluded that the thermal unfolding, which includes a change in the secondary IgG structure, affects at least two IgG domains.

Characterization of the F_{ab} and F_c units

The yields of the F_{ab} and F_c fragments obtained after IgG digestion were too low to allow for a thorough investigation by DSC. The amount of the F_{ab} fragment was so low that we could not perform a DSC experiment at all. Therefore, characterization of these fragments was done mainly by CD spectroscopy, for which much lower sample concentrations suffice. Fig. 5 shows temperature scans of the isolated F_{ab} and F_c fragments as measured by CD spectroscopy. Each curve is characterized by one step at which the ellipticity decreases strongly. The temperature at which this transition occurs is about 60°C for the F_{ab} and 71°C for the F_c fragment. The transition observed for the F_{ab} fragment shows a slight shoulder at about 55°C. This shoulder is most probably caused by a low concentration digestion by-product that has a molecular weight half that of the F_{ab}, as was observed in the SDS-PAGE experiments. This product was also detected by immunoblotting using an antibody specific for the F_{ab} fragment, whereas it was not detected using an antibody specific for the F_c fragment.

For a better comparison between the CD spectra of the F_{ab} and F_c fragments and that of the intact IgG molecule, all spectra were normalized to zero millidegrees at 35°C. Comparing Fig. 4 with Fig. 5 reveals that the change in ellipticity observed for the intact IgG at 61°C and 71°C is equal to two-thirds of the change observed for the F_{ab} and one-third of the F_c, respectively. Since the mass of one IgG molecule is equal to the sum of that of two F_{ab} and one F_c fragments, it can be concluded that the overall CD spectrum can be recalculated from the spectra of the two isolated fragments, and that the unfolding behavior of the fragments is hardly affected by the digestion. The temperatures at which the heat-induced changes in the secondary structure of the F_{ab} and the F_c fragment occur are the same as those where the two transitions in intact IgG were observed.

This coincidence of temperature-induced structural changes, in F_{ab} and F_c on the one hand and in intact IgG on the other, has been further confirmed by DSC. Unfortunately, as mentioned above, only the F_c fragment was obtained in an amount that was sufficient for one DSC experiment. The thermogram shows only one, irreversible, denaturation peak with a maximum at 71°C and a transition enthalpy of 18 J/g (Fig. 6). This enthalpy is somewhat higher than that calculated for the F_c transition in intact IgG (about 13.5 J/g F_c). The difference may be explained by a higher degree of freedom of the isolated fragment. Because intact IgG molecules form aggregates when the F_{ab} parts denature at a temperature of 61°C, the unfolding of the F_c unit (which occurs at 71°C) may be hindered when these units become locked in the aggregates (Vermeer and Norde, 2000a). Thus, this DSC experiment confirms the CD results

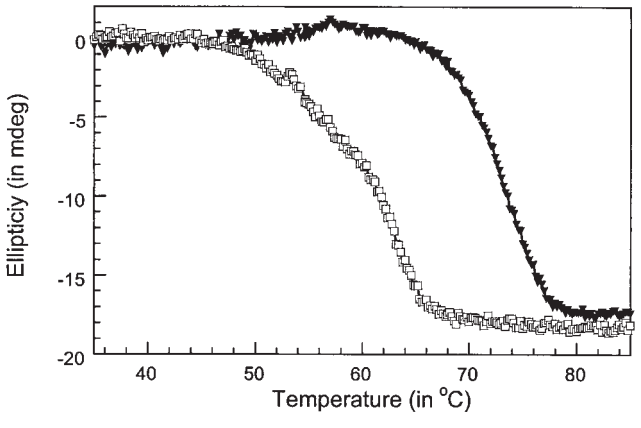

FIGURE 5 CD (206.5 nm) temperature scan of the F_{ab} (□) and F_c (▼) fragment (0.2 mg/ml) in a 10 mM phosphate buffer, pH 8.1. Heating rate 0.5°C/min.

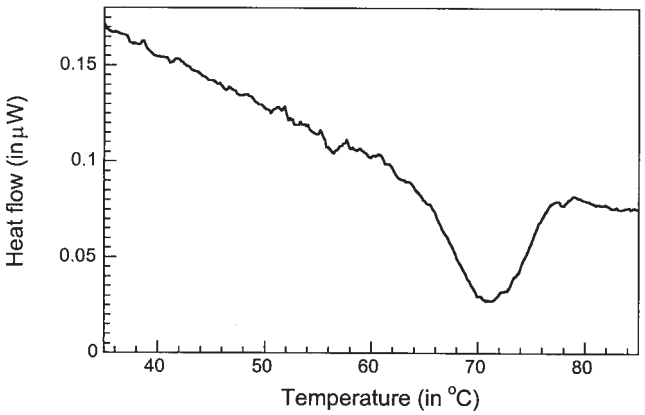

FIGURE 6 DSC thermogram of the F_c fragment (3.8 mg/ml) in a 0.1 M phosphate buffer, pH 8.1. Heating rate 0.5°C/min.

and strongly indicates that the second peak in the thermogram of IgG (Fig. 4) represents the F_c part, leaving the peak at 61°C to be caused by structural rearrangements in the F_{ab} parts.

CONCLUSIONS

Stable F_{ab} and F_c fragments were isolated from the enzymatic digest of IgG of isotype 2b. In spite of the fact that some further digestion products were still present in both F_{ab} and F_c batches, it was shown to be possible to use these fragments to assign the transitions in intact IgG to its different domains. Comparison of DSC and CD data for whole, intact IgG of isotype 2b on the one hand with those for isolated F_{ab} and F_c fragments on the other hand revealed that the structural transition of IgG at 61°C occurs in its F_{ab} segment, and the transition at 71°C occurs in its F_c segment. Both CD and DSC confirmed that the F_{ab} and F_c behave as independent subunits within this IgG, as was previously suggested based on isothermal calorimetry and DSC experiments (Vermeer and Norde, 2000a). The results further suggest that the different functions of the F_{ab} and F_c parts of the IgG molecule, i.e., antigen binding and adsorption to receptors/tissues, respectively, may be studied independently, using the isolated F_{ab} and F_c fragments instead of intact IgG molecule.

We thank Mr. Carlos Manuel F. M. Ribeiro and Mrs. Luciënne B. J. M. Berendsen at ATO-DLO for their technical assistance with the enzymatic digestion of IgG and the subsequent isolation of the fragments.

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