The Thermal Stability of Immunoglobulin: Unfolding and Aggregation of a Multi-Domain Protein

Arnoldus W. P. Vermeer and Willem Norde
Laboratory for Physical Chemistry and Colloid Science, Wageningen Agricultural University, Dreijenplein 6, 6703 HB Wageningen, The Netherlands

ABSTRACT The denaturation of immunoglobulin G was studied by different calorimetric methods and circular dichroism spectroscopy. The thermogram of the immunoglobulin showed two main transitions that are a superimposition of distinct denaturation steps. It was shown that the two transitions have different sensitivities to changes in temperature and pH. The two peaks represent the F_{ab} and F_c fragments of the IgG molecule. The F_{ab} fragment is most sensitive to heat treatment, whereas the F_c fragment is most sensitive to decreasing pH. The transitions were independent, and the unfolding was immediately followed by an irreversible aggregation step. Below the unfolding temperature, the unfolding is the rate-determining step in the overall denaturation process. At higher temperatures where a relatively high concentration of (partially) unfolded IgG molecules is present, the rate of aggregation is so fast that IgG molecules become locked in aggregates before they are completely denatured. Furthermore, the structure of the aggregates formed depends on the denaturation method. The circular dichroism spectrum of the IgG is also strongly affected by both heat treatment and low pH treatment. It was shown that a strong correlation exists between the denaturation transitions as observed by calorimetry and the changes in secondary structure derived from circular dichroism. After both heat- and low-pH-induced denaturation, a significant fraction of the secondary structure remains.

INTRODUCTION

The concept that proteins may comprise of a number of independent, compact globular regions, called domains, has become widely accepted (Hardie and Coggins, 1986). Sometimes these domains can be isolated as stable fragments. These fractions, or subunits, are generally associated with different functions. Interactions between the domains may provide the intra-protein communication that is necessary to coordinate the various protein functions. In some cases the functions of the protein are found to be connected to different sequences within a single polypeptide chain. Goto and Hamaguchi (1982) demonstrated that a polypeptide chain segment corresponding to a single domain can be refolded independently of the rest of the protein. However, independent unfolding of different domains is not a general phenomenon. Several proteins show cooperativity in the unfolding transition due to heat or pH treatment (Takahashi and Fukada, 1985; Solís-Mendiola et al., 1993; Protasevich et al., 1997).

An important class of proteins that conform to a common subunit structure are the immunoglobulin Gs (IgGs). These molecules have domains that are structurally independent, compact globular regions consisting of continuous stretches of the polypeptide chain approximately 100 amino acids long, with a characteristic fold (Chothia et al., 1985; Edmundson and Ely, 1986; Padlan, 1997). This fold contains two β-sheets and essentially no α-helices (Amzel and Poljak, 1979). A predominant feature of these globular protein structures is that nonpolar residues are sequestered into a core, where they largely avoid contact with water. Immunoglobulins, or antibodies, show a strong structure-function relation in the different domains, which makes these molecules excellent systems for various diagnostic tests. The domains of the antibodies with a high specificity to bind analytes (antigens) assure that these immunoglobulins can be used for a reliable and fast determination of low concentrations of analyte, whereas other domains of the immunoglobulin promote protein binding to a surface in the proper orientation, i.e., with its binding sites accessible to the antigen.

Studying the structure of immunoglobulins in more detail reveals that these proteins are composed of four polypeptide chains that are connected by disulphide bonds and noncovalent forces. The four polypeptide chains are grouped together in different fragments, two identical F_{ab} segments and one F_c segment, thus forming a Y-shaped conformation. The antigen binding sites are located on the far ends of the F_{ab} segments. The F_{ab} segments are linked to the F_c by the hinge region, which varies in length and flexibility in the different antibody classes and isotypes. Data reported by Oi et al. (1984) suggested that immunoglobulin G (IgG) of isotype 2b exhibits considerable segmental flexibility, whereas, for example, IgG of isotype 1 is rather rigid. Both F_{ab} and F_c fragments consist of four of the above mentioned globular regions.

Changes in the secondary structure of IgG as a function of temperature and, e.g., pH, can be studied by circular dichroism (CD) spectroscopy (Fasman, 1996). The major

Received for publication 2 February 1999 and in final form 7 October 1999.

Address reprint requests to Dr. Vermeer's present address: Bayer AG, ZF-FP Biophysik (Building E41), D-51368 Leverkusen, Germany. Tel.: 49-214-3023988; Fax: 49-214-3050698; E-mail: Ronald.Vermeer.RV@BAYER-AG.DE.

0006-3495/00/01/394/11 $2.00

advantages of this technique are that the spectroscopic signal is not affected by the presence of the surrounding solution and that well-defined procedures are available to elucidate the secondary structure based on reference spectra of the different structure elements (de Jongh et al., 1994). The fractions of the secondary structural elements can be obtained from the CD spectra. CD has been used previously to study the secondary structure of IgG (Rousseaux et al., 1982; Kats et al., 1995; Tetin and Linthicum, 1996; Kats et al., 1997); however, these authors did not calculate the fractions of the structural elements.

The stability of multi-domain proteins is commonly investigated using differential scanning calorimetry (DSC). One of the great advantages of DSC is that it can detect fine-tuning of interactions between the individual domains of a protein (Privalov and Putekhin, 1986). Numerous studies on the thermal denaturation of immunoglobulins are available, reporting on the effects of pH (Tischenko et al., 1982; Buchner et al., 1991; Martsev et al., 1995; Vlasov et al., 1996), pre-heating (Lindström et al., 1994; Vlasov et al., 1996), and, for example, the cooperativity between the IgG fragments (Tischenko et al., 1982; Shimba et al., 1995). However, based on the literature mentioned above, it is far from clear to what extent the denaturation of the different isotypes of immunoglobulin G is reversible, how it depends on the solution conditions, and whether the denaturation of the domains is cooperative.

Goto et al. (1988) mentioned that the constant domain of isolated F_{ab} denatures more easily than the variable domain and that the denaturation of the isolated domains can be described as a two-state process. This supports the idea that the denaturation of a multi-domain protein can be described by the denaturation of the individual domains. If we assume for the moment that the various domains in a multi-domain protein denature independently, different denaturation routes may exist, whereby the different domains are affected in different orders. Depending on the conditions, either of the routes may be preferred.

The possibility that partly denatured intermediates of immunoglobulins exist was first suggested by Rowe and Tanford (1973). There is indirect evidence for the existence of such intermediates (Tischenko et al., 1982; Buchner et al., 1991; Martsev et al., 1995; Shimba et al., 1995; Vlasov et al., 1996). DSC allows individual domains to be seen as they undergo thermal unfolding. If the domain transitions are well separated along the temperature axis, the thermodynamic parameters of each transition can be derived. Although some immunoglobulins show several transitions in their DSC thermogram (Tischenko et al., 1982; Shimba et al., 1995), the overlap of the corresponding peaks is, generally, too strong to allow for a rigorous thermodynamic analysis of the individual transitions. However, even when these transitions overlap considerably, available software allows rapid deconvolution of the transition envelope to obtain estimates of the individual domains' transitions (Buchner et al., 1991; Martsev et al., 1995; Vlasov et al., 1996). The existence of denaturation intermediates has also been reported by Brody (1997), who detected a ladder of denaturation intermediates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after exposure of the IgG to sodium dodecyl sulfate, urea, or heat, and by Hughes and coworkers (Hughes and Richberg, 1993; Alexander and Hughes, 1995), who described IgG ladder formation by capillary electrophoresis. The different intermediates were formed, as proposed by Brody, due to differences in sensitivity of the disulphide bonds in the different IgG domains. However, stable IgG intermediates have not yet been obtained.

From this short overview it may be concluded that the presence of different domains in immunoglobulins has a strong impact on the overall behavior of these proteins. It was suggested that, at least to some extent, the domains denature independently and that changes in the solution conditions may effect the individual domains differently. In this paper, the unfolding process of immunoglobulin G, induced by heat, will be investigated in detail by differential scanning calorimetry, the kinetics of the process will be studied by isothermal calorimetry, and the effect of the denaturation process on the secondary structure will be monitored by circular dichroism. In addition to the heat-induced transitions, the effect of pH on the secondary structure will be studied. The results obtained by DSC and CD are consistent and they indicate different domains that denature independently and irreversibly. Furthermore, it will be shown that the denaturation method affects the structure of the aggregates formed.

MATERIALS AND METHODS

All chemicals were of analytical grade and were used without further purification. The water was purified by percolation through a mixed bed of ion exchangers followed by an activated carbon column and a microfilter.

Immunoglobulin

The immunoglobulin is a monoclonal mouse anti-rat antibody of isotype 2b, specific for the glycosylated N-terminal part of the β-chain of human hemoglobin A1c.

The degree of aggregation of the immunoglobulin in a 10-mM phosphate buffer, pH 8.1, was measured by Gel Permeation Chromatography using a Sephadex 200 HR 10/30 column on an Äkta explorer (Pharmacia Biotech, Uppsala, Sweden). The protein concentration was measured by UV-spectroscopy at a wavelength of 280 nm. The IgG was monomeric; only one peak was observed, representing molecules with a molecular weight of 150 kd. The isoelectric point (iep) of the molecules was measured on a PhastSystem (Pharmacia LKB, Uppsala, Sweden), using a Phastgel with a pH range of 3.5 to 8.65. The iep of the IgG molecules ranges from pH 6.0 to 7.0.

Differential scanning calorimetry

The DSC experiments at low heating rate (<3°C/min) were done using a Setaram Micro-DSC III (Seta-rau, 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 inside the calorimeter for 1 h at 20°C before heating up to the final temperature at a given rate. Subsequent cycles of cooling and reheating of the samples were performed as indicated. The denaturation temperature, T_m, corresponding to the maximum of the transition peak, was determined from at least two replicate runs and varied not more than 0.25°C.

The DSC experiments at a heating rate of 5°C/min were performed on a Perkin-Elmer DSC7, which is preferably used at faster heating rates because of the low thermal inertia. The samples were measured in aluminium cells containing 20 μl of a 20 mg/ml IgG solution. As a reference the same amount of the buffer solution was used.

Isothermal calorimetry

Isothermal calorimetry (IC) experiments were done in a 2277 Thermal Activity Monitor (LKB, Bromma, Sweden). The calorimeter contains a 4-ml reference cell and a 4-ml sample cell. The reference cell was filled with 2.000 $\pm$ 0.001 g nano-pure water and sealed using a Teflon interface. The other cell was filled with about 2 g of the sample (mass determination within $\pm$0.001 g). The cells were brought to the experimental temperature by equilibrating for 20 min (Hoffmann and van Mil, 1997) in the temperature equilibration position. Immediately after the cells were brought into their final position the difference in heat flow between the sample and reference cell was monitored. This difference corresponds to the heat generated in, or consumed by, the sample. The heat flow of the samples is always compared with the heat flow of a reference-reference measurement at comparable temperature. The reproducibility of the heat flow in the first hour of the experiment was very poor and depended strongly on the sample treatment before the IC experiment. The reproducibility in the absolute heat flow values was about 1 μW and the baseline stability, at longer time scales, was within 0.2 μW.

Circular dichroism measurements

The CD spectra were measured with a JASCO spectropolarimeter, model J-715 (JASCO International Co., Tokyo, Japan). For the far-UV and near-UV measurements quartz cuvettes having light path lengths of 0.1 and 1.0 cm, respectively, were used. Temperature regulation was carried out using a PTC-348WI thermocouple (JASCO). Comparison of the actual temperature in the cell with the temperature set by the Peltier element showed that the deviation of the actual temperature was $<$0.1°C. For the far-UV measurements the concentration of IgG was 0.2 mg/ml. In the 190- to 260-nm wavelength region (0.2 nm resolution) 16 or 32 scans were accumulated with a scan rate of 100 nm/min and a time constant of 0.125 s. The final spectra are the average of these scans. In the near-UV region, 250–350 nm (0.2 nm resolution), the concentration of IgG was 1.0 mg/ml. The final spectrum was the average of 64 scans. All the other settings were the same as for the far-UV measurements. In addition to these wavelength scans, temperature scans at a given wavelength were recorded. For these experiments the temperature was increased at a given heating rate (°C/min) using the Peltier thermocouple with a resolution of 0.2°C and a time constant of 16 s.

Circular dichroism spectral analysis

The CD curves of poly-L-lysine containing varying amounts of α-helix, β-sheet, β-turn, and randomly coiled conformations have been applied for determining the content of the structural components of the immunoglobulin. The measured CD curves for the IgG are a superposition of these four structure elements. The poly-L-lysine reference spectra were measured (de Jongh et al., 1994) as described by Greenfield and Fasman (1969) and Chang et al. (1978). Fitting of the spectra was performed by a nonlinear regression procedure, making use of the Gauss-Newton algorithm (de Jongh et al., 1994). The reference spectra (data from Chang et al., 1978) were fitted independently from 190 to 240 nm with 1-nm resolution. No constraints were used in the fit procedure. The quality of the fit was expressed using the definition of the normalized root-mean-square (RMS) error as described by Brahms and Brahms (1980). A fit was considered as reliable only if the RMS error was $<$10.

RESULTS AND DISCUSSION

Heat-induced denaturation of immunoglobulin G

Thermal denaturation of immunoglobulin G was studied by differential scanning calorimetry. A typical thermogram of IgG in a 10-mM phosphate buffer, pH 8.1, is shown in Fig. 1. The IgG concentration of the sample was 6 mg/ml and the heating rate was 0.5°C/min. The curve shows two transitions, one with a denaturation temperature, T_m, of 61°C, and with a denaturation enthalpy, Δ_dH, of 12.5 J/g, and a second at 71°C with an enthalpy of 4.5 J/g. As has already been discussed in the introduction of this paper, IgG can be described as a multi-domain protein. The presence of these two peaks may therefore indicate that at least two domains, or groups of domains, exist that denature under distinct conditions. In a previous paper (Vermeer et al., 1998) we reported the thermogram of IgG of isotype 1, which showed only one transition peak. This difference between the two IgG isotopes may arise from the higher flexibility of the hinge region of the IgG used in this investigation, as has

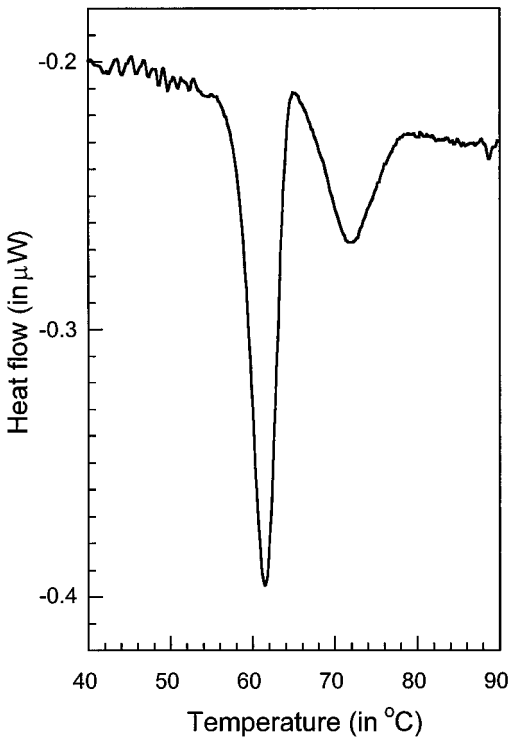

FIGURE 1 DSC thermogram of IgG (6 mg/ml) in a 10-mM phosphate buffer, pH 8.1. Heating rate was 0.5°C/min.

been proposed by Oi et al. (1984). As reported in a separate paper (Vermeer, Norde, and van Amerongen, unpublished manuscript), we isolated the F_{ab} and F_c fragments of the present IgG as major products from papain digests. These authors report that the isolated F_{ab} fragment showed only one transition at 61°C. The F_c fragment showed a single transition at 71°C. Moreover, the denaturation enthalpies of both fragments were comparable to the enthalpies of the two peaks observed in Fig. 1. These results show that the transitions at 61°C and 71°C for whole IgG represent the denaturation of the F_{ab} and F_c domains, respectively. After cooling the IgG sample, the thermogram of a subsequent cycle did not show any peak, indicating irreversible denaturation. Although under the experimental conditions no aggregation peak was observed in the thermogram, aggregates were observed to be formed after the heat treatment in DSC and a gel-like structure resulted.

A simple model that is consistent with irreversible protein denaturation is a reversible unfolding step followed by an irreversible process that locks the unfolded protein in a state from which it does not refold (Lepock et al., 1992; Castronouvo, 1991). It seems reasonable that the observed aggregation is the step that induces the irreversibility of the overall denaturation process; this has also been suggested by several other authors (Augener and Grey, 1970; Oreskes and Mandel, 1983; McCarthy and Drake, 1989). Thus, the denaturation of the IgG domains should be described as a three-state process rather than a two-state process.

It has been demonstrated (Hu and Sturtevant, 1987; Sanchez-Ruiz et al., 1988) that irreversible DSC thermograms can be interpreted in terms of reversible thermodynamics, provided that T_m and Δ_dH are independent of the heating rate. However, the data in Table 1 show a heating rate dependency, indicating that the protein denaturation is kinetically controlled. To explain the heating rate dependence of T_m, we follow the theory of Sanchez-Ruiz et al. (1988) and assume that the irreversible denaturation reaction can be represented by

$$N \underset{k_2}{\overset{k_1}{\rightleftarrows}} U \xrightarrow{k_3} D$$

where D is the final (aggregated) state of the IgG irreversibly arrived at from the (reversible) unfolded state U, and k is a first-order kinetic constant that changes with temperature according to the Arrhenius equation. If $k_3 \gg k_2$, all unfolded molecules will be converted into aggregates instead of returning to their native state. Based on this kinetic model, T_m should vary with heating rate (υ) according to

$$\ln\left(\frac{\nu}{T_m^2}\right) = \ln\left(\frac{AR}{E_a}\right) - \frac{E_a}{RT_m} \quad (1)$$

where $A(min^{-1})$ is the frequency factor, $E_a(kJ\ mol^{-1})$ is the activation energy for the denaturation step, and R the gas constant. Evidently, the slope of the plot of $\ln(\upsilon/T_m^2)$ as a function of $1/T_m$ equals E_a/R.

In Fig. 2 the apparent heat capacities curves for IgG measured at various heating rates are shown. From the data given in Table 1, three major effects are inferred: (1) the peak temperature of both peaks increases with increasing heating rate, the effect being most pronounced for the first transition; (2) the enthalpy of, especially, the first transition decreases with increasing heating rate; and (3) an exothermic gelation peak appears at higher heating rates. The latter phenomenon has also been found for other proteins (Barone et al., 1992), such as bovine serum albumin and human serum albumin.

The fact that the denaturation of IgG has to be described by a three-state process follows also from the change in the shape of the thermogram when varying the heating rate. For a two-state irreversible denaturation process an increase in heating rate is expected to shift T_m to a higher value, whereas the influence on Δ_dH is expected to be small (Lepock et al., 1992). For a three-state process the effects of increasing the heating rate strongly depend on both the rate of the unfolding step and the rate of the irreversible aggregation step.

The values calculated for the activation energy, E_a for the two transitions are 456 kJ/mol (9.1 J/g) for the first transition, and 692 kJ/mol (13.8 J/g) for the second. These values are reasonable for the denaturation of globular proteins (Haynes and Norde, 1995, and references therein).

In order to investigate whether the two endothermic transitions are related to each other, a thermogram of an IgG sample, with a concentration of 20 mg/ml, was determined after incubation for 40 h at 55°C. As a result of this incubation the least thermostable domains will be affected. The result is shown in Fig. 3. It is observed that the peak at 61°C has disappeared, whereas the peak at 71°C is still present. It indicates that the F_{ab} and F_c fragments denature independently of each other. Further, it can be seen that, at this high IgG concentration, a shoulder appears in the peak at 71°C. It suggests that, although only two major peaks are observed in the thermogram shown in Fig. 1, several transitions take place that are likely to be related to different domains of the IgG molecule. It has been discussed by Tischenko et al. (1982) that in the pH range 2.5 to 5.5, the F_{ab} and F_c fragments of rabbit IgG are thermodynamically

TABLE 1 Thermodynamic parameters as a function of heating rate

	Peak 1		Peak 2	
Heating rate	T_m (°C)	Δ_dH (J/g)	T_m (°C)	Δ_dH (J/g)
0.2°C/min	59.9	12.1	70.7	5.3
0.5°C/min	61.5	12.2	71.8	4.9
1°C/min	63.0	11.3	72.6	4.2
1.5°C/min	63.9	9.6	73.5	4.4
5°C/min	66.5	8.3	—	—

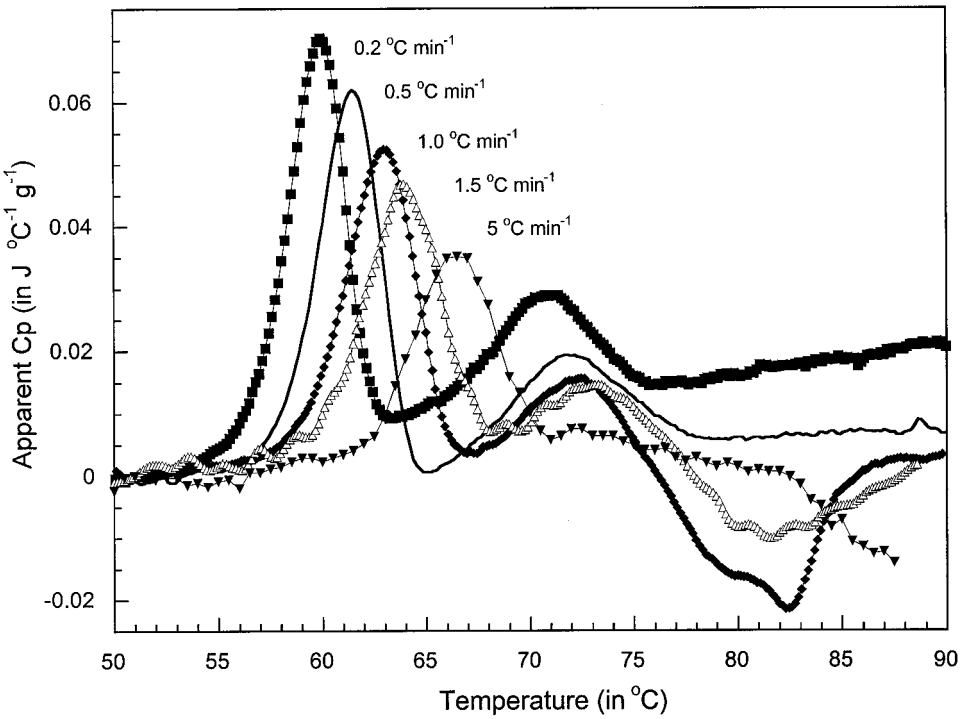

FIGURE 2 The apparent heat capacity of IgG in a 10-mM phosphate buffer, pH 8.1. Heating rates are indicated in the figure.

independent subunits. These authors further showed that the thermogram of the intact IgG is the sum of those of its individual fragments. Using a deconvolution procedure it was revealed that the F_{ab} fragment exhibits three cooperative melting transitions, whereas the F_c fragment contains four transitions. These transitions were ascribed to different globular domains (or parts of a domain in the case of the F_c fragment). This picture corresponds well with the results of the thermograms described above.

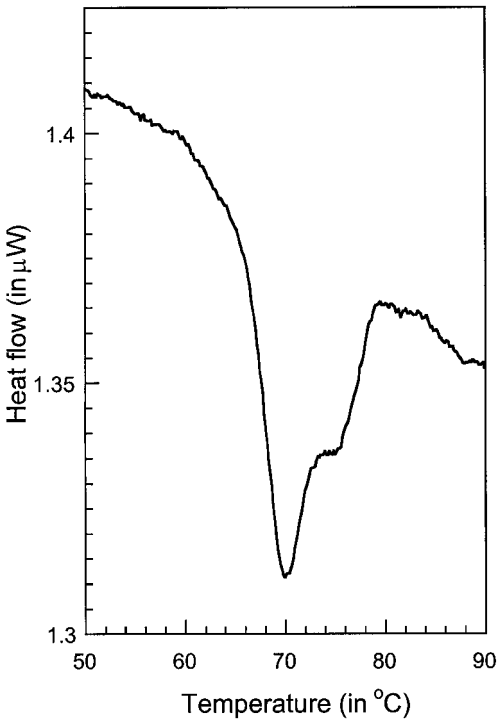

FIGURE 3 DSC thermogram of IgG (20 mg/ml) in a 10-mM phosphate buffer, 0.015 M NaCl, 0.01% sodium-Azide, pH 7.4, measured after incubation for 40 h at 55°C. Heating rate was 0.5°C/min.

Rate of denaturation of immunoglobulin G

As mentioned above, the rates of both the unfolding and the subsequent aggregation step affect the overall denaturation process. It may well be that unfolding and aggregation of IgG take place on a much longer time scale than that in the DSC experiment discussed above. Then, it is virtually impossible to derive a detailed picture of the kinetics of these processes based on DSC experiments only. To study the complex reactions involved in IgG denaturation, IC may be more useful because it gives more insight into the mechanism, especially in the kinetics of the reaction (Hoffmann and van Mil, 1997). With IC the heat flow at constant temperature is monitored as a function of time.

Isothermal calorimetry data at 41, 55, 60, and 70°C are shown in Fig. 4. The heat flow at 41°C did not deviate from the blank curve, indicating that neither an endothermic nor an exothermic process occurred. A DSC thermogram of the sample that was determined directly after the IC experiment was identical to the one shown in Fig. 1. At 55°C an endothermic process occurs which indicates that native IgG is transferred into an unfolded conformation. At $t = 0$ (this is after 20 min of thermal equilibration) this endothermic

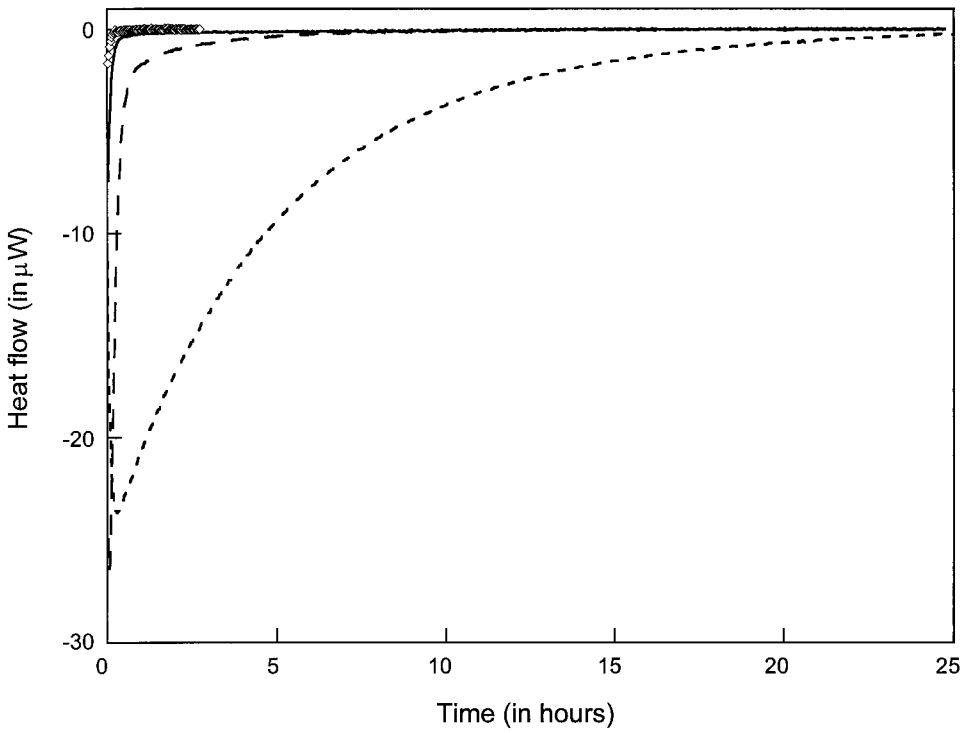

FIGURE 4 IC thermograms of IgG (20 mg/ml) in a 10 mM phosphate buffer, 0.015 M NaCl, 0.01% sodium-Azide, pH 7.4. The signals at 41°C (◇), 55°C (*dotted line*) , 60°C (*dashed line*), and 70°C (*solid line*) were obtained after subtraction of the blank.

effect is very strong, implying that the concentration of the native IgG molecules is still relatively high. After about 25 h, the heat flow has returned to the blank value, indicating that the IgG is essentially completely denatured. After this time period the sample had formed a gel. The formation of this gel may be explained as follows: upon unfolding the hydrophobic patches become exposed to the solution (Arntfield et al., 1989); this triggers the formation of aggregates through intermolecular hydrophobic binding (Augener and Grey, 1970; Rosenqvist et al., 1987; López-Bote et al., 1993; McCarthy and Drake, 1989; Oreskes and Mandel, 1983). In the DSC thermogram determined after 40 h at 55°C (Fig. 3) the peak at 61°C has vanished, whereas the peak at 71°C representing the F_c fragment is still present. This picture is in good agreement with the results of McCarthy and Drake (1989), who mentioned that in IgG aggregates produced by heating at 63°C the F_{ab} fragments are associated, leaving the F_c fragments exposed. Light microscopy revealed that the resulting gel is composed of closely packed aggregates of about 1 μm diameter that could easily be disrupted into fragments, which demonstrates that attraction between the aggregates is only weak.

The IC thermogram at 60°C shows that the endothermic heat flow decreases strongly over the first hour, after which the signal is almost equal to the blank. Because the enthalpy of the denaturation process at 55°C and 60°C should be equal, we must conclude that a significant fraction of the IgG is already denatured within 20 min equilibration time. Hence, at a temperature this close to T_m, the kinetics of denaturation are fast as compared to 55°C. The DSC thermogram measured after incubation at 60°C was comparable to that after incubation at 55°C, i.e., only the second peak was present. Light microscopy showed closely packed aggregates of about 1 μm diameter. However, under these conditions the gel could not easily be broken into smaller fragments, indicating that the attraction between the aggregates is strong. Clearly, when the rate of unfolding relative to the rate of aggregation becomes higher, a more coarse and strong aggregate results.

Finally, at 70°C the measured heat flow in the IC experiment was again comparable to the blank. The DSC thermogram did not show any peak, indicating that the IgG was completely denatured within 20 min equilibration time.

Based on the calorimetric experiments, the following denaturation scheme is proposed. At low heating rate and/or incubation at a constant temperature considerably below T_m the concentration of (partially) unfolded IgG molecules is low, so that aggregation of the unfolded IgG molecules will be slow. The result is a relatively open aggregate structure. At higher heating rate and/or incubation around or above T_m the unfolding occurs at a high speed, leading to a high concentration of (partially) unfolded IgG molecules. The rate of aggregation is correspondingly faster, and it could well be that at such high aggregation rates IgG molecules are incorporated in the aggregate before they have had sufficient time for complete unfolding. The decrease in the transition enthalpy with increasing heating rate as shown in Fig. 2 is probably caused by native domains being locked in aggregates.

Effect of solution conditions on the heat-induced denaturation of IgG

DSC thermograms were determined at various pH values (buffered at pH 2.0, 3.5, 6.0, 7.0, and 8.1), without buffer, and as a function of salt concentrations 0.01 M Na_2HPO_4 and 0.1 M (0.01 M Na_2HPO_4 + NaCl), at a constant heating rate of 0.5°C/min. At pH 6.0, 7.0, and 8.1 the thermograms showed, within experimental error, two peaks at the same temperatures and with comparable enthalpies, as indicated in Table 1. The thermograms were identical to the one shown in Fig. 1. However, at more extreme pH values the appearance of the thermogram was significantly altered. In a glycine buffer, pH 3.5, the thermogram of the IgG contains only the peak characterized by a T_m of 61°C and a $\Delta_d H$ of about 12 J/g (Fig. 5). The second peak at 71°C has disappeared. As discussed above, the denaturation of the F_{ab} and F_c fragments is independent. The isolated F_{ab} fragment showed only a transition at 61°C. Under these conditions an aggregation peak was observed at 61.5°C. After heat treatment in the DSC the IgG aggregated as judged by the presence of large flocs. At pH 2.0 both peaks were no longer present. After heat treatment at this pH the protein solution was still clear, implying that if aggregation has occurred, it has formed small units that are invisible to the naked eye.

From these results it is inferred that pH and heat treatment influence the IgG structure in different ways. Temperature-induced denaturation primarily affects the domains that cause the transition at 61°C, whereas the domains corresponding to the second thermal transition are more sensitive to low pH. The exothermic transition at pH 3.5 and the variation in the appearance of aggregates at different heating rates may be explained in terms of differences in the aggregation mechanism following the unfolding of the IgG. This supports the conclusions drawn from the experiments where the heating rate was varied.

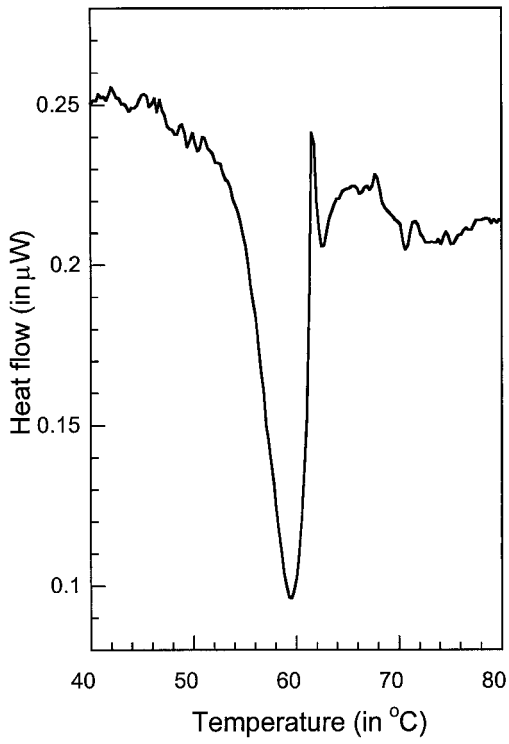

FIGURE 5 DSC thermogram of IgG (6 mg/ml) in a 0.1 M glycine buffer, pH 3.5. Heating rate was 0.5°C/min.

Heat-induced changes in the secondary structure

The endothermic enthalpy change observed in a DSC experiment is ascribed to unfolding of a part of the protein molecule. However, DSC does not give detailed information, i.e., on a molecular scale, about the change in protein structure. In a previous paper (Vermeer et al., 1998) it was shown that a combination of DSC and CD spectroscopy results in a better understanding of the processes that are involved in the denaturation of IgG. The most common CD technique is to measure the ellipticity as a function of the wavelength at a given temperature. In addition to these wavelength scans the ellipticity as a function of temperature at a given wavelength may also be measured. With these temperature scans the heating rate can be varied and spectra can be obtained under conditions that are comparable to those of the DSC thermograms.

The CD reference spectra (Chang et al., 1978) show that at a wavelength of 206.5 nm the intensity due to the β-sheets is essentially zero, whereas the other structural elements significantly contribute. Thus, by measuring the ellipticity at 206.5 nm as a function of temperature, one monitors the changes in β-turn, α-helix, and random coil contents. At this wavelength, an increase in β-turns shifts the ellipticity in a positive direction, whereas increased α-helix and random coil contents cause a negative shift.

The CD temperature scans of IgG in a 10-mM phosphate buffer, pH 8.1, at heating rates the same as those in the DSC experiments, are given in Fig. 6. All ellipticity-temperature profiles display two steps at which the intensity strongly decreases, indicating two distinct temperatures where changes in secondary structure occur. These temperatures are summarized in Table 2. The major changes at, for example, a heating rate of 0.5°C/min occur at 60°C, followed by a second step at about 70°C. These temperatures correspond unambiguously with those observed for the structural transitions observed in the DSC experiment (Fig. 2 and Table 1). The decrease in ellipticity at 206.5 nm suggests that the fraction of random coil structures increases with increasing temperature; however, the contributions from a variation in α-helix and β-turn may not be neglected. Thus, by evaluating the far-UV wavelength spectra, changes in the secondary structure involved in heat denaturation can be assessed.

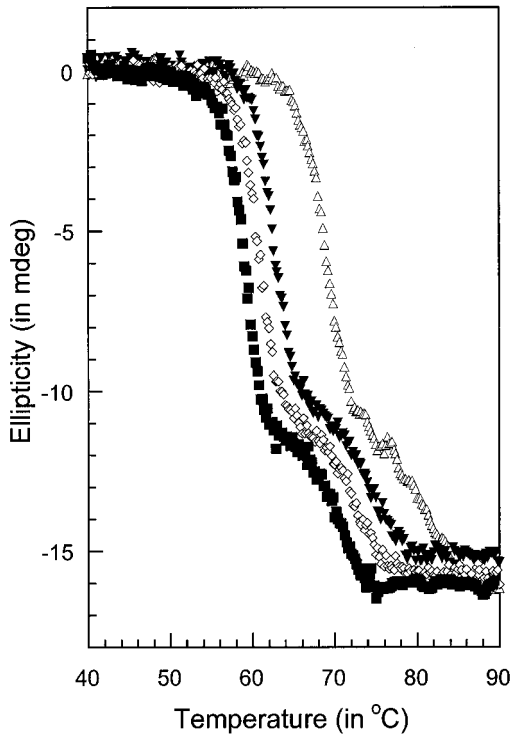

FIGURE 6 CD temperature scans of IgG (0.2 mg/ml) in a 10-mM phosphate buffer, pH 8.1, at 206.5 nm, as a function of the heating rate. ■ = 0.2°C/min, ◇ = 0.5°C/min, ▼ = 1.0°C/min, and △ = 5.0°C/min.

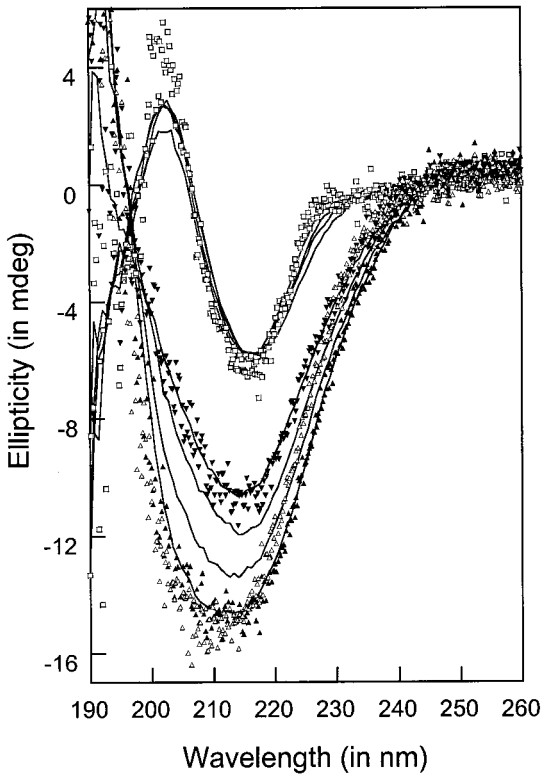

FIGURE 7 Far-UV CD spectra of IgG (0.2 mg/ml) in a 10-mM phosphate buffer, pH 8.1. The experimental data are shown for three temperature values, □ = 20°C, ▲ = 60°C, and ▲ = 75°C. The solid curves are smoothed through the experimental data measured at 20, 35, 50, 55, 60, 65, 70, and 75°C, respectively. Note that at temperatures <60°C, the experimental data are underestimated, especially in the wavelength range around 208 nm, as is shown for 20°C. △ = 20°C after cooling down.

CD spectra of intact and heat-denatured IgG in a 10-mM phosphate buffer, pH 8.1, were examined for both the far-UV and near-UV regions (Figs. 7 and 8). For reasons of clarity, the results obtained at some temperatures (i.e., 35, 50, 55, 65, and 70°C) are represented by smooth curves only. It is observed that at temperatures >55°C the smoothed curves match the experimental data very well (60°C and 75°C). The smoothed curve, representing the data at 20°C, however, deviates somewhat from the experimental data in the wavelength range of 200 to 210 nm. Nevertheless, the effect of temperature is evident. The CD spectra of the intact IgG ($T \leq 55$°C) are that of a typical immunoglobulin, with a negative band at 217 nm and a zero intensity at a wavelength of 206 nm, representing a high content of β-sheet, and several smaller positive and negative bands in the near-UV region between 260 and 300 nm. The ellipticity in the far-UV region is affected only slightly by temperature changes between 20 and 55°C, indicating that the IgG secondary structure is stable within this temperature range. At higher temperatures the spectra change gradually with increasing temperature. The minimum at 217 nm broadens and shifts to a lower wavelength, and a shoulder appears at about 208 nm. The wavelength corresponding to zero intensity also shifts to a lower value. The IgG sample measured at 75°C was cooled and again a wavelength scan was measured. This scan is also shown in Fig. 7 (*open symbols*). The irreversibility of the denaturation indicated by the DSC measurements is confirmed by the CD data.

TABLE 2 Transitions observed in CD experiments at 206.5 nm as a function heating rate

	Transition 1		Transition 2	
Heating rate	T_t (°C)	$\Delta_{ellipticity}$ (mdeg)	T_t (°C)	$\Delta_{ellipticity}$ (mdeg)
0.2°C/min	59	11	71	5
0.5°C/min	62	11	72	5
1°C/min	63	10	74	5
5°C/min	68	10	77	4

The structural changes reflected in the near-UV region of the CD spectra are usually associated with reorientation of the aromatic amino acids tyrosine and tryptophan, and also from the asymmetric environment of disulphide linkages. It is observed that the negative band at 270 nm gradually decreases with increasing temperature. This may be explained by an increasing degree of freedom of the side chains of the aromatic amino acids. Around 300 nm (see CD temperature scan shown in *inset*, Fig. 8) a strong decrease in the ellipticity occurs at a temperature of about 55°C. It

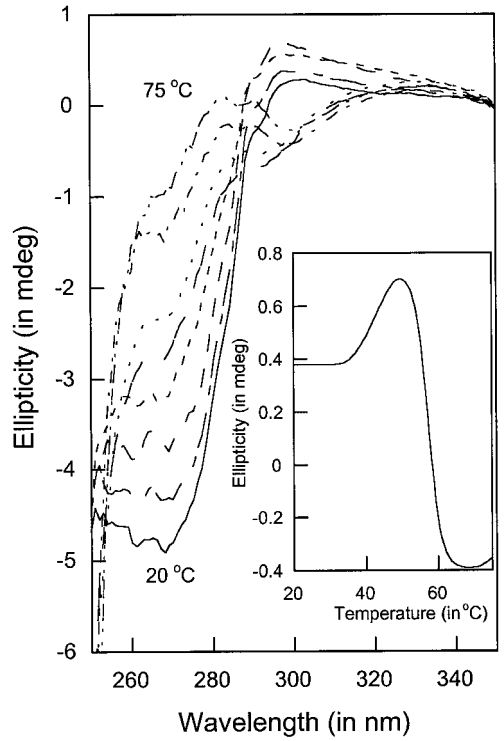

FIGURE 8 Near-UV CD spectra of IgG (1.0 mg/ml) in a 10-mM phosphate buffer, pH 8.1. The *solid curves* are smoothed through the experimental data measured at 20, 35, 50, 55, 60, 65, 70, and 75°C, respectively. The *inset* shows a CD temperature scan of IgG measured at 300 nm.

suggests rupture of disulphide bonds due to the denaturation process.

Comparison of the CD results with the DSC thermograms shown in Fig. 1 helps to relate the changes in the secondary structure to the overall denaturation of the F_{ab} and F_c fragments. Thus, the differences between the spectra at 55°C and 60°C are ascribed to the denaturation of the F_{ab} segment, whereas the gradual change between 60°C and 75°C is caused by denaturation of the F_c fragment.

To estimate the content of the various structural elements above, a described fitting procedure was applied. Table 3 summarizes the trends that were observed with increasing temperature. The structural composition calculated for temperatures ≤50°C compare well with those reported in literature (Byler and Susi, 1986; Buijs et al., 1996). With increasing temperature (>50°C) the fraction of α-helices and random coils increases, whereas the fraction of β-sheets and β-turns decreases.

TABLE 3 Content (in %) of structure elements in IgG at different temperatures

Temperature (°C)	α-helix	β-sheet	β-turn	random coil	RMS error
50	0	66	22	12	8.5
55	0	59	16	25	8.8
60	4	44	8	44	4.5
65	7	43	6	44	6.7
70	9	39	6	46	7.3
75	9	38	6	47	7.2

Medium, 10 mM phosphate buffer, pH 8.1.

The α-helix induction observed with increasing temperature may be ascribed to peptide units arriving in a nonaqueous environment upon aggregation of the IgG. For dissolved, native proteins it is more favorable for the peptide units at the aqueous periphery to form hydrogen bonds with water molecules than among each other. As hydrophobic interaction is a major force of protein folding (Dill, 1990), it may be expected that upon denaturation part of the hydrophobic interior of the IgG will be exposed to the solution, which, in turn, causes the formation of aggregates. Now, at the interfaces between the building blocks of the aggregate, hydrogen bonds between peptide units in the polypeptide chain may be formed, inducing the formation of α-helixes. This explanation is supported by the experimental observation that α-helixes are induced in proteins that adsorb at hydrophobic surfaces (Maste et al., 1996; Zoungrana and Norde, 1997; Vermeer et al., 1998).

Although the IgG is completely denatured at 75°C, a significant fraction of ordered secondary structural elements remain, i.e., about 50% of the polypeptide chain is present in β-sheets, α-helix, and β-turn conformations. This is in contrast to the denaturation of IgG induced by guanidine hydrochloride, where only the random coiled conformation was observed (Buchner et al., 1991; Goto et al., 1988).

pH-induced changes in the secondary structure

It was already concluded from the DSC thermograms that the low-pH-induced denaturation of IgG has a different effect on the domains of the protein as compared to heat-induced denaturation. Upon decreasing the pH, the F_c fragment is primarily affected followed by the denaturation of the F_{ab} fragment at even more extreme conditions. To study the effect of pH on the secondary structure of IgG, CD experiments were performed. Fig. 9 shows the results of IgG in a glycine buffer at pH 3.5, for different temperatures. The spectra are significantly altered relative to those for the native IgG at pH 8.1. The secondary structure obtained by the fitting procedure is summarized in Table 4. Even at 20°C a significant fraction of the IgG is denatured, which is reflected by the low β-sheet content. This observation is supported by Buchner et al. (1991), who concluded in their paper that the formation of a new, well-defined IgG structure occurs at low pH values. The protonation of amino acid side chains was supposed to cause the reorganization of the native state into the so-called A-state. This A-state was found to be relatively compact, which supports our observation that the IgG, after heat treatment at pH 2.0, is not strongly aggregated. This structural rearrangement may also explain the presence of an exothermic aggregation peak in

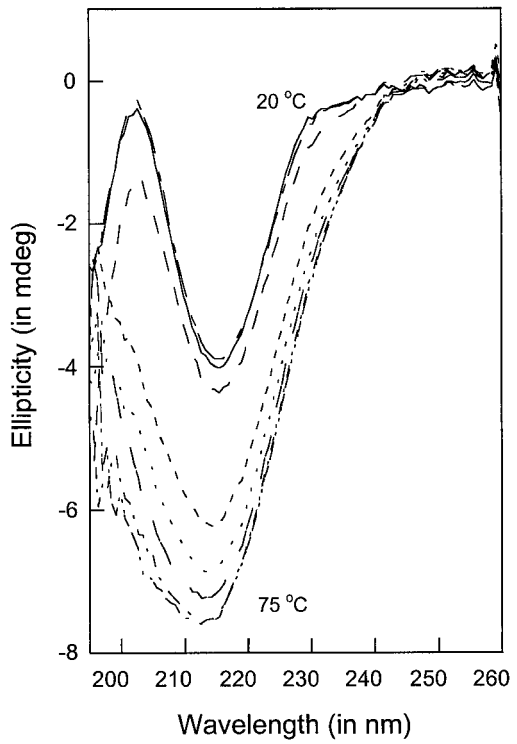

FIGURE 9 Far-UV CD spectra of IgG (0.2 mg/ml) in a 0.1 M glycine buffer, pH 3.5. The *solid curves* are smoothed through the experimental data measured at 20, 35, 50, 55, 60, 65, 70, and 75°C, respectively.

the thermogram at pH 3.5, whereas it was not present in the phosphate buffer. As for the thermal denaturation, the pH-induced structural changes in IgG observed with CD are consistent with the calorimetric data.

CONCLUSIONS

The unfolding of immunoglobulin G is a complex process, in which the denatured state is obtained from the native protein through several, at least partly independent, intermediate states. Moreover, depending on the type of denaturing stress the denaturation process follows different paths: the F_{ab} fragment is most sensitive to heat treatment, whereas the F_c fragment is most sensitive to lowering the pH.

At temperatures lower than the temperature of the first peak observed in DSC (and for low heating rates), the concentration of unfolded IgG is low; consequently, so is the rate of aggregation. At those conditions, most IgG molecules will be completely unfolded before they are incorporated in the aggregates. At higher temperatures (high heating rates) the unfolding occurs at a high speed, leading to a high concentration of (partially) unfolded IgG molecules. The rate of aggregation is correspondingly faster and it could well be that at such high aggregation rates IgG molecules are incorporated in the aggregate before they had sufficient time for complete unfolding. Furthermore, the route and method of denaturation affect the structure of the aggregates formed.

TABLE 4 Content (in %) of structure elements in IgG at different temperatures

Temperature (°C)	α-helix	β-sheet	β-turn	random coil	RMS error
20	0	47	16	37	15.0
35	0	48	16	36	14.8
50	0	47	14	39	13.4
55	1	44	10	45	8.4
60	2	43	9	46	8.4
65	2	42	9	47	6.4
70	3	41	9	47	7.2
75	4	40	9	47	7.3

Medium, 0.1 M glycine buffer, pH 3.5.

This work has been made possible by the support of Bayer AG (Leverkusen, Germany), who provided us with the materials. We thank Dr. H. H. J. de Jongh of the Centre for Protein Technology (Wageningen, The Netherlands) for his assistance with carrying out the CD experiments and the data processing.

REFERENCES

Alexander, A. J., and D. E. Hughes. 1995. Monitoring of IgG antibody thermal stability by micellar electrokinetic capillary chromatography and matrix-assisted laser desorption/ioniztion mass spectrometry. *Anal. Chem.* 67:3626–3632.

Amzel, L. M., and R. J. Poljak. 1979. Three-dimensional structure of immunoglobulins. *Annu. Rev. Biochem.* 48:961–997.

Arntfield, S. D., E. D. Murray, M. A. H. Ismond, and A. M. Bernatsky. 1989. Role of the thermal denaturation-aggregation relationship in determining the rheological properties of heat induced networks for Ovalbumin and Vicilin. *J. Food Sci.* 54:1624–1631.

Augener, W., and H. M. Grey. 1970. Studies on the mechanism of heat aggregation of human IgG. *J. Immunol.* 4:1024–1030.

Barone, G., C. Giancola, and A. Verdoliva. 1992. DSC studies on the denaturation and aggregation of serum albumins. *Thermochim. Acta.* 199:197–205.

Brahms, S., and J. Brahms. 1980. Determination of protein secondary structure in solution by vacuum ultraviolet Circular Dichroism. *J. Mol. Biol.* 138:149–178.

Brody, T. 1997. Multistep denaturation and hierarchy of disulfide bond cleavage of a monoclonal antibody. *Anal. Biochem.* 247:247–256.

Buchner, J., M. Renner, H. Lilie, H.-J. Hinz, R. Jaenicke, T. Kiefhaber, and R. Rudolph. 1991. Alternatively folded states of an immunoglobulin. *Biochemistry.* 30:6922–6929.

Buijs, J., W. Norde, and J. W. T. Lichtenbelt. 1996. Changes in the secondary structure of adsorbed IgG and $F(ab')_2$ studied by FTIR spectroscopy. *Langmuir.* 12:1605–1613.

Byler, D. M., and H. Susi. 1986. Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers.* 25:469–487.

Castronouvo, G. 1991. Proteins in aqueous solutions: calorimetric studies and thermodynamic characterization. *Thermochim. Acta.* 193:363–390.

Chang, C. T., C.-S. C. Wu, and J. T. Yang. 1978. Circular dichroic analysis of protein conformation: inclusion of the β-turns. *Anal. Biochem.* 91: 13–31.

Chothia, C., J. Novotny, R. Bruccoleri, and M. Karplus. 1985. Domain association in immunoglobulin molecules: the packing of variable domains. *J. Mol. Biol.* 186:651–663.

de Jongh, H. H. J., E. Goormaghtigh, and J. A. Killian. 1994. Analysis of circular dichroism spectra of oriented protein-lipid complexes: towards a general application. *Biochemistry.* 33:14521–14528.

Dill, K. A. 1990. Dominant forces in protein folding. *Biochemistry.* 29: 7133–7155.

Edmundson, A. B., and K. R. Ely. 1986. Determination of the three-dimentional structures of immunoglobulins. *In* Handbook of Experimental Immunology (4 vol), ch. 15. D. M. Weir, editor. Blackwell Scientific Publications, Oxford.

Fasman, G. D. 1996. Circular Dichroism and the Conformational Analysis of Biomolecules. Plenum Press, New York.

Goto, Y., N. Ichimura, and K. Hamaguchi. 1988. Effects of ammonium sulfate on the unfolding and refolding of the variable and constant fragments of an immunoglobulin light chain. *Biochemistry.* 27: 1670–1677.

Goto, Y., and K. Hamaguchi. 1982. Unfolding and refolding of the reduced constant fragment of immunoglobulin light chain, kinetic role of the intrachain disulphide bond. *J. Mol. Biol.* 156:911–926.

Greenfield, N., and G. D. Fasman. 1969. Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry.* 8:4108–4116.

Hardie, D. G., and J. R. Coggins. 1986. Multidomain proteins: structure and evolution. Elsevier, Amsterdam.

Haynes, C. A., and W. Norde. 1995. Structures and stabilities of adsorbed proteins. *J. Colloid Interface Sci.* 169:313–328.

Hoffmann, M. A. M., and P. J. J. M. van Mil. 1997. Heat induced aggregation of β-lactoglobulin: role of the free thiol group and disulphide bonds. *J. Agric. Food Chem.* 45:2941–2948.

Hu, C. Q., and J. M. Sturtevant. 1987. Thermodynamic study of yeast phosphoglycerate kinase. *Biochemistry.* 26:178–182.

Hughes, D. E., and P. Richberg. 1993. Capilary micellar electrokinetic, sequential multiwavelength chromatographic characterization of a chimeric monoclonal antibody-cytotoxin conjugat. *J. Chromatogr.* 635: 313–318.

Kats, M., P. C. Richberg, and D. E. Hughes. 1995. Conformational diversity and conformational transitions of a monoclonal antibody monitored by circular dichroism and cappillary electrophoresis. *Anal. Chem.* 67: 2943–2948.

Kats, M., P. C. Richberg, and D. E. Hughes. 1997. pH-Dependent isoform transitions of a monoclonal antibody monitored by micellar electrokinetic capillary chromatography. *Anal. Chem.* 69:338–343.

Lepock, J. R., K. P. Ritchie, M. C. Kolios, A. M. Rodahl, K. A. Heinz, and J. Kruuv. 1992. Influence of transition rates and scan rate on kinetic simulations of differential scanning calorimetry profiles of reversible and irreversible protein denaturation. *Biochemistry.* 31:12706–12712.

Lindström, P., M. Paulsson, T. Nylander, U. Elofsson, and H. Lindmark-Månsson. 1994. The effect of heat treatment on bovine immunoglobulins. *Milchwissenschaft.* 49:67–71.

López-Bote, J. P., C. Langa, P. Lastres, C. Ruis, A. Marquet, R. Ramos-Ruiz, and C. Bernabéu. 1993. Aggregated human immunoglobulins bind to modified proteins. *Scand. J. Immunol.* 37:593–601.

Martsev, S. P., Z. I. Kravchuk, A. P. Vlasov, and G. V. Lyakhnovich. 1995. Thermodynamic and functional characterization of a stable IgG conformer obtained by renaturation from a partially structured low pH-induced state. *FEBS Lett.* 361:173–175.

Maste, C. L. M., E. H. W. Pap, A. van Hoek, W. Norde, and A. J. W. G. Visser. 1996. Spectroscopic investigations of the structure of a protein on a hydrophobic latex. *J. Colloid Interface Sci.* 180:632–633.

McCarthy, D. A., and A. F. Drake. 1989. Spectroscopic studies on IgG aggregate formation. *Mol. Immunol.* 26:875–881.

Oi, V. T., T. M. Vuong, R. Hardy, J. Reidler, J. Dangl, L. A. Herzenbrg, and L. Stryer. 1984. Correlation between segmental flexibility and effector function of antibodies. *Nature.* 307:136–140.

Oreskes, I., and D. Mandel. 1983. Size fractionation of thermal aggregates of immunoglobulin G. *Anal. Biochem.* 134:199–204.

Padlan, E. A. 1997. Anatomy of the antibody molecule. *Mol. Immunol.* 31:169–217.

Privalov, P. L., and S. A. Putekhin. 1986. Scanning microcalorimetry in studying temperature-induced changes in proteins. *Methods Enzymol.* 131:4–51.

Protasevich, I., B. Ranjbar, V. Lobachov, A. Makarov, R. Gilli, C. Briand, D. Lafitte, and J. Haiech. 1997. Conformation and thermal denaturation of apocalmodulin: rol of electrostatic mutations. *Biochemistry.* 36: 2017–2024.

Rosenqvist, E., T. Jossang, and J. Feder. 1987. Thermal properties of human IgG. *Mol. Immunol.* 24:495–501.

Rousseaux, J., J. P. Aubert, and M. Loucheux-Lefebvre. 1982. Comparative study of the conformational features of rat immunoglobulin G subclasses by circular dichroism. *Biochim. Biophys. Acta.* 701:93–101.

Rowe, E. S., and C. Tanford. 1973. Equilibrium and kinetics of the denaturation of a homogeneous human immunoglobulin light chain. *Biochemistry.* 12:4822–4827.

Sanchez-Ruiz, J. M., J. L. López-Lacomba, M. Cortijo, and P. L. Mateo. 1988. Differential scanning calorimetry of the irreversible thermal denaturation of thermolysin. *Biochemistry.* 27:1648–1652.

Shimba, N., H. Torigoe, H. Takahahi, K. Masuda, I. Shimada, Y. Arata, and A. Sarai. 1995. Comparative thermodynamic analyses of the Fv, Fab* and Fab fragments of anti-dansyl mouse monoclonal antibody. *FEBS Lett.* 360:247–250.

Solís-Mendiola, S., A. Rojo-Domínguez, and A. Hernández-Arana. 1993. Cooperativity in the unfolding transitions of cysteine proteinases. Calorimetric study of the heat denaturation of chymopapain and papain. *Biochim. Biophys. Acta.* 1203:121–125.

Takahashi, K., and H. Fukada. 1985. Multi-state transition for the thermal unfolding of certain globular proteins as evaluated from the analysis of DSC curve. *Thermochim. Acta.* 88:229–233.

Tetin, S. Y., and D. S. Linthicum. 1996. Circular dichroism spectroscopy of monoclonal antibodies that bind a superpotent guanidinium sweetener ligand. *Biochemistry.* 35:1258–1264.

Tischenko, V. M., V. P. Zav'yalow, G. A. Medgyesi, S. A. Potekhin, and P. L. Privalov. 1982. A thermodynamic study of cooperative structures in rabbit Immunoglobulin G. *Eur. J. Biochem.* 126:517–521.

Vermeer, A. W. P., M. G. E. G. Bremer, and W. Norde. 1998. Unfolding of IgG induced by heat treatment and adsorption onto a hydrophobic surface studied by circular dichroism. *Biochim. Biophys. Acta.* 1425: 1–12.

Vlasov, A. P., Z. I. Kravchuk, and S. P. Martsev. 1996. Non-native conformational states of immunoglobulins: Thermodynamic and functional studies of rabbit IgG. *Biochemistry (Moscow).* 61:155–171.

Zoungrana, T., and W. Norde. 1997. Thermal stability and enzymatic activity of α-chymotrypsin adsorbed on polystyrene surfaces. *Colloids Surfaces B.* 9:157–167.