Changes in Calorimetric Parameters and Solvent Accessibility of Hydrophobic Groups in Native and Chemically Modified Immunoglobulin G

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Structural rearrangements and aggregation resulting from covalent modification of human IgG by caprylic ester of *N*-hydroxysuccinimide were studied by differential scanning calorimetry, dynamic light scattering, and ANS-binding spectrofluorimetry. The thermogram for the native IgG displays only one transition peak ($T_{max} = 68$ °C, $\Delta H = 20.9 \pm 0.3$ J/g). For the chemically modified IgG the temperatures corresponding to the initial and maximal deviations of the heat flow as well as the area under the transition peak decreased as the number of attached alkyl groups increases. This finding may be explained by weakening the intramolecular interactions responsible for the rigidity of the IgG molecular structure and by an increase in the protein−protein interactions for the modified IgG. Dynamic light scattering data indicate spontaneous aggregation of the modified IgG molecules in aqueous solution; the size of aggregates depends on the modification degree. These data correlate with a drastic increase in the surface hydrophobicity index for IgG molecules with an increase in the number of attached alkyl chains.

Introduction

Immunoglobulins are designed by nature to defend against viruses, bacteria, and parasites and are used as a unique analytical reagent armed with extraordinary specificity and binding affinity, which are the basis of various types of immunoassays. Immunoglobulin G (IgG) molecules are composed of four polypeptide chains: two "heavy" with 446 amino acid residues (∼50 kDa) and two "light" with 214 amino acid residues (∼25 kDa). These chains are cross-linked by disulfide bonds into the T- or Y-shaped structure, which is characterized by three fragments (two F_{ab} and one F_c). The presence of "hinges", which are located between these fragments, leads to flexibility of the IgG molecule and to easiness of the transition between these structures.[1,2] The IgG molecule consists of different structure elements (domains) such as antiparallel β-sheets (47 and 45% for the F_{ab} and F_c fragments, respectively), short stretches of α-helices (2 and 7% for the F_{ab} and F_c fragments, respectively) and β-turns, which are stabilized by intramolecular hydrogen bonding.[3,4]

It was found that chemical hydrophobization of IgG by attachment of alkyl chains leads to significant changes in its surface activity and functional properties. Hydrophobized human IgG spontaneously aggregates in aqueous solutions, and the size of the aggregates (colloidal clusters) depends both on the number of attached alkyl chains and on their length.[5] It displays higher affinity for hydrophobic surfaces (polystyrene and silica coated by phosphatidylcholine monolayer) and forms more compact surface layers as compared with the native protein.[5,6] Modified IgG decreases the surface tension at the air/water interface more effectively than the native protein, and this decrease correlates with an increase in the surface hydrophobicity index (SHI) evaluated with the use of fluorescent hydrophobic probe, 8-anilino-1-naphthalenesulfonate (ANS).[7] In addition, hydrophobized human IgG retains high specific recognition ability in ELISA tests.

An important question is how the increase in the IgG hydrophobicity due to the chemical modification affects the conformational stability of the protein.

There are several experimental approaches providing information on the conformational changes in the protein molecules (optical rotation dispersion, far-UV circular dichroism, Raman spectroscopy), on the environment of specific residues (solvent perturbation technique, differential UV spectroscopy, fluorimetry, near-UV circular dichroism, NMR), or on the global accessibility of the amino acid residues to the solvent (hydrogen/deuterium exchange).[8,9] High sensitivity differential scanning calorimetry (DSC) allows the determination of the thermodynamic functions of the conformational transitions directly from the heat capacity curves. This method provides information on the thermodynamic features of protein unfolding and on the contribution of various forces determining the protein stability.[8]

The heat conformational stability of IgG and changes in interaction of its domains at acid pHs as well as heat-induced and adsorption-induced denaturation have been the focus of several studies.[9−12]

The aim of the present study was to combine differential scanning calorimetry (DSC) and ANS-binding spectrofluorimetry to compare heat-induced structural changes and relative changes in the surface hydrophobicity of the native and chemically modified IgG molecules. In addition, dynamic light scattering was used for evaluation of the modified IgG aggregation in solution.

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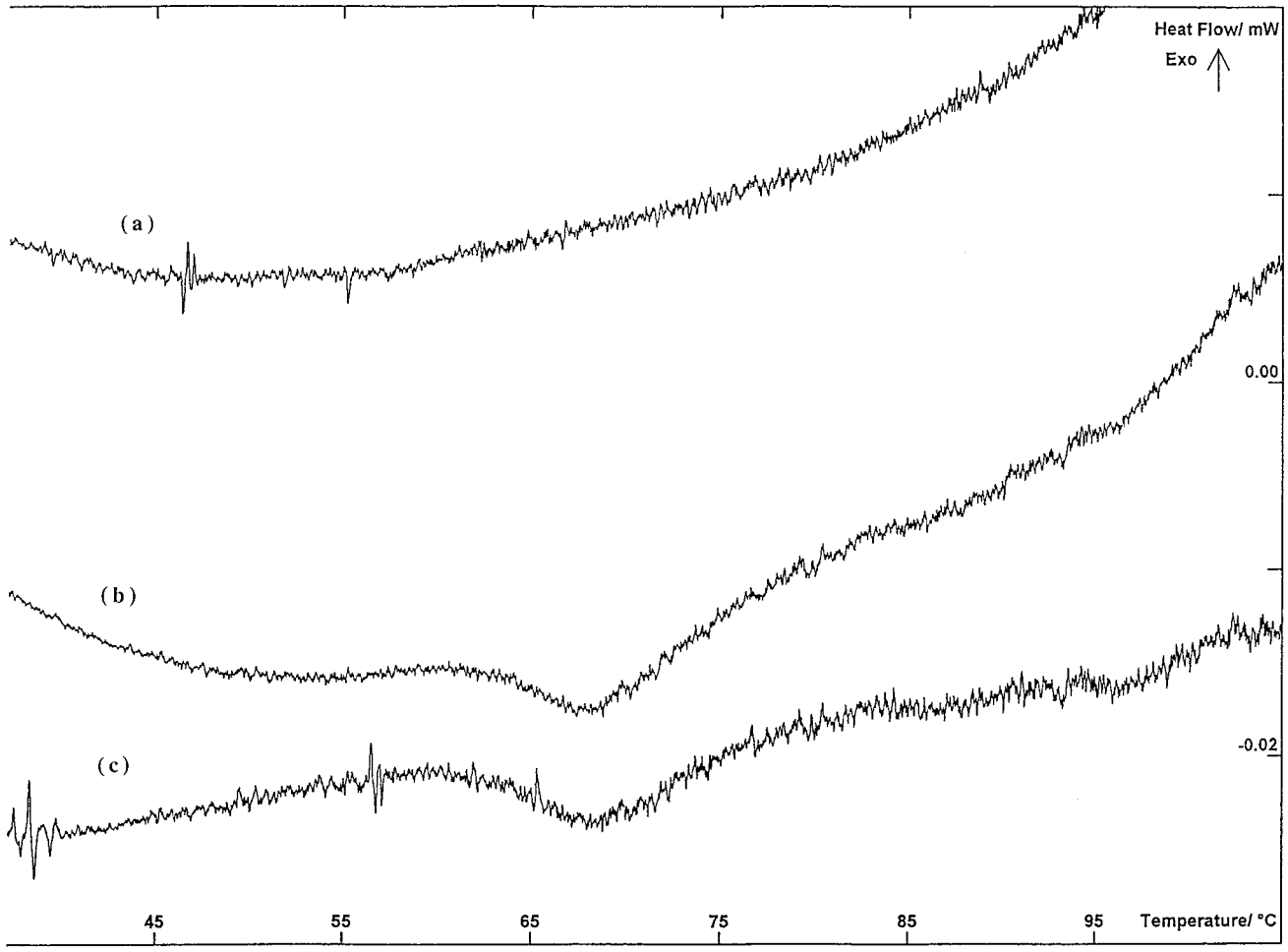

Figure 1. Thermograms obtained with the native and modified IgGs (2.8 mg·mL^{-1} of protein in 0.1 M PBS, pH 7.4; heating from 10 to 100 °C at 0.1 °C·min^{-1}). Thermograms of the first heatings of buffer (a) and native IgG before (b) and after subtraction of the buffer one (c).

Experimental Section

IgG Isolation and Modification. IgG was isolated from human serum by ammonium sulfate precipitation, purified by caprylic acid, and concentrated by additional ammonium sulfate precipitation.[13] The IgG modification was performed with *N*-hydroxysuccinimide ester of caprylic acid.[5] The ester was synthesized and isolated according to Lapidot et al.[14]

The number of covalently bound alkyl chains after modification was determined by the TNBS method[15,16] and was found to be 4 ($4C_8$-IgG), 12 ($12C_8$-IgG), and 19 ($19C_8$-IgG) at the initial molar ratios of IgG to ester in the reaction mixture 1:10, 1:20, and 1:30, respectively.

Protein concentrations were determined spectrophotometrically (Hitachi double-beam spectrophotometer, model U-2000) by the Bradford method[17] or by the improved Lowry method[18] using Bio-Rad protein assay reagents.

Differential Scanning Calorimetry. Micro-DSC III (Setaram, Caluire-France) was used to monitor the heat-induced structural changes in the native and chemically modified IgG. The protein solution (2.8 mg·mL^{-1}) in 0.1 M PBS (phosphate buffer saline), pH 7.4, was placed in 1 mL vessel, scanned from 10 °C up to 100 °C (0.1 °C.min^{-1}), cooled at the highest rate allowed by this equipment (2 °C.min^{-1}), and then reheated at the same rate as in the first cycle. The same buffer was used as a reference. The calorimetric parameters of heat-induced structural changes in the IgG molecules were determined on the basis of thermogram of the first scan with the use of Setaram Software after subtraction of the sample baseline.[19] The heat conformational stability of the native and chemically modified IgGs was evaluated from the values of the initial (T_i) and maximum (T_{max}) deviation of the heat flow signal and also from the value of specific enthalpy transition (ΔH), which was calculated from the area under the transition peak with the use of a straight baseline between the initial and final temperatures of the transition peak.

Protein Surface Hydrophobicity. Protein surface hydrophobicity was determined according to procedures described elsewhere.[7,20−22] Fifteen microliters of 8 mM ANS solution in absolute ethanol was added to 2 mL of nonheated or preheated native or modified IgG solution (0.1−0.6 mg.mL^{-1}) in 0.1 M PBS, pH 7.4, and after vigorous stirring for 10 min, the fluorescence intensity at 470 nm was performed at 20 °C (excitation wavelength 380 nm). The fluorescence spectra were recorded with the use of AMINCO-Bowman luminescence spectrometer (band-passes 4 nm) for the nonheated solutions and with Perkin-Elmer Luminescence spectrometer LS-5 (excitation band-pass 5 nm, emission band-pass 2.5 nm) for preheated solutions. The SHI values were quantified from the slope of the fluorescence intensity versus protein concentration plots. The fluorescence intensity values were corrected taking into account the fluorescence of the protein itself and of ANS in PBS.

Particles Size Measurements. The average particle size of the protein samples was measured by dynamic light scattering

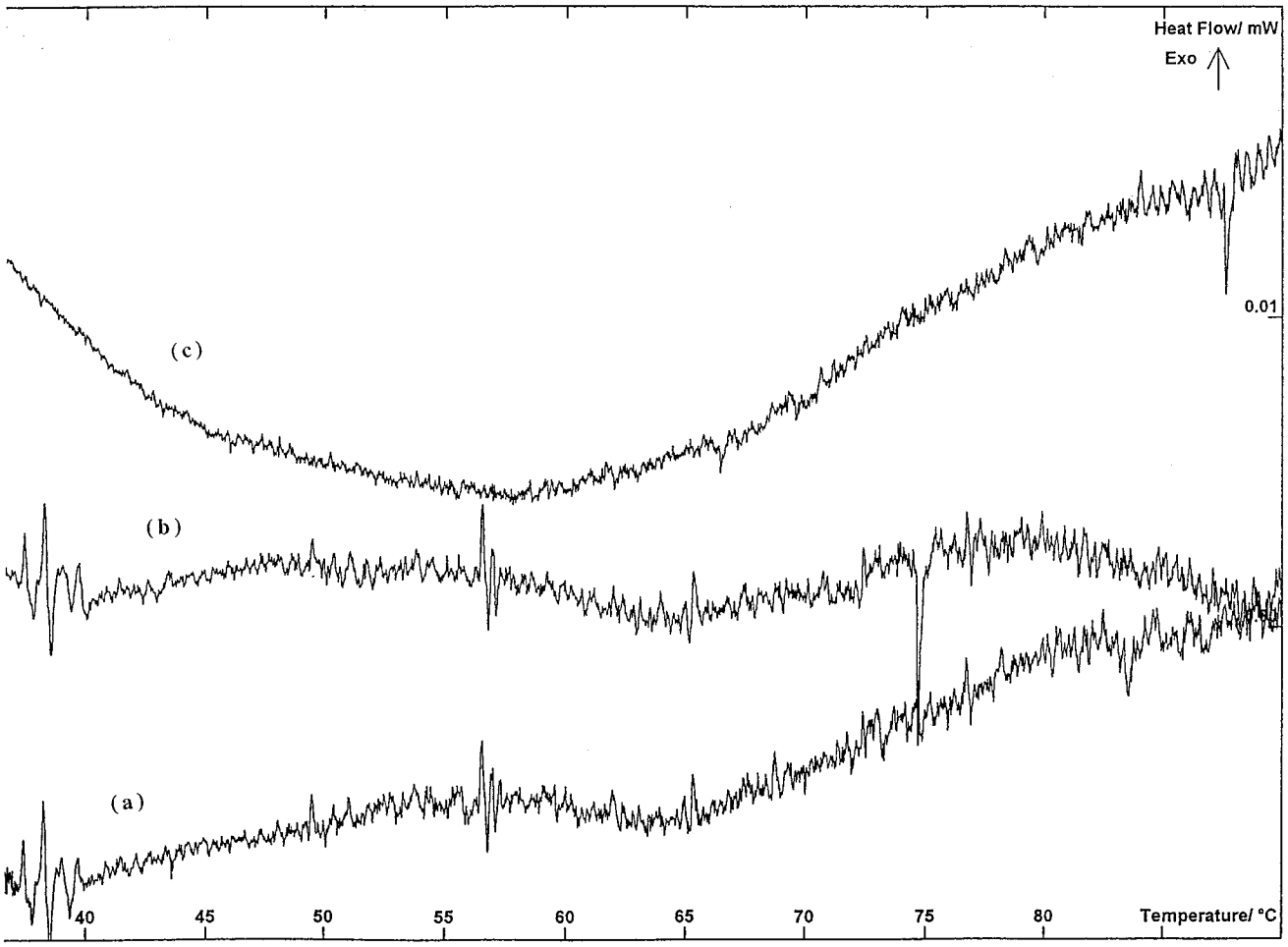

Figure 2. Thermograms obtained with $4C_8$-IgG (a) and $12C_8$-IgG (b) after subtraction of the buffer thermogram and for $19C_8$-IgG without subtraction of the buffer thermogram (c).

with a Zetasizer 3000, Malvern Instruments, UK (Ar^+-laser, wavelength 488 nm, power 70 mW; detector angle 90°; temperature 25 °C, dispersant viscosity 0.89 cP; dispersant refractive index 1.33, sample refractive index 1.40, absorbance 0.00; Auto:CONTIN analysis). The particles size was taken as a mean value of four measurements.

Results and Discussion

Heat-Induced Transition. The curves in Figure 1 present examples of first heating thermograms obtained for 0.1 M PBS pH 7.4 (a), native IgG solution (2.8 $mg.mL^{-1}$) before (b), and after (c) subtraction of the PBS thermogram. The transition peak was located between 60 and 80 °C. The thermograms obtained with 4C8- and 12C8-IgG, after subtraction of PBS thermogram are shown in Figure 2, a and b, respectively. It is seen that the DSC transition peak is the most noticeable for native IgG sample. The thermograms obtained on reheating native, 4C8-, and 12C8-IgG were different to that of PBS: they all presented a weak and very large endothermic peak lying between 20 and 85 °C, likely for the first heating thermogram of 19C8-IgG (Figure 2c). The nonsymmetrical shape of the native transition peak (Figure 1c) is a reflection of a multistep behavior, as expected for such multidomain protein as IgG. Similar thermograms have been reported for thermal denaturation of rabbit and monoclonal mouse IgGs at neutral pH.[9,12] The temperature of maximum heat flow deviation ($T_{max} = 68$ °C) and specific transition enthalpy ($\Delta H = 20.9 \pm 0.3$ $J{\cdot}g^{-1}$) calculated from the peak area between 60 and 80 °C are in good agreement with previous results. The transition peak obtained with $4C_8$-IgG and $12C_8$-IgG samples was located between 55 and 80 °C, and the corresponding values of ΔH decreased more for 12C8 than for 4C8, relative to unmodified IgG. The peak transition obtained with 19C8-IgG, after subtraction of the PBS signal, was not repeatable and we considered its ΔH value equal to zero. Upon heat treatment, the change in the native IgG molecular structure causes increased exposure of both initially buried apolar and polar groups to the solvant molecules. This may lead to interaction forces between those protein groups and water molecules which can have positive (for apolar groups) and negative (for polar groups) contributions on the heat capacity change, ΔC_N^D between the initial (native) and final (denatured) states. Those mechanisms have been shown to decrease (polar groups hydration) and to increase (apolar groups hydration) as the temperature increases.[23] For globular proteins, the enthalpy change of heat-induced denaturation may also be affected by subsequent interactions between proteins as they denature.[8,19,22] That last negative contribution of protein/protein interactions to the overall calorimetric heat of reaction can be confirmed by performing DSC experiments at various scan rates. However, as previously reported for bovine serum albumin,[24] monoclonal mouse IgG,[9] and ovalbumin[25] solutions which have been heated at relatively higher scan rates (0.5 to 1 $°C{\cdot}min^{-1}$) and in large volume cells (1 mL), we observed a large distortion of the thermogram by an exothermic peak (Figure 3). At a high scan rate, the kinetics of heat-induced aggregation is enhanced, compared to that of denaturation even for unmodified IgG. Therefore, on the basis of the overall calorimetric heat of reaction reported in Figure 4 (as determined from the thermo-

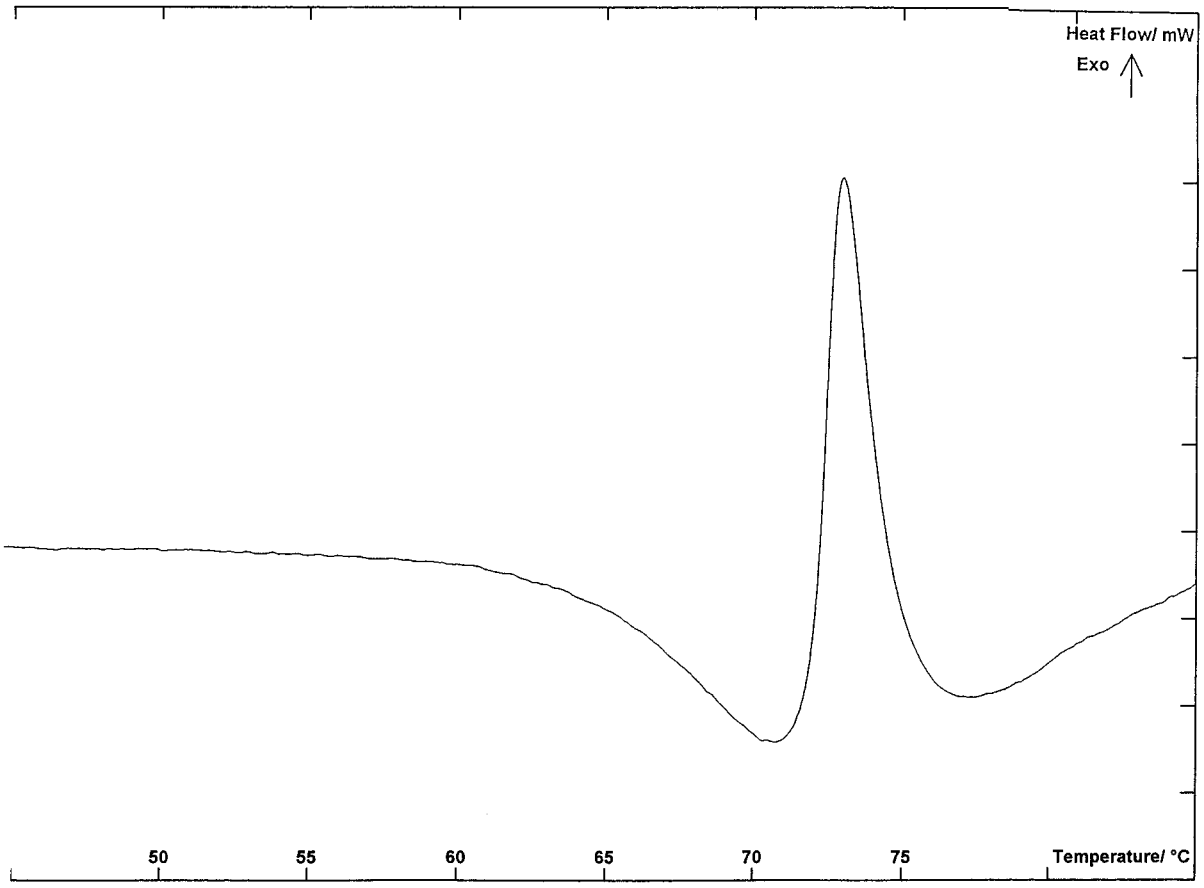

Figure 3. Thermogram obtained with the native IgG (2.8 mg·mL^{-1} of protein in 0.1 M PBS, pH 7.4; heating from 10 to 100 °C at 1 °C·min^{-1}).

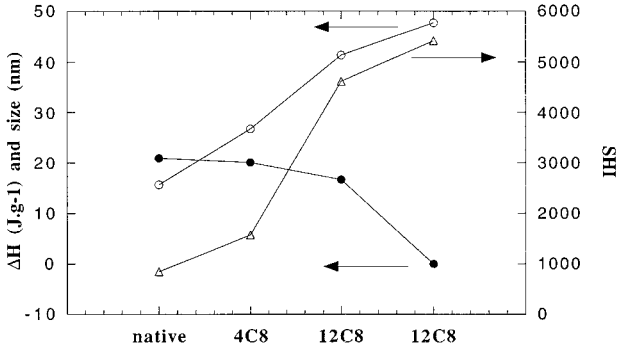

Figure 4. Specific heat of reaction (ΔH in J·g^{-1}, filled circles), surface hydrophobicity index (SHI in au, triangles), and size of aggregates (in nm, open circles) for native and chemically modified IgG.

grams obtained at 0.1 °C·min^{-1} and where no exothermic distortion was detected between 55 and 80 °C), and following the last considerations we may postulate that the tertiary structure of 4C_8-IgG and the level of heat-induced protein/protein aggregation are close to those of the native IgG. For 12C_8-IgG, which could be less rigid, and 19C8-IgG which could be highly changed after attachment of 19 alkyl chains, less and more heat-induced aggregation could take place during the DSC scan.

Surface Exposition of Hydrophobic Groups. The surface hydrophobicity of the modified enzyme in this study was evaluated by using a fluorescent probe, ANS. This method is based on the rule that dyes with higher dipole moments in the excited state than in the ground state show emission spectra, which depend on the polarity of environment.[24,25] Generally, ANS transfer from a polar to a nonpolar medium results in a blue shift in the fluorescence emission maximum and in a significant increase in the quantum yield.

Addition of ANS to solutions of chemically modified IgG led to noticeable changes in the ANS fluorescence spectra (blue shift of the wavelength of fluorescence maximum and an increase in the fluorescence intensity), as previously reported.[7] The same results were obtained in the present study. In addition, heat treatment of the protein (from 20 to 90 °C, incubation at 90 °C for 60 min) produced analogous effect on the fluorescence spectra. These results reflect changes in relative hydrophobicity of the solvent-accessible protein surface upon chemical and/or thermal modifications. The relative change in hydrophobicity was evaluated from the dependence of the emission intensity at 470 nm on the protein concentration. This wavelength did not coincide with the maximum in the emission spectra of ANS in the presence of the native IgG but it was chosen for several reasons. First, the emission of the protein itself at 470 nm is very low. Second, the fluorescence bands of ANS in the presence of different concentrations of the native IgG were rather wide, and the difference in the emission intensities at 470 and 520 nm (band maximum for ANS bound to the native protein) was not significantly pronounced (corrections were made for all measured intensities). In addition, 470 nm is a generally used wavelength for measuring fluorescence of ANS bound to various proteins.[7,20−22]

The linear plots of the fluorescence intensity vs concentration obtained at protein concentrations ranging from 0.1 to 0.6 mg·mL^{-1} are presented in Figure 5, a (unheated samples) and b (preheated samples), respectively. The values of SHI presented in Figure 4 were estimated from the slope of these plots. The native IgG was characterized by the lowest SHI value, indicating the lowest surface hydrophobicity. An increase in the number

of attached C8 groups resulted in an increase in the surface hydrophobicity and, therefore, in an increase in solvent accessibility of the protein hydrophobic groups. For unheated $4C_8$-IgG, the SHI was approximately 1.8 times higher than for the native IgG, while its ΔH value did not significantly change, compared to unmodified protein. This could be explained by a change in surface hydrophobicity as a result of attachment of 4C8 groups to surface exposed lysine groups, without significant changes in the protein internal structure and in protein/protein interactions. For unheated $12C_8$-IgG and $19C_8$-IgG, SHI was approximatly 5.5 and 6.4 higher than that of native IgG, while the corresponding ΔH values were different from those to 4C8 and native IgG. These results may indicate that (i) up to a certain degree of chemical modification, although there is a relatively low increase in surface hydrophobicity, the heat conformational change of IgG is not greatly affected, and (ii) further increase in the degree of chemical modification leads to decrease in the initial internal structure stability, increase in the index of the surface hydrophobicity, and/or enhancement of heat-induced aggregation between chemically modified proteins. The curves in Figure 4 indicated that the trend of heat-induced calorimetric reaction (ΔH) and accessibility of hydrophobic groups to the solvent (SHI), relative to unmodified IgG may be related. A similar conclusion[22] has been drawn for a low molecular weight globular protein β-lactoglobulin, for which structural modification by preheating at various temperatures has been shown to result in an increase in solvent accessibility of hydrophobic groups and in a significant decrease in the residual specific enthalpy after heating to 80 °C. After heat-induced modification was performed at $T > 80$ °C, SHI was shown to increase much more, in parallel with the decreasing of ΔH to zero value.[22] The similar trend in SHI and ΔH values, obtained in the present study, (Figure 4) may be explained, as in the previous one, by increased heat destabilization of the packed structure of IgG molecule, as a result of alkyl groups attachment, increased accessibility of the hydrophobic groups for a solvent, and enhancement of protein−protein interactions.

When ANS was added to preheated samples of the native or chemically modified IgG, the slope values of the plots in Figure 5b were much greater as compared with the corresponding unheated samples. After heat treatment, the native and $4C_8$-IgG molecules on one hand, and $12C_8$-IgG and $19C_8$-IgG on the other hand, exhibited a similar solvent accessibility of hydrophobic groups with an increase in SHI of 9 and 13 times relative to the unheated native IgG. Native and 4C8-IgG molecules, which presented similar ΔH values (as determined from the first heating scan) and different SHI values before heating, seemed to present a similar surface hydrophobicity after previous heating at 90 °C for 60 min, cooling to 20 °C, and dilution. 12C8- and 19C8-IgG presented different first heating DSC signals and different SHI values before heat modification. These results may be explained by a much more open structure for unheated 19C8-IgG. After cooling and dilution of the heated samples, there is an apparent similarity between their SHI values, which seemed to be much higher than those of native and 4C8-IgG after heat treatment. For all protein samples, the second heating thermograms, although there was a lack of repeatability, were more curved than that of PBS one. These results could reflect the formation of different local structures after heating and dilution, although on reheating in the calorimeter the DSC signals for all samples were similar. Further investigations are needed to clarify this point.

It is expected that when more hydrophobic groups are exposed to the aqueous medium, spontaneous aggregation may take place. Dynamic light scattering measurements were performed only with unheated native and chemically modified IgG, since preheating led to formation of turbid solutions. The average sizes of the IgG aggregates (from number distribution) are presented Figure 4. As seen, the mean size of the aggregates gradually increases with an increase in the degree of modification. This finding clearly indicates that in accordance with the results obtained with the use of the polarity-sensitive ANS probe, the protein−protein hydrophobic interactions occur before and after heat treatment. Furthermore, in accordance with the trend observed for the specific enthalpy changes as a function of the modification degree, it may be suggested that the chemical attachment of hydrophobic chains to the protein causes destabilization of the protein conformation. Such a destabilization becomes distinctly apparent for the IgG molecules with 12 and 19 covalently bound alkyl chains.

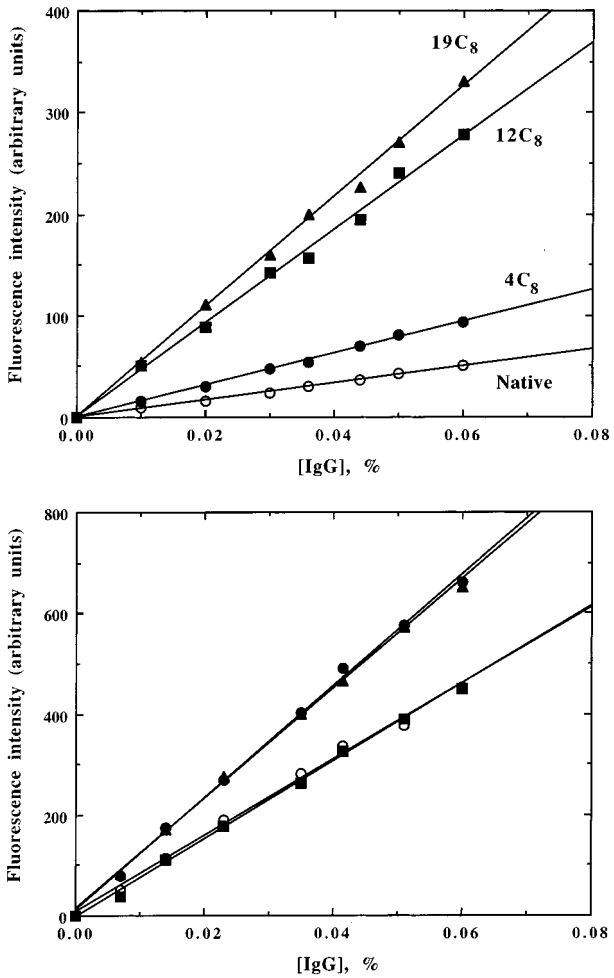

Figure 5. Variation of ANS fluorescence intensity at 470 nm as a function of the protein concentration: (a) nonheated native IgG, $4C_8$-IgG, $12C_8$-IgG, and $19C_8$-IgG (from bottom to top) and (b) preheated native IgG, $4C_8$-IgG, $19C_8$-IgG, and $12C_8$-IgG (from bottom to top).

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