Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Biologicals 34 (2006) 5–14
IABs BIOLOGICALS
www.elsevier.com/locate/biologicals

The effect of solvent environment on the conformation and stability of human polyclonal IgG in solution

Árpád Szenczi [a,b], József Kardos [c], György A. Medgyesi [d], Péter Závodszky [a,*]

[a] *Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, H-1113, Karolina út 29, Budapest, Hungary*
[b] *Quality Assurance Division, Teva Pharmaceutical Works Co. Ltd., H-2100, Táncsics Mihály út 82, Gödöllő, Hungary*
[c] *Department of Biochemistry, Eötvös Loránd University, Pázmány P. sétány 1/C, Budapest H-1117, Hungary*
[d] *National Blood Transfusion Service, H-1518, P.O. Box 44, Budapest, Hungary*

Received 5 February 2004; revised 19 May 2005; accepted 23 June 2005

Abstract

Stability of therapeutic IgG preparations is an important issue as adequate efficacy and safety has to be ensured throughout a long shelf life. To this end, denaturation and aggregation have to be avoided. In many cases sugars are applied for stabilizing IgG in relatively high concentration (5–10%). However, certain sugars (sucrose, maltose) are responsible for adverse effects including renal failure.

In this work we reassessed the effect of pH and stabilizers to optimize the solvent environment and minimize the amount of additives without endangering quality and stability. Since both biological function and aggregation depend on the conformational properties of individual IgG molecules, two sensitive and rapid physical methods were introduced to assess conformational changes and structural stability as a function of pH and addition of standard stabilizers.

It was observed that the conformational stability decreases with decreasing pH, while the resistance against aggregation improves. The optimum pH range for storage is 5.0–6.0, as a compromise between conformational stability and the tendency for oligomerization. Intriguingly, additives in physiologically acceptable concentration have no effect on the thermal stability of IgG. On the other hand, glucose or sorbitol, even at a concentration as low as 1%, have significant effect on the tertiary structure as revealed by near-UV-CD spectroscopy, reflecting changes in the environment of aromatic side-chains. Although, 0.3% leucine does not increase conformational stability, it decreases the aggregation tendency even more efficiently than 1% glucose or sorbitol.

Both pH and storage temperature are decisive factors for the long-term stability of IgG solutions. An increase in the dimer content was observed upon storage at 5 °C which was partly reverted upon incubation at 37 °C. Storage at temperatures higher than 5 °C may help to maintain an optimal proportion of dimers. Regarding the known side effects, and their limited stabilizing capacity at low concentration, it is advisable to omit sugars at intravenous immunoglobulin (IVIG) formulation. Hydrophobic amino acids give promising alternatives.

Keywords: Immunoglobulin G; Differential scanning calorimetry; Circular dichroism

1. Introduction

Intravenous immunoglobulins (IVIGs) are widely used therapeutic agents applied not only for substitution in antibody deficiency but also for immunomodulation in certain autoimmune conditions [1,2]. The active

* Corresponding author. Tel.: +36 1 2093535; fax: +36 1 4665465.
E-mail address: zxp@enzim.hu (P. Závodszky).

1045-1056/06/$32.00
doi:10.1016/j.biologicals.2005.06.007

ingredient of IVIG, polyclonal IgG is subject to dimerization/oligomerization in solution. Dimers are in the focus of interest since they have an important role in the mechanism of action of IVIG. First, they were implicated in hypotension as observed in a rat model [3–5] and more recently, in the reversal of platelet depletion in mouse models for idiopathic thrombocytopenic purpura (ITP) [5]. Moreover, dimers fractionated from IVIG are more potent activators of human granulocytes than monomers [7]. These effects presumably depend on the interaction of dimers with low affinity Fcγ-receptors of macrophages and granulocytes [4–7]. Prospective of expanding application of IVIG and biological effects of dimers pose the question, how does the formulation influence the dimer content of the solution and the structural stability of IgG. Commercially available IVIG preparations contain variable amounts of IgG dimers in the range of 5–15% [4]. The requirement of European Pharmacopoea is to limit the oligomer (but not the dimer) content (<3% for polymers with >90% for monomers plus dimers) in IVIG products to avoid the undesirable complement activation effect and to achieve intravenous tolerability.

IgG-dimer formation stems from the special function and the heterogeneity of the protein solution. IgG is a multidomain protein, four domains can be distinguished on each of the two heavy chains and two on each light chains. Distinct domain surfaces are engaged in intramolecular interactions, while others are prone to combine to other molecules. Polyclonal IgG is a multitude of closely related proteins. As an expression of the huge Ig-gene repertoire, the variable region of the IgG molecule is extremely heterogeneous, giving rise to 10^9–10^{12} idiotypes. This heterogeneity is further increased by the heavy and light chain isotypes, by the numerous allotypes and by its diversified oligosaccharide components. Batches of IVIG products are derived from at least 1000 blood donations. The great variability results in a significant propensity for oligomerization, i.e. certain IgG molecules recognize other IgG molecules as their antigen counterparts (idiotype–anti-idiotype interaction) and form reversible complexes through secondary interactions [8]. The IgG molecules constituting dimers are thus connected through their variable regions and mainly through their both Fab arms [9]. Driving forces of antigen–antibody binding are electrostatic [10] and hydrophobic [11,12], where the binding energy is locally strong enough to overcome the overall macroscopic hydrophilic repulsion [13]. Hydrophobic interactions thus play a primary role in the formation of idiotype–anti-idiotype complexes as well. The rate of dimerization is significantly lower at acidic pH values [8] and the frequent IVIG additives, sugars and the recently investigated amphipathic compounds exert a similar effect [12]. The mechanism of these interactions has not been fully understood. On the other hand, renal failure as adverse effect of IVIG administration has been observed in certain cases that are thought to be associated with carbohydrate containing products [14,15]. Therefore, avoiding sugar additives or decreasing their concentration has a primary importance in IVIG formulation.

In this study we present a systematic approach for a "protein drug" formulation challenge by using rapid and sensitive physico-chemical and biophysical methods. We investigated the effect of additives on IgG including frequently applied sugars and evaluated their efficiency. Conformational stability and pH optimum are basic parameters in protein formulation, therefore structural characteristics of IgG in various solvent environments were compared by the use of differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy. Correlation of the physico-chemical parameters with dimerization was examined and pH dependence of temperature induced structural transitions has been recorded. Dimer and polymer content of IgG in solution was determined as a function of additives and storage time using size exclusion chromatography.

2. Materials and methods

2.1. IgG preparation

Cohn fraction II paste, prepared from a plasma pool of 6000 blood donations [16], and virus inactivated purified IgG precipitate were provided by Teva Co. Ltd., Gödöllő, Hungary. The purified IgG was derived from fraction II paste through a 10 h heat treatment at 60 °C and a polyethylene glycol precipitation process – samples were derived from the manufacture of Humaglobin (IVIG) – based on the purification method as described previously [17]. Proper amount of PEG precipitate was dissolved in 6 times amount of 40 mM Na-acetate buffer, pH 5.5. Protein concentration – determined by measuring optical density at 280 nm, using an extinction coefficient of 14 (1%, 1 cm) – was adjusted by dilution with sodium-acetate buffer. Alternatively, fraction II paste was purified by anion exchange chromatography as described previously [18]; 2.5 g of fraction II paste was dissolved in and dialyzed against 70 mM sodium-acetate, pH 5.2 and was applied to a DEAE Sepharose CL 6B (Pharmacia) column equilibrated in the same buffer. Breakthrough fraction was collected. The IgG samples were clarified by passing through 0.2 μm filters.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reduced SDS-PAGE gels were prepared to quantitatively assess the purity of samples and the effect

of solvent environment on IgG. A 5 μg protein load per well was used on a Bio-Rad 5–20% Tris–glycine gradient gel and a 10% gel. Gels were run at 30 mA for 30 min at constant voltage of 40 V and then for 1.5 h at 100 V, subsequently stained using a 0.1% Coomassie Blue staining solution.

2.3. Differential scanning calorimetry

For calorimetric measurements purified IgG fractions were dialyzed overnight at 4 °C against different solutions without additives, against either 60 mM citrate-phosphate or 40 mM sodium-acetate buffers (pH 4.0–7.0). Glucose, sorbitol and leucine were added to the dialyzing buffer at pH 6.0 in 10, 10 and 3 mg/ml concentrations, respectively, and a combination of sorbitol and leucine was also applied (10 and 3 mg/ml). After the removal from the dialysis tubing, protein concentration was adjusted to 0.2–40 mg/ml by dilution with the proper buffer. Calorimetric studies were performed with a MicroCal VP-DSC differential scanning calorimeter using a digital acquisition data software provided by the manufacturer. Samples were degassed before injection into the calorimeter cells (511 μl cell volume). The protein samples were placed in the calorimeter into the sample cell against the reference cell that was filled with the appropriate blank solution. The scan rate was 1 °C/min in all cases. Data processing of the DSC-curves was carried out by MicroCal ORIGIN software. The melting temperature, T_m, corresponding to the maximum of the transition peak was determined by deconvolution analysis.

2.4. Circular dichroism (CD) spectroscopy

Circular dichroism spectra were measured with a JASCO spectropolarimeter (Japan Spectroscopic Co., – model J-720). Quartz cuvette with a cell length of 1 mm was used. CD spectra were recorded at 25 °C with the help of a thermostatically controlled cell holder attached to a Neslab RTE-111 thermostat with an accuracy of ±0.1 °C. IgG was dissolved and dialyzed as for the DSC experiments, but for CD measurements in the far-UV region (200–250 nm) 5 mM Na-acetate buffer was applied. The concentration of IgG was 0.2 mg/ml and six scans were accumulated with a scan rate of 20 nm/min and 4 s response time. During measurements in the near-UV region (250–300 nm) 40 mM Na-acetate buffer and a protein concentration of 10 mg/ml were applied.

2.5. Size exclusion chromatography

SEC-HPLC was performed using a TSK SW 7.5 × 75 mm precolumn and a TSK G3000SW 7.5 × 600 mm gel filtration column (TosoHaas) equilibrated with 40 mM sodium-phosphate, 200 mM NaCl, pH = 7.0 buffer operated by a Merck-Hitachi equipment. Samples were diluted to a concentration of 2 mg/ml with physiological saline solution and filtered through 0.2 μm membranes before injections. An injection volume of 20 μl solution was analysed in all cases using a flow rate of 1 ml/min. Column eluates were monitored at 280 nm and peak integration was performed by the software.

2.6. Dimerization-kinetics experiments

The PEG precipitate of IgG was dissolved in deionized water (IVIG-A) and 40 mM Na-acetate (IVIG-B) as described above. The protein concentration was adjusted to 50 mg/ml by dilution and the pH was corrected to 6.0. Residual PEG content was below 1 mg/ml in all samples. D-glucose, D-sorbitol, L-leucine and the combination of sorbitol and leucine were dissolved in IVIG-A solutions at the concentrations of 10 – 10, 3 and 10 + 3 mg/ml, respectively. There were no pH shifts of IVIG-A solutions due to these additives. Samples were sterile filtered into type I vials and incubated at 5 °C and 37 °C. Samples were also derived from the manufacture of Humaglobin after aseptic filling before freeze-drying (IVIG-C) and incubated at 5 °C in liquid form. Molecular-size distribution was determined by SEC-HPLC as a function of time.

Glucose, sorbitol and leucine solutions were added to IgG solutions at 100 mg/ml protein concentration at room temperature and changes in dimer content were measured with SEC-HPLC. Molar ratio of stabilizers and IgG were 10:1.

2.7. Turbidity

Turbidity was determined as a function of time measuring the absorbance of IVIG-A solutions at 600 nm, 75 °C. A Nicolet Evolution 300 instrument with a thermostat was used for these experiments.

2.8. Isoelectric focusing

Isoelectric focusing was carried out in the pH range 3–10 using Bio-Rad Mini IEF cell (Model 111). The gel was prefocused using 200 V, 6 mA, 1 W for 30 min. Some 50 μg of each of the IgG samples and p*I* markers in the range 4.6–9.0 were then applied to the gel. Samples were desalted by dialysis against deionized water. The gel was then focused using 450 V, 4 mA, 2 W for 1 h. The separated protein components were stained with Coomassie Brilliant Blue G-250, and quantified by densitometry.

2.9. Anticomplementary activity

Anticomplementary activity (AcA) of the preparations containing additives was estimated by the method described in the European Pharmacopoeia. A defined amount of test material (10 mg IgG) and a defined amount of complement (20 CH50 (quantity of complement required for 50% lysis)) were diluted with 0.6 ml of barbital-buffered saline containing optimal levels of Ca^{2+} and Mg^{2+} and incubated for 60 min at 37 °C. Then, 0.2 ml of this mixture was diluted with 9.8 ml barbital buffer. The remaining complement activity was titrated and 0.2 ml of buffer containing 5×10^8/ml sheep erythrocytes sensitized with anti-sheep red cell antibodies from rabbit (E-As) was added. The suspension was then incubated for 60 min at 37 °C. After centrifugation, the absorbance of the supernatant was measured at 541 nm and the degree of haemolysis was evaluated, using 100% lysis obtained from 1.3 ml distilled water and 0.2 ml E-As as reference. In parallel, a complement control solution was titrated, using barbital buffer instead of IgG solution. The AcA of preparation to be examined was calculated in relation to the complement control considered as 100%.

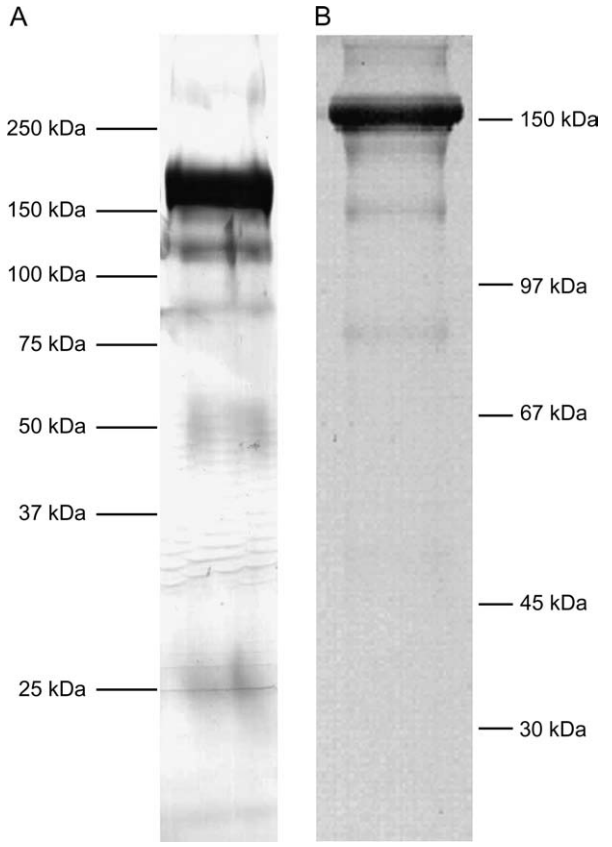

Fig. 1. SDS-PAGE of polyclonal IgG on a 5–20% gradient gel (A) and a 10% gel (B). Intact IgGs appear as a single major band at 160 kDa representing monomeric IgG.

3. Results

3.1. Effect of pH on the structure and stability of IgG

Protein purity of the preparations was checked by SDS-PAGE (Fig. 1). IgG content of the preparation using either of the two purification processes was >95%. With varying pH no change in IgG gel image occurred.

DSC thermograms of IgG at pH 6.0 were obtained at protein concentration between 0.2 and 10 mg/ml. No difference was detected in the heat-stability of IgG obtained by the two methods of purification (data not shown). Heat capacity drops above 70 °C due to thermal aggregation, particularly at high protein concentration, therefore, comparison of thermograms recorded at varying pH was carried out at an IgG concentration of 0.2 mg/ml.

Thermograms show significant complexity in accordance with previous results with monoclonal IgG [19–23], upon deconvolution 4–5 cooperative transitions remain distinct (Fig. 2). Melting temperature (T_m) values associated with these components are summarized in Table 1 and a pH dependent shift was observed for the deconvoluted components.

At lower pH, especially below pH 5.0 the stability of IgG decreases with respect to heat denaturation; T_m values are shifted to a lower temperature range (Table 1, Fig. 2A). Melting temperature values of the first two transitions are less clear-cut than those of the other transitions.

IgG shows the greatest stability at pH 7.0, but increased aggregation tendency appears due to the proximity of the isoelectric point of the major portion of the molecules. According to our IEF experiments, isoelectric point of polyclonal IgG varied between 4.7 and 7.5 in equal distribution (Fig. 3).

In the descending phase of the DSC-curves higher rate of aggregation occurs simultaneously with unfolding. During aggregation, where the associated state is the energetically favorable, heat capacity drops significantly (exothermic process), while unfolding remains incomplete. Therefore, ΔH values, the result of deconvolution, could not be considered as the real calorimetric enthalpy changes of the proper transitions.

The effect of pH on the CD spectrum of IgG is shown in Fig. 4. The CD spectra of IgG are that of a typical immunoglobulin, with a negative band at 217 nm, representing a high content of β-sheet and several positive and negative bands in the near-UV region. These spectra were similar to the results observed earlier [22,23]. In the far-UV region there is a hardly measurable difference in the CD spectra recorded at pH 4.0, 5.0,

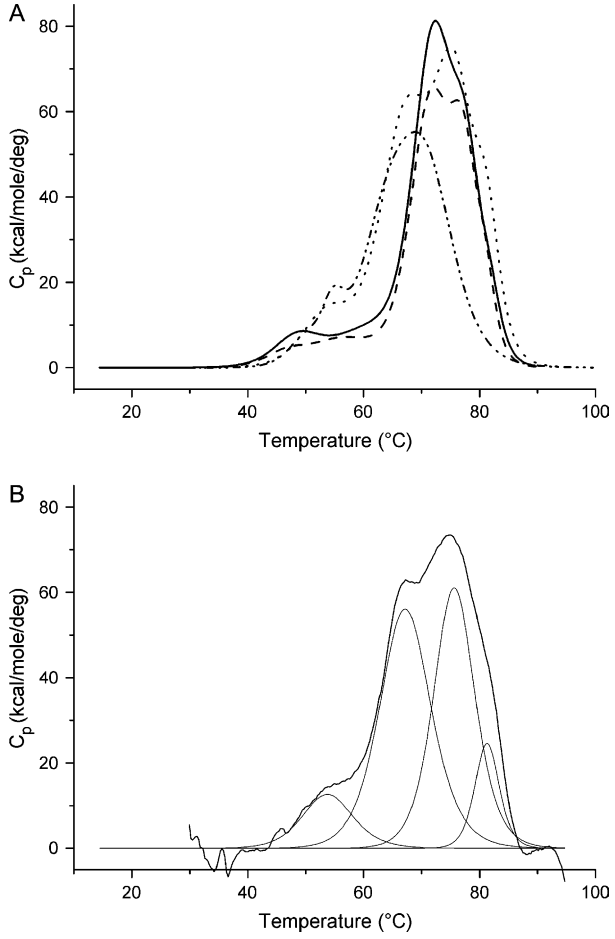

Fig. 2. (A) DSC thermograms of polyclonal IgG at pH 7.0 (solid line), pH 6.0 (dashed), pH 5.0 (dotted), pH 4.0 (dash–dot). (B) Deconvolution of the excess heat capacity functions of IgG at pH 5.0.

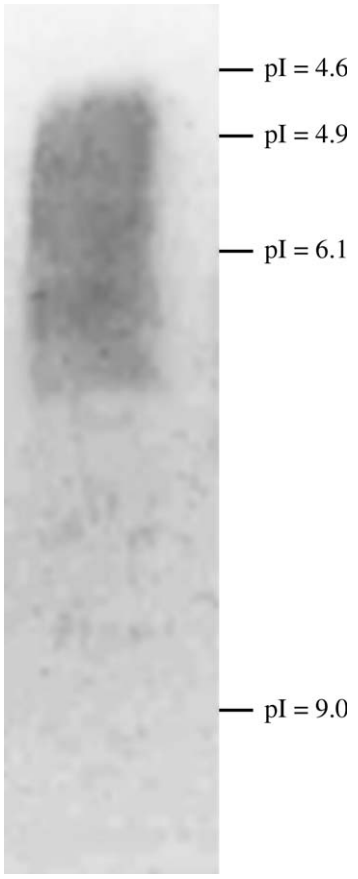

Fig. 3. Isoelectric focusing of polyclonal IgG that appears in a wide band corresponding to the p*I* range of 4.7–7.5.

and 7.0, although slight increase of the CD-signal in the range of above 210 nm is detected at pH 6.0. It indicates that, at pH 6.0 the population of IgG molecules is enriched in secondary structure and compactness. Near-UV-CD spectra of IgG at different pH were characterized by the presence of two minima at 262 and 268 nm as well as a shoulder at 286 nm, characteristics of aromatic chromophores and disulfide bonds. Near-UV-CD spectra show slight alterations (i.e. tertiary structure alterations) with varying pH.

Table 1
Transition temperatures obtained by deconvolution of excess heat capacity curves

	T_{max} (°C)	T_{m1} (°C)	T_{m2} (°C)	T_{m3} (°C)	T_{m4} (°C)	T_{m5} (°C)
pH 7.0	72.2	48.7 ± 1.1	60.7 ± 1.5	72.1 ± 0.3	77.8 ± 0.3	81.9 ± 0.7
pH 6.0	72.3	46.7 ± 1.9	56.7 ± 1.6	71.6 ± 0.3	77.3 ± 0.3	81.0 ± 0.5
pH 5.0	75.0	–	53.8 ± 0.6	67.3 ± 0.4	75.7 ± 0.3	81.3 ± 0.3
pH 4.0	69.2	–	49.9 ± 1.0	54.8 ± 0.2	64.5 ± 0.6	71.1 ± 0.4

First transition disappeared due to the effect of acidic pH. Protein concentration is 0.2 mg/ml.

3.2. Effect of additives

Since pH 6.0 seems to be appropriate to reconcile the aspects of isoelectric precipitation and conformational stability, the effect of stabilizers was investigated at pH 6.0 in all cases. After addition of glucose or sorbitol or leucine to the IgG solution, the different transition temperature values are only slightly shifted to higher temperature (data not shown). However, no characteristic difference between the DSC thermograms of stabilizer-free and stabilized solutions was observed.

In the far-UV-CD region (data not shown) alteration, i.e. loss of secondary structure is not detectable after addition of stabilizers. The effect of excipients on the near-UV-CD spectra is shown in Fig. 5. Changes in the tertiary structure were observed: leucine slightly increases CD-signal in the 272–287 nm band and sugars shift the whole spectra, however, simultaneous application of leucine and sorbitol results in restoring certain ranges of the near-UV-CD spectra (mainly in the 287–300 nm band). Our findings indicate that combination of

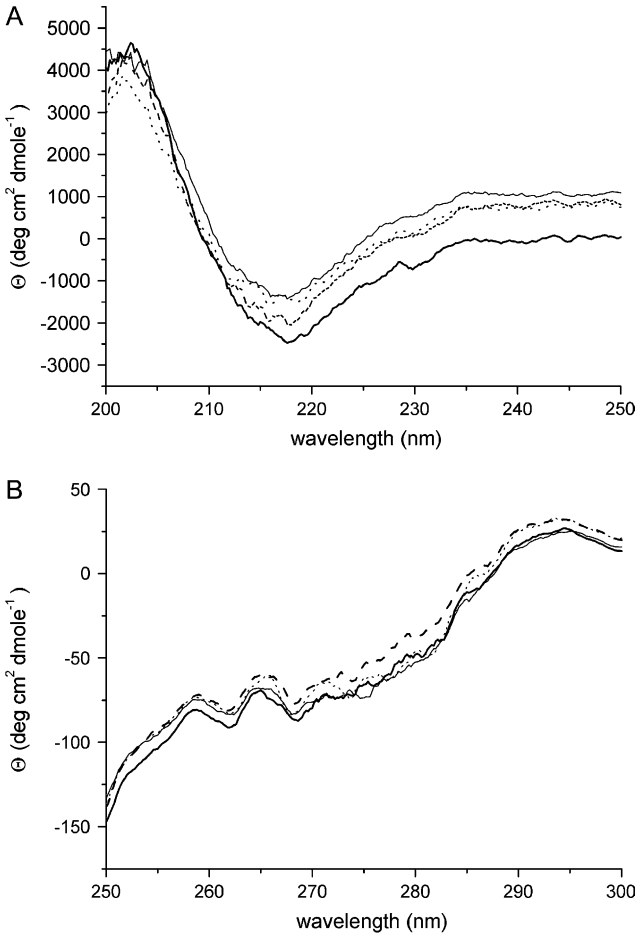

Fig. 4. CD spectra of polyclonal IgG at pH 7.0 (dotted line), pH 6.0 (solid), pH 5.0 (dashed), pH 4.0 (thick solid), (A) far-UV-CD spectra, (B) near-UV-CD spectra.

sorbitol and leucine could preserve a greater part of IgG in native structure.

Thermal aggregation properties of IgG at 75 °C in the presence of additives were evaluated by turbidity measurements. The results of these aggregation kinetic experiments are shown in Fig. 6. All the investigated additives elicit a delay in the aggregation process, in these cases, aggregation occurs later compared to the stabilizer-free reference. Sugars, in lowered concentration, have only little effect, but leucine delays the aggregation to a higher extent. Increased amount of sorbitol (5%) produced the same effect as 0.3% leucine. Interestingly, simultaneous addition of 1% sorbitol and 0.3% leucine slightly decreases the aggregation delaying effect of leucine.

Anticomplementary activities of IgG samples were measured to evaluate the extent of soluble IgG aggregates. AcA results of IgG solutions are summarized in Table 2. AcA levels remained low in all cases (below 0.3 CH50/mg Ig) and no significant effect of any of the additives was observable.

3.3. Reversible dimerization at low temperature

The mean polymer and dimer contents of IVIG-C solutions prepared in year 2001 and its freeze-dried form after reconstitution (Humaglobin) are shown in Table 3.

Storage stability studies at 5 °C of IVIG-C solution revealed an increase in the dimer content from 5% to 14% in 27 months (Fig. 7). Composition of IVIG-C (equivalent to Humaglobin) is shown in Table 4.

Different IgG samples were prepared from IVIG-A solution in order to test the effect of solution environment on IgG. Molecular-size distribution of these IgG samples was measured immediately after preparation and after 1, 2, 6 and 18 months of incubation at 5 °C (Table 5). The dimer proportion increased from 4.4–5.6% to 8.4–10.2% during storage at 5 °C. Polymer content did not change significantly and was below 2% in all samples. Oligomerization was not decreased significantly by additives applied in this low concentration.

After four weeks of storing at 5 °C samples were moved to 37 °C for a further four weeks and the molecular-size distribution was measured again (Table 5). At this elevated temperature dimer content decreased in all cases, became significantly lower than in samples stored continuously at low temperature.

Oligomerization pattern at 37 °C was also investigated in detail using a fresh Humaglobin IVIG after reconstitution and an aged IVIG-B preparation (6 months, 5 °C) (Fig. 8). The relatively high dimer content (~12–16%) decreased rapidly, and after a day a minimum (~6–7%) was attained. During a more prolonged storage at this temperature, a slow increase of the dimer content was observed.

Effect of additives on the induction of dimerization or dimer dissociation in IgG solution was also determined in the case of glucose, sorbitol and leucine. Stabilizers were added directly to the IgG solutions with 5% (w/v) protein content and no sizeable difference in the rate of dimerization was observed in the presence of any of the compounds investigated.

4. Discussion

The aggregation properties and long-term stability of the IgG solutions, regarding the molecular level, are dependent on the conformational stability and structural properties of the individual IgG molecules. Numerous immunoglobulin preparations with different formulations are produced and applied in clinical practice, however, little is known about the effect of different parameters on the conformational stability and dimerization of IgG. We introduced rapid and sensitive biophysical techniques, differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy to investigate the effect of the solvent environment on the

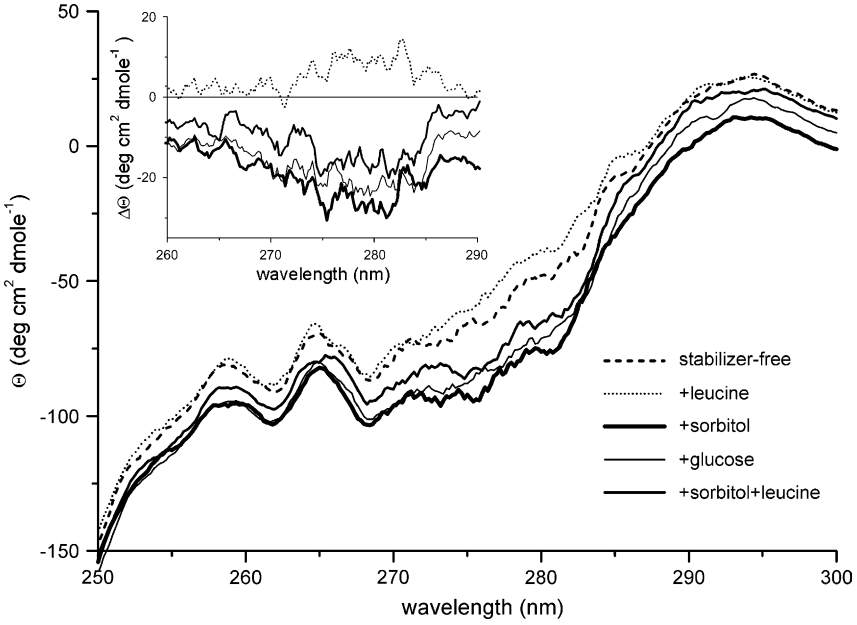

Fig. 5. Effect of stabilizers on the near-UV-CD spectrum of IgG at pH 6.0. Differential spectra are shown in the inset. Sugars have the most significant effect on the tertiary structure of IgG, but in the case of sorbitol + leucine the spectrum tends back to the reference.

conformational stability and structural characteristics of IgG solutions.

During the heating process the unfolding signal measured by DSC is perturbed due to the aggregation of IgG. In order to minimize this effect a protein concentration as low as 0.2 mg/ml was used. The highest conformational stability was detected in the pH range 6.0–7.0. The generally accepted concept – which assumes that the pH, where the melting temperature is the highest, gives pH optimum of the dissolved protein – is not valid in this case [24]. It is known that oligomerization/aggregation of polyclonal IgG decreases at lower pH, when moving away from the isoelectric range, while the tendency of denaturation increases. This increased resistance for aggregation at a more acidic pH can be concluded indirectly from DSC measurements: the aggregation signal at lower pH and at higher protein concentration significantly decreases if compared to aggregation observed at higher pH. Decrease of T_m and ΔH values with decreasing pH indicates enhanced flexibility of certain solvent accessible amino acid residues.

Stabilizers involved in this study did not affect the dimerization and the conformational stability of IgG, however, they were able to prevent aggregation. Glucose and sorbitol increase the hydrophilicity of the protein surface, because their affinity for water is greater than that for the co-solvent due to preferential hydratation [25,26]. This effect is clearly reflected in near-UV-CD spectra (Fig. 5).

The interaction between the amphipathic leucine and IgG is rather different when compared to sugar–protein interactions. Leucine is of hydrophobic nature and is able to cover hydrophobic surfaces [12]. Surfactant–protein interactions are primarily hydrophobic and

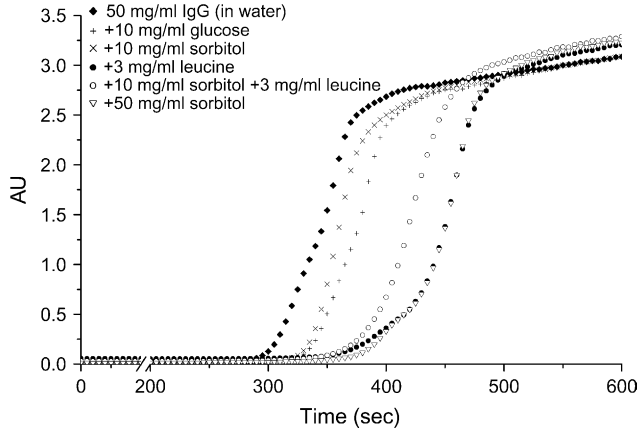

Fig. 6. Effect of excipients on the turbidity of IgG at 75 °C, 600 nm as a function of time. Aggregation process is delayed due to the stabilizers.

Table 2
Effect of different additives on AcA of IgG solution

IgG in	AcA (CH50/mg Ig)
Water	0.211
+Glucose	0.133
+Sorbitol	0.164
+Leucine	0.155
+Sorbitol + leucine	0.248

The figures represent the average of 4 measurements.

Table 3
Dimer and polymer content of IVIG-C and its freeze-dried form after reconstitution with water for injection (Humaglobin)

	Before freeze-drying (IVIG-C)	Freeze-dried, reconstituted (Humaglobin)
Polymer	0.3 ± 0.3%	0.8 ± 0.7%
Dimer	7.1 ± 1.7%	8.2 ± 1.3%

The data are annual mean values (mean ± SD, $n = 33$).

Table 4
Composition of IVIG-C and Humaglobin after reconstitution with water for injection

Total protein	50 mg/ml
D-glucose	15 mg/ml
Glycine	15 mg/ml
pH	6.8
PEG	<5 mg/ml

therefore the amphipathic additive protects the protein from aggregation [27], i.e. leucine, by interacting directly with the hydrophobic residues of the protein, blocks protein–protein interactions. This co-solvent–protein interaction is reflected in the CD spectra of IgG molecules as a consequence of subtle changes in the tertiary structure. On the other hand, CD spectral changes induced by addition of sorbitol can be partially reversed by simultaneous addition of leucine (Fig. 5).

In the heat aggregation tests leucine exhibits a favorable and significant protection at a concentration of 0.3% (W/V), which is equivalent to the effect of a sorbitol concentration as high as 5% (Fig. 6). In case of simultaneous addition, these agents appear to act antagonistically on the kinetics of heat aggregation (Fig. 6).

In addition to heat aggregation, oligomerization of polyclonal immunoglobulins also occurs as a consequence of storage at low temperature (5 °C). Dimer content of IVIG was found to vary in a range of 5–15% whilst polymer content remained below 2%. According to our storage stability tests, two types of IgG oligomers (primarily dimers) may be distinguished. In the first type IgG molecules are weakly associated and dissociate at physiological temperature, while in the second type such dissociation does not occur (Table 5, Fig. 8). Both types of oligomers are present in polyclonal IgG preparations in proportions depending on the storage circumstances.

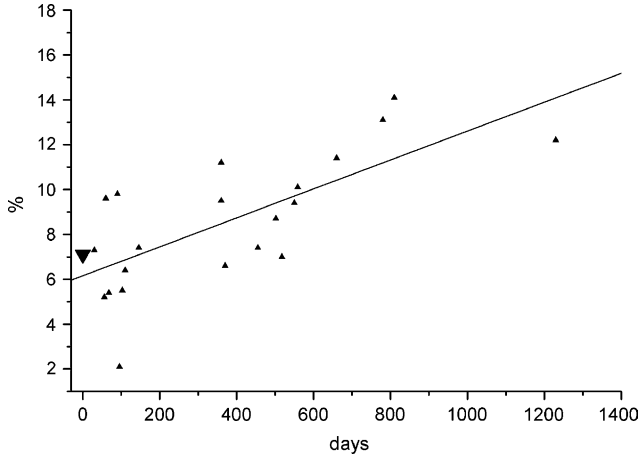

Fig. 7. Increase of dimer content of different IVIG-C solutions during storage at 5 °C. Down triangle represents the annual mean value.

The acidic pH conditions disfavor these aggregation processes in polyclonal IgG solutions [8].

Obviously, the rate and extent of oligomerization in soluble IgG samples are to be controlled. While the content of oligomers is to be kept at a minimum level to avoid serious side effects [4,5], the presence of dimers appears to be necessary for effective immunomodulatory action [6,7]. In a guidance document of the EU Committee for Proprietary Medicinal Products (Core SPC for human normal immunoglobulin for intravenous administration [IVIg], CPMP/BWG/859/95 rev. 1) the warning "Rarely, human normal immunoglobulin can induce a fall in blood pressure with anaphylactic reaction, even in patients who had tolerated previous treatment with human normal immunoglobulin" is read. Although the relevance of results obtained in rat model for adverse effects observed in patients have not been directly established, avoiding excessive dimer content in intravenously administered IgG solutions appears desirable. The importance of warming the solution to physiological temperature prior to administration is emphasized, and storage temperatures higher than the usual 2–8 °C may be considered. Using up to date, validated aseptic manufacturing technologies may obviate the necessity of low temperature storage.

From our studies we can conclude that adjusting the pH in the optimal range is essential for the stability of IgG in solution. Sugars, such as glucose and sorbitol, which were involved in this study, exerted no relevant effect either on oligomerization partially, or on the conformational stability of IgG. This observation raises

Table 5
Dimer content of IVIG-A solutions immediately after preparation (A), after 1, 2, 6 and 18 months of incubation at 5 °C (B, C, D and E) and after 4 weeks of storage at 5 °C followed by a 4 week storage at 37 °C (F)

	Dimer content (%)					
	A	B	C	D	E	F
Water	5.6	6.5	7.8	9.1	9.1	4.9
+Glucose	5.1	6.0	7.2	7.5	10.2	4.5
+Sorbitol	4.7	5.7	6.9	8.1	8.8	4.4
+Leucine	4.6	5.6	6.6	8.3	8.4	4.6
+Sorbitol + leucine	4.4	5.7	6.5	8.8	8.7	4.3

Protein concentration was 50 mg/ml and the pH of the solutions was 6.0.

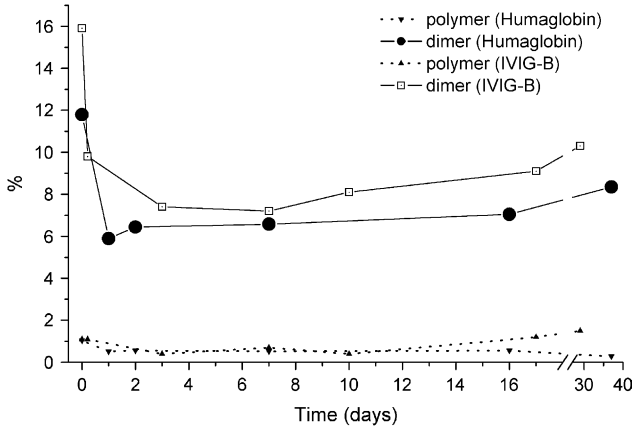

Fig. 8. Influence of the incubation at 37 °C on the molecular-size distribution of an aged IVIG-B solution and a fresh Humaglobin preparation with 50 mg/ml protein concentration. After rapid dissociation of dimers, slow dimerization/aggregation occurs again.

the question of their necessity. Hydrophobic amino acids could provide efficient additives against aggregation.

The development of recombinant DNA technology and biotechnology has led to an increasing number of protein-based medicines and highlighted issues such as their long-term stability and means of efficacious delivery avoiding adverse side effects [28]. Our systematic approach may provide a solution for such formulation problems in the future.

Acknowledgements

We would like to thank Dr. Ferenc Schnitzer and Ms. Györgyi Szabó for encouragement and interest, Dr. András Szilágyi and Mr. Csaba Pogány for their help in the preparation of the manuscript and Mr. Levente Dajka for his help in IEF experiments. The technical assistance of colleagues of biochemical laboratory at Teva is greatly appreciated. This work was supported by OTKA T46412 and the Hungarian Ministry of Health (EU Council grant #555/2003).

References

[1] Kazatchkine MD, Kaveri SV. Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. N Engl J Med 2001;345:747–55.
[2] Emmi L, Chiarini F. The role of intravenous immunoglobulin therapy in autoimmune and inflammatory disorders. Neurol Sci 2002;23:S1–8.
[3] Jenei B, Lázár G, Bartha K, Medgyesi GA. Hypotensive action of IgG preparations containing aggregates is suppressed by PAF-receptor antagonist BN 52021 and by gadolinium chloride (an agent blocking Kupffer cell function). Agents Actions 1991;32:333–8.
[4] Bleeker WK, Teeling JL, Verhoeven AJ, Rigter GMM, Agterberg J, Tool ATJ, et al. Vasoactive side effects of intravenous immunoglobulin preparations in a rat model and their treatment with recombinant platelet-activating factor acetylhydrolase. Blood 2000;95:1856–61.
[5] Kroez M, Kanzy EJ, Gronski P, Dickneite G. Hypotension with intravenous immunoglobulin therapy: importance of pH and dimer formation. Biologicals 2003;31:277–86.
[6] Teeling JL, Jansen-Hendriks T, Kuijpers TW, de Haas M, van de Winkel JGJ, Hack CE, et al. Therapeutic efficacy of intravenous immunoglobulin preparations depends on the immunoglobulin G dimers: studies in experimental immune thrombocytopenia. Blood 2001;98:1095–9.
[7] Nemes E, Teichman F, Roos D, Maródi L. Activation of human granulocytes by intravenous immunoglobulin preparations is mediated by FcgammaRII and FcgammaRIII receptors. Pediatr Res 2000;47:357–61.
[8] Tankersley DL, Preston MS, Finlayson JS. Immunoglobulin G dimer: an idiotype–anti-idiotype complex. Mol Immunol 1988;25:41–8.
[9] Roux KH, Tankersley DL. A view of the human idiotypic repertoire – electron microscopic and immunologic analyses of spontaneous idiotype–anti-idiotype dimers in pooled human IgG. J Immunol 1990;144:1387–95.
[10] Mariuzza RA, Poljak RJ, Schwarz FP. The energetics of antigen–antibody binding. Res Immunol 1994;145:70–2.
[11] Schwarz FP, Tello D, Goldbaum FA, Mariuzza RA, Poljak RJ. Thermodynamics of antigen–antibody binding using specific anti-lysozyme antibodies. Eur J Biochem 1995;228:388–94.
[12] Bolli R, Spycher MO, Brügger R, Wüst B, Gennari K. IgG-dimer formation in liquid immunoglobulin preparations is inhibited by nicotinamide and other amphiphilic compounds. J Autoimmun 1999;96(Suppl.):96.
[13] van Oss CJ. Hydrophobic, hydrophilic and other interactions in epitope–paratope binding. Mol Immunol 1995;32:199–211.
[14] Nydegger UE, Sturzenegger M. Adverse effects of intravenous immunoglobulin therapy. Drug Saf 1999;21:171–85.
[15] Knezevic-Maramica I, Kruskall MS. Intravenous immune globulins: an update for clinicians. Transfusion 2003;43:1460–80.
[16] Cohn EJ, Strong LE, Hughes WL, Mulford DJ, Ashworth JN, Melin M, et al. Preparations and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. J Am Chem Soc 1946;68:459–75.
[17] Uemura Y, Uriyu K, Hirao Y, Takechi K, Ishikawa H, Nakajima T, et al. Inactivation and elimination of viruses during the fractionation of an intravenous immunoglobulin preparation: liquid heat treatment and polyethylene glycol fractionation. Vox Sang 1989;56:155–61.
[18] Friesen AD, Bowman JM, Bees WCH. Column ion exchange chromatographic production of human immune globulins and albumin. In: Curling JM, editor. Separation of plasma proteins. Uppsala: Pharmacia Fine Chemicals AB; 1983. p. 117–26.
[19] Závodszky P, Jaton JC, Venyaminov SY, Medgyesi GA. Increase of conformational stability of homogeneous rabbit immunoglobulin G after hapten binding. Mol Immunol 1981;18:39–46.
[20] Tischenko VM, Zav'yalov VP, Medgyesi GA, Potekhin SA, Privalov PL. A thermodynamic study of cooperative structures in rabbit immunoglobulin G. Eur J Biochem 1982;126:517–21.
[21] Závodszky P, Kilár F, Török J, Medgyesi GA. Dynamic aspects of signal transfer in antibody molecules. In: Celada F, Schumaker VN, Sercarz EE, editors. Protein conformation as an immunological signal. New York: Plenum Press; 1983. p. 15–29.
[22] Welfle K, Misselwitz R, Hausdorf G, Höhne W, Welfle H. Conformation, pH-induced conformational changes, and thermal unfolding of anti-p24 (HIV-1) monoclonal antibody CB4-1 and its Fab and Fc fragments. Biochim Biophys Acta 1999;1431:120–31.
[23] Vermeer AWP, Norde W. The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein. Biophys J 2000;78:394–404.

[24] Remmele Jr RL, Nightlinger NS, Srinivasan S, Gombotz WR. Interleukin-1 receptor (IL-1R) liquid formulation development using differential scanning calorimetry. Pharm Res 1998;15:200–8.
[25] Timasheff SN, Lee JC, Pittz EP, Tweedy N. The interaction of tubulin and other proteins with structure-stabilizing solvents. J Colloid Interf Sci 1976;55:658–63.
[26] Arakawa T, Timasheff SN. Stabilization of protein structure by sugars. Biochemistry 1982;21:6536–44.
[27] Arakawa T, Kita Y. Protection of bovine serum albumin from aggregation by Tween 80. J Pharm Sci 2000;89:646–51.
[28] Frokjaer S, Otzen DE. Protein drug stability: a formulation challenge. Nat Rev Drug Disc 2005;4:298–306.