

DSC IV - Antibody studies

Ahrer K., Buchacher A., Iberer G., and Jungbauer A. (2006) Thermodynamic stability and formation of aggregates of human immunoglobulin G characterised by differential scanning calorimetry and dynamic light scattering. *J Biochem Biophys Methods* **66**, 73-86.

Abstract: The final process step of polyclonal human immunoglobulin G is formulation with agents such as sugars, polyols, amino acid and salts. Often the most stable formulations were empirically identified. Physicochemical methods, such as differential scanning calorimetry and dynamic light scattering, provide a deeper insight on the biophysical properties of such a protein solution. The combination of these methods proved to be sensitive enough to detect fine differences in the properties relevant for the development of stable protein solutions. The influence of additives, such as maltose and glycine in combination with water or low concentrations of salts, on human immunoglobulin preparations was analysed. Differential scanning calorimetry illustrated that 0.2 M glycine had better stabilising effects compared to 10% maltose. Dynamic light scattering and differential scanning calorimetry revealed that solutions preventing aggregation were not optimal in terms of thermodynamic stability. Aggregation was minimised with increasing ionic strength, shown by dynamic light scattering, whereas thermodynamic stability for heat sensitive parts of human immunoglobulin G, analysed with differential scanning calorimetry, was decreased.

Arutyunova E. I., Arutyunov D. Y., Pleten' A. P., Nagradova N. K., and Muronetz V. I. (2004) Antibodies specific to modified glyceraldehyde-3-phosphate dehydrogenase induce inactivation of the native enzyme and change its conformation. *Biochim Biophys Acta* **1700**, 35-41.

Abstract: The antibodies specific to an inactive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Bacillus stearothermophilus* prepared by the treatment of the tetrameric holoenzyme with glutaraldehyde were obtained. They were purified from the pool of polyclonal rabbit antibodies to GAPDH with the use of immobilized GAPDH cross-linked by glutaraldehyde as an affinity sorbent. Such antibodies were capable of interacting with the native enzyme, inducing its time-dependent inactivation; the effect was different with the apo- and holoenzyme forms. Differential scanning calorimetry of the purified [GAPDH].[antibody] complex revealed a large shift of the temperature corresponding to the maximal heat capacity of the holoenzyme towards the lower temperature. Again, the effect appeared to be different with the apoenzyme. Together, the results are consistent with the hypothesis that a specific antibody is able to exercise a certain strain on the target protein, altering its conformation toward the structure of the species which served to select the antibody. The possibility of preparing selective enzyme inhibitors based on the antibodies specific to inactive enzyme conformations is considered.

Arutyunova E. I., Pleten A. P., Nagradova N. K., and Muronetz V. I. (2006) Antibodies to Inactive Conformations of Glyceraldehyde-3-phosphate Dehydrogenase Inactivate the Apo- and Holoforms of the Enzyme. *Biochemistry (Mosc)* **71**, 685-691.

Abstract: Polyclonal antibodies produced after the immunization of a rabbit with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Bacillus stearothermophilus* were used to isolate two types of antibodies interacting with different non-native forms of the antigen. Type I antibodies were purified using Sepharose-bound apo-GAPDH that was treated with glutaraldehyde to stabilize the enzyme in the tetrameric form. Type II antibodies were isolated using immobilized denatured monomers of the enzyme. It was shown that the type I antibodies bound to the native holo- and apoforms of the enzyme with the ratio of one antibody molecule per GAPDH tetramer. While interacting with the native holoenzyme, the type I antibodies induce a time-dependent decrease in its activity by 80-90%. In the case of the apoenzyme, the decrease in the activity constitutes only 25%, this indicating that only one subunit of the tetramer is inactivated. Differential scanning calorimetry experiments showed that the formation of the complex between both forms of the enzyme and the type I antibodies resulted in a shift of the maximum of the thermal capacity curves ($T(m)$ value) to lower temperatures. The extremely stable holoenzyme was affected to the greatest extent, the shift of the $T(m)$ value constituting approximately 20 degrees C. We assume that the formation of the complex between the holo- or apo-GAPDH and the type I antibody results in time-dependent conformational changes in the enzyme molecule. Thus, the antibodies induce the structural rearrangements yielding the conformation that is identical to the structure of the antigen used for the selection of the antibodies (i.e., inactive). The interaction of the antibodies with the apo-GAPDH results in the inactivation of the subunit directly bound to the antibody. Virtually complete inactivation of the

holoenzyme by the antibodies is likely due to the transmission of the conformational changes through the intersubunit contacts. The type II antibodies, which were selected using the immunosorbent with unfolded enzyme form, do not affect the activity of native holo- and apo-GAPDH, but prevent the reactivation of the denatured GAPDH, binding the denatured forms of the enzyme.

Atwell S., Ridgway J. B., Wells J. A., and Carter P. (1997) Stable heterodimers from remodeling the domain interface of a homodimer using a phage display library. *J Mol Biol* **270**, 26-35.

Abstract: Structure-guided phage display was used to select for combinations of interface residues for antibody C(H)3 domains that promote the formation of stable heterodimers. A C(H)3 "knob" mutant was made by replacement of a small residue, threonine, with a larger one, tryptophan: T366W. A library of C(H)3 "hole" mutants was then created by randomizing residues 366, 368 and 407, which are in proximity to the knob on the partner C(H)3 domain. The C(H)3 knob mutant was fused to a peptide flag and the C(H)3 hole library was fused to M13 gene III. Phage displaying stable C(H)3 heterodimers were recovered by panning using an anti-flag antibody. Phage-selected C(H)3 heterodimers differed in sequence from the previously designed heterodimer T366W-Y407'A, and most clones tested were more stable to guanidine hydrochloride denaturation. The thermal stability of individual C(H)3 domains secreted from *Escherichia coli* was analyzed by differential scanning calorimetry. One heterodimer, T366W-T366'S:L368'A:Y407'V, had a T_m of 69.4 degrees C, which is 4.0 deg.C higher than that for the designed heterodimer and 11.0 deg.C lower than that for the wild-type homodimer. The phage-selected C(H)3 mutant maintained the preference for forming heterodimers over homodimers as judged by near-quantitative formation of an antibody/immunoadhesin hybrid in a cotransfection assay. Phage optimization provides a complementary and more comprehensive strategy to rational design for engineering homodimers for heterodimerization.

Badkar A., Yohannes P., and Banga A. (2006) Application of TZERO calibrated modulated temperature differential scanning calorimetry to characterize model protein formulations. *Int J Pharm* **309**, 146-156.

Abstract: The objective of this study was to evaluate the feasibility of using T(ZERO) modulated temperature differential scanning calorimetry (MDSC) as a novel technique to characterize protein solutions using lysozyme as a model protein and IgG as a model monoclonal antibody. MDSC involves the application of modulated heating program, along with the standard heating program that enables the separation of overlapping thermal transitions. Although characterization of unfolding transitions for protein solutions requires the application of high sensitive DSC, separation of overlapping transitions like aggregation and other exothermic events may be possible only by use of MDSC. A newer T(ZERO) calibrated MDSC model from TA instruments that has improved sensitivity than previous models was used. MDSC analysis showed total, reversing and non-reversing heat flow signals. Total heat flow signals showed a combination of melting endotherms and overlapping exothermic events. Under the operating conditions used, the melting endotherms were seen in reversing heat flow signal while the exothermic events were seen in non-reversing heat flow signal. This enabled the separation of overlapping thermal transitions, improved data analysis and decreased baseline noise. MDSC was used here for characterization of lysozyme solutions, but its feasibility for characterizing therapeutic protein solutions needs further assessment.

Bliznukov O. P., Kozmin L. D., Klimovich V. B., Tsybovsky Y. I., Kravchuk Z. I., and Martsev S. P. (2001) Thermodynamic stability and functional activity of tumor-associated antibodies. *Biochemistry (Mosc)* **66**, 27-33.

Abstract: Tumor-associated antibodies of human IgG1 subclass were eluted from cell-surface antigens of human carcinoma cells and studied by differential scanning calorimetry and binding to local conformational probes, protein A from *Staphylococcus aureus* and a monoclonal antibody targeted to the CH2 domain of the Fc fragment. At pH 2.0-7.0, we observed virtually identical enthalpies of thermal unfolding for IgG1 from normal human sera and tumor-associated IgG1. The exact values of calorimetric enthalpy (Δh) at pH 7.0 were 6.1 and 6.2-6.3 cal/g for IgG1 from normal serum and IgG1 from carcinoma cells, respectively. The affinity constants of protein A binding to the CH2--CH3 domain interface demonstrated differences between serum IgG1 and tumor associated IgG1 that did not exceed 3-8-fold. The binding affinity toward the anti-CH2 monoclonal antibody determined for serum IgG1 and IgG1 from carcinoma cells differed not more than 2.5-fold. The thermodynamic parameters of IgG1 from carcinoma cells strongly suggest that protein conformational stability was essentially unaltered and that the Fc fragment of the tumor-derived IgG1 preserved its structural integrity.

Breen E. D., Curley J. G., Overcashier D. E., Hsu C. C., and Shire S. J. (2001) Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation. *Pharm Res* **18**, 1345-1353.

Abstract: PURPOSE: To determine the effect of moisture and the role of the glass transition temperature (T_g) on the stability of a high concentration, lyophilized, monoclonal antibody. METHODS: A humanized monoclonal antibody was lyophilized in a sucrose/histidine/polysorbate 20 formulation. Residual moistures were from 1 to 8%. T_g values were measured by modulated DSC. Vials were stored at temperatures from 5 to 50 degrees C for 6 or 12 months. Aggregation was monitored by size exclusion chromatography and Asp isomerization by hydrophobic interaction chromatography. Changes in secondary structure were monitored by Fourier transform infrared (FTIR). RESULTS: T_g values varied from 80 degrees C at 1% moisture to 25 degrees C at 8% moisture, there was no cake collapse and there were no differences in the secondary structure by FTIR. All formulations were stable at 5 degrees C. High moisture cakes had higher aggregation rates than drier samples if stored above their T_g values. Intermediate moisture vials were more stable to aggregation than dry vials. High moisture samples had increased rates of Asp isomerization at elevated temperatures both above and below their T_g values. Chemical and physical degradation pathways followed Arrhenius kinetics during storage in the glassy state. Only Asp isomerization followed the Arrhenius model above the T_g value. Both chemical and physical stability at T > or = T_g were fitted to Williams-Landel-Ferry (WLF) kinetics. The WLF constants were dependent on the nature of the degradation system and were not characteristic of the solid system. CONCLUSION: High moisture levels decreased chemical stability of the formulation regardless of whether the protein was in a glassy or rubbery state. In contrast, physical stability was not compromised, and may even be enhanced, by increasing residual moisture if storage is below the T_g value.

Chumanevich A. A., Kravchuk Z. I., Vlasov A. P., Zhorov O. V., and Martsev S. P. (1998) Thermodynamic stability of immunoglobulins and allosteric interactions with ferritin and protein A: distinct properties of the two antibodies of IgG2a subclass. *Biochemistry (Mosc)* **63**, 476-484.

Abstract: The two anti-ferritin monoclonal antibodies of mouse IgG2a subclass, G10 and F11, are described that have similar affinity to human spleen ferritin and identical protein A-binding affinity. Antigen binding was shown to change significantly the protein A-binding parameters of the IgG2a antibodies. Antigen-induced conformational changes result in enhanced protein A-binding affinity of the G10 antibody while reduced affinity of the F11 antibody. Antigen binding does not change inherently low affinity of the anti-ferritin IgG1 antibody C5 to protein A. Differential scanning calorimetry revealed that the enthalpy and Gibbs free energy of denaturation for G10 was respectively by 19 and 29% higher than the corresponding parameters for F11. The lower structural energetics of F11 is associated with the lack of a calorimetrically revealed folding unit that may be responsible for distinct interaction between the antigen-binding and protein A-binding sites. This work provides experimental demonstration of the fact that functionally significant interactions between the two spatially remote recognition sites in antibodies of the same heavy chain isotype can be modulated by relatively small structural variations that also result in different thermodynamic stability.

Chung C. M., Chiu J. D., Connors L. H., Gursky O., Lim A., Dykstra A. B., Liepnieks J., Benson M. D., Costello C. E., Skinner M., and Walsh M. T. (2005) Thermodynamic stability of a kappa1 immunoglobulin light chain: relevance to multiple myeloma. *Biophys J* **88**, 4232-4242.

Abstract: Immunoglobulin light chains have two similar domains, each with a hydrophobic core surrounded by beta-sheet layers, and a highly conserved disulfide bond. Differential scanning calorimetry and circular dichroism were used to study the folding and stability of MM-kappa1, an Ig LC of kappa1 subtype purified from the urine of a multiple myeloma patient. The complete primary structure of MM-kappa1 was determined by Edman sequence analysis and mass spectrometry. The protein was found to contain a cysteinyl post-translational modification at Cys(214). Protein stability and conformation of MM-kappa1 as a function of temperature or denaturant conditions at pH 7.4 and 4.8 were investigated. At pH 4.8, calorimetry demonstrated that MM-kappa1 undergoes an incomplete, cooperative, partially reversible thermal unfolding with increased unfolding temperature and calorimetric enthalpy as compared to pH 7.4. Secondary and tertiary structural analyses provided evidence to support the presence of unfolding intermediates. Chemical denaturation resulted in more extensive protein unfolding. The stability of MM-kappa1 was reduced and protein unfolding was irreversible at pH 4.8, thus suggesting that different pathways are utilized in thermal and chemical unfolding.

Demarest S. J., Hopp J., Chung J., Hathaway K., Mertsching E., Cao X., George J., Miatkowski K., LaBarre M. J., Shields M., and Kehry M. R. (2006) An intermediate pH unfolding transition abrogates the ability of IgE to interact with its high affinity receptor FcepsilonRIalpha. *J Biol Chem* **281**, 30755-30767.

Abstract: The interaction between IgE-Fc (Fcepsilon) and its high affinity receptor FcepsilonRI on the surface of mast cells and basophils is a key event in allergen-induced allergic inflammation. Recently, several therapeutic strategies have been developed based on this interaction, and some include Fcepsilon-containing moieties. Unlike well characterized IgG therapeutics, the stability and folding properties of IgE are not well understood. Here, we present comparative biophysical analyses of the pH stability and thermostability of Fcepsilon and IgG1-Fc (Fcgamma). Fcepsilon was found to be significantly less stable than Fcgamma under all pH and NaCl conditions tested. Additionally, the Cepsilon3Cepsilon4 domains of Fcepsilon were shown to become intrinsically unfolded at pH values below 5.0. The interaction between Fcepsilon and an Fcgamma-FcepsilonRIalpha fusion protein was studied between pH 4.5 and 7.4 using circular dichroism and a combination of differential scanning calorimetry and isothermal titration calorimetry. Under neutral pH conditions, the apparent affinity of Fcepsilon for the dimeric fusion protein was extremely high compared with published values for the monomeric receptor ($K_D < 10^{-12}$ M). Titration to pH 6.0 did not significantly change the binding affinity, and titration to pH 5.5 only modestly attenuated affinity. At pH values below 5.0, the receptor binding domains of Fcepsilon unfolded, and interaction of Fcepsilon with the Fcgamma-FcepsilonRIalpha fusion protein was abrogated. The unusual pH sensitivity of Fcepsilon may play a role in antigen-dependent regulation of receptor-bound, non-circulating IgE.

Demarest S.J., Chen G., Kimmel B.E., Gustafson D., Wu J., Salbato J., Poland J., Elia M., Tan X., Wong K., Short J., and Hansen G. (2006) Engineering stability into Escherichia coli secreted Fabs leads to increased functional expression. *Protein Eng Des Sel.* **19**, 325-36.

Abstract: The recombinant expression of immunoglobulin domains, Fabs and scFvs in particular, in Escherichia coli can vary significantly from antibody to antibody. We hypothesized that poor Fab expression is often linked to poor intrinsic stability. To investigate this further, we applied a novel approach for stabilizing a poorly expressing anti-tetanus toxoid human Fab with a predisposition for being misfolded and non-functional. Forty-five residues within the Fab were chosen for saturation mutagenesis based on residue frequency analysis and positional entropy calculations. Using automated screening, we determined the approximate midpoint temperature of thermal denaturation (TM) for over 4000 library members with a maximum theoretical diversity of 855 unique mutations. This dataset led to the identification of 11 residue positions, primarily in the Fv region, which when mutated enhanced Fab stability. By combining these mutations, the TM of the Fab was increased to 92 degrees C. Increases in Fab stability correlated with higher expressed Fab yields and higher levels of properly folded and functional protein. The mutations were selected based on their ability to increase the apparent stability of the Fab and therefore the exact mechanism behind the enhanced expression in E.coli remains undefined. The wild-type and two optimized Fabs were converted to an IgG1 format and expressed in mammalian cells. The optimized IgG1 molecules demonstrated identical gains in thermostability compared to the Fabs; however, the expression levels were unaffected suggesting that the eukaryotic secretion system is capable of correcting potential folding issues prevalent in E.coli. Overall, the results have significant implications for the bacterial expression of functional antibody domains as well as for the production of stable, high affinity therapeutic antibodies in mammalian cells.

Deutscher S. L., Crider M. E., Ringbauer J. A., Komissarov A. A., and Quinn T. P. (1996) Stability studies of nucleic acid-binding Fab isolated from combinatorial bacteriophage display libraries. *Arch Biochem Biophys* **333**, 207-213.

Abstract: This study examined the global stability and activity properties of recombinant DNA-binding antibody fragments that were obtained from a bacteriophage combinatorial display library. The goal of this study was to determine whether the combinatorial approach of heavy and light chain assembly in E. coli and subsequent affinity selection preferentially selects for antibody fragments with unusual structural stabilities. Specifically, the binding properties and stability of recombinant antibody fragments with or without a C-terminal His tag to temperature, pH, and guanidine-HCl were examined. Both Fab exhibited almost identical K_d (120-130, 140-170, and 450-560 nM) and maximal fluorescence quenching (20-25%)

values for binding to (dT)20, (dT)15, and (dT)10, respectively. Thermal denaturation data obtained by CD spectroscopy demonstrated that both Fab possessed structural properties comparable to well-folded proteins with defined tertiary structures which were stable below 70 degrees C (T_m 73 degrees C). These results were confirmed by differential scanning calorimetry. Both Fab exhibited the same rate of irreversible thermal inactivation (0.061-0.069 min⁻¹) at 75 degrees C and could be reversibly renatured from guanidine-HCl and pH extremes. Crystallization trials with one recombinant DNA-binding Fab yielded diffraction quality crystals also suggesting a well-defined tertiary structure.

Ejima D., Tsumoto K., Fukada H., Yumioka R., Nagase K., Arakawa T. and Philo J. S. (2007) Effects of acid exposure on the conformation, stability, and aggregation of monoclonal antibodies. *Proteins* **66**, 954-962.

Abstract: Exposure of antibodies to low pH is often unavoidable for purification and viral clearance. The conformation and stability of two humanized monoclonal antibodies (hIgG4-A and -B) directed against different antigens and a mouse monoclonal antibody (mIgG1) in 0.1M citrate at acidic pH were studied using circular dichroism (CD), differential scanning calorimetry (DSC), and sedimentation velocity. Near- and far-UV CD spectra showed that exposure of these antibodies to pH 2.7-3.9 induced only limited conformational changes, although the changes were greater at the lower pH. However, the acid conformation is far from unfolded or so-called molten globule structure. Incubation of hIgG4-A at pH 2.7 and 3.5 at 4 degrees C over the course of 24 h caused little change in the near-UV CD spectra, indicating that the acid conformation is stable. Sedimentation velocity showed that the hIgG4-A is largely monomeric at pH 2.7 and 3.5 as well as at pH 6.0. No time-dependent changes in sedimentation profile occurred upon incubation at these low pHs, consistent with the conformational stability observed by CD. The sedimentation coefficient of the monomer at pH 2.7 or 3.5 again suggested that no gross conformational changes occur at these pHs. DSC analysis of the antibodies showed thermal unfolding at pH 2.7-3.9 as well as at pH 6.0, but with decreased melting temperatures at the lower pH. These results are consistent with the view that the antibodies undergo limited conformational change, and that incubation at 4 degrees C at low pH results in no time-dependent conformational changes. Titration of hIgG4-A from pH 3.5 to 6.0 resulted in recovery of native monomeric proteins whose CD and DSC profiles resembled those of the original sample. However, titration from pH 2.7 resulted in lower recovery of monomeric antibody, indicating that the greater conformational changes observed at this pH cannot be fully reversed to the native structure by a simple pH titration.

Garber E. and Demarest S. J. (2007) A broad range of Fab stabilities within a host of therapeutic IgGs. *Biochem Biophys Res Commun* **355**, 751-757.

Abstract: Although the functional properties of IgGs are well known, little has been published concerning the stability of whole IgG molecules. Stability is, however, a requirement for the development of antibodies for therapeutic or diagnostic applications. The hypervariable antigen-binding region (F_v) is responsible for stability variations between IgGs of identical subclass. To determine the range of stabilities that may be expected for human(ized) antibodies, differential scanning calorimetry was performed on 17 human(ized) antibodies from various in-house programs. The antigen-binding fragments (Fabs) of these antibodies exhibited thermal unfolding transitions with midpoints (T_ms) varying from 57 to 82 degrees C. Antibodies with very low Fab stabilities were found to aggregate and express poorly. Fab instability was often associated with high levels of uncommonly observed amino acids or CDR loop lengths particularly within the variable heavy chain domain. Overall, the study provides a thermostability range for IgGs and suggests possible stability guidelines for developing antibody diagnostics or therapeutics.

Gombotz W. R., Pankey S. C., Phan D., Drager R., Donaldson K., Antonsen K. P., Hoffman A. S., and Raff H. V. (1994) The stabilization of a human IgM monoclonal antibody with poly(vinylpyrrolidone). *Pharm Res* **11**, 624-632.

Abstract: An IgM anti-group B Streptococcus monoclonal antibody (4B9) was found to undergo irreversible heat-induced aggregation at 50 degrees C. A variety of excipients was tested for their ability to inhibit antibody aggregation. The amount of 4B9 aggregation, which was determined by analysis on a size-exclusion HPLC, was significantly reduced in the presence of low concentrations [between 0.1 and 1.0% (w/v)] of poly(vinylpyrrolidone) (PVP) molecules ranging in molecular weight from 10 to 40 kDa. When the PVP concentration was greater than 1.0%, antibody aggregation was enhanced, and with the highest molecular weight PVP, antibody precipitation occurred. HPLC was used to show that more PVP was

associated with the 4B9 at 50 degrees C than at 25 degrees C. Differential scanning calorimetry revealed that PVP concentrations greater than 2.0% decreased the antibody thermal transition temperature. Enzyme-linked immunosorbent assays were used to assess the effects of PVP on the antigen binding capacity of 4B9 and on 4B9 quantitation. At 4 degrees C, PVP solutions of up to 5.0% had no effect on either 4B9 quantitation or antigen binding. At 50 degrees C, however, less 4B9 was detected in the 5.0% PVP solution. The heat stabilization of the 4B9 antibody by low concentrations of PVP can be explained by a weak binding of PVP to the native protein. The PVP may sterically interfere with protein-protein interactions, thus reducing aggregation. Higher concentrations of PVP lead to protein aggregation and precipitation, probably by a volume-exclusion mechanism.(ABSTRACT TRUNCATED AT 250 WORDS).

Gonzalez M., Murature D. A., and Fidelio G. D. (1995) Thermal stability of human immunoglobulins with sorbitol. A critical evaluation. *Vox Sang* **68**, 1-4.

Abstract: The effect of the additive sorbitol on the thermal stabilization of human IgG was investigated by differential scanning calorimetry and size exclusion chromatography. In the presence of 33% sorbitol, the temperature at which denaturation of IgG began (Ti) was increased from 52 to 65 degrees C. Similarly, the temperature of the maximum heat capacity (Tmax) was increased from 69 to 76 degrees C. Sorbitol also decreased dimer aggregation and the extent of oligomerization during heating compared with IgG dissolved in phosphate buffer. Sorbitol at 33% prevented massive protein denaturation but a 10-15% of oligomerization of high molecular weight aggregates with turbidity could not be avoided when heating for 10 h at 60 degrees C. The use of sorbitol 33% to avoid heat denaturation of human IgG during viral inactivation did not prevent protein aggregation or the appearance of turbidity. Consequently, further processing will be required to achieve a product suitable for pharmaceutical use.

Gupta S. and Kaisheva E. (2003) Development of a multidose formulation for a humanized monoclonal antibody using experimental design techniques. *AAPS PharmSci* **5**, E8.

Abstract: The purpose of this study was to identify optimal preservatives for a multidose formulation of a humanized monoclonal antibody using experimental design techniques. The effect of antimicrobial parenteral preservatives (benzyl alcohol, chlorobutanol, methylparaben, propylparaben, phenol, and m-cresol) on protein stability was assessed using size-exclusion chromatography, differential scanning calorimetry, right-angle light scattering, UV spectroscopy, and potency testing using a cell-based fluorescence-activated cell sorting method. A quick, cost-effective preservative screening test was designed. Combinations of preservatives were examined using an I-optimal experimental design. The protein was most stable in the presence of methylparaben and propylparaben, and was compatible with benzyl alcohol and chlorobutanol at low concentrations. Phenol and m-cresol were not compatible with the protein. The I-optimal experimental design indicated that as an individual preservative, benzyl alcohol was promising. The model also indicated several effective combinations of preservatives that satisfied the antimicrobial efficacy and physical stability constraints. The preservative screening test and the experimental design approach were effective in identifying optimal concentrations of antimicrobial preservatives for a multidose protein formulation; (1) benzyl alcohol, and (2) the combination of methylparaben and chlorobutanol were screened as potential candidates to satisfy the regulatory requirements of various preservative efficacy tests.

Harn N., Allan C., Oliver C. and Middaugh C. R. (2007) Highly concentrated monoclonal antibody solutions: direct analysis of physical structure and thermal stability. *J Pharm. Sci* **96**, 532-546.

Abstract: Virtually all current analytical methods employed in the development of highly concentrated monoclonal antibody (MAb) formulations require dilution of the sample before acquiring data. Thus, there is an unmet need for methods to study proteins directly at high concentration, since extrapolation of stability indicating parameters obtained from dilute studies may not be representative of the high concentration solution. Only slight or no modifications of biophysical methods including fluorescence, UV absorbance, circular dichroism, and FTIR (ATR and transmittance) spectroscopies as well as differential scanning calorimetry (DSC) are described here that permit the direct study of immunoglobulins (and other proteins) at high concentrations. Although FTIR spectra show differences that are dependent upon sampling geometry, other spectroscopic data from two different recombinant MAbs suggests that structure of each antibody exists in a physically similar state in the concentration range of 0.1-190 mg/mL in 40 mM pH 6 citrate-phosphate buffer. Upon thermally stressing these proteins, spectroscopic techniques that probe tertiary structure demonstrate a decrease in the apparent thermal melting temperature of approximately 5-20

degrees C of both proteins with increasing concentration. In contrast, DSC thermograms and CD thermal experiments suggest a minor degree of stabilization (approximately 2 degrees C) for both antibodies although protein association could be responsible for these observations. Empirical phase diagrams produced from spectroscopic data also suggest (1) the existence of similar structural states at low temperatures independent of concentration and (2) a decrease in the temperature at which phase changes are observed with increasing concentration. The decrease in structural stability observed in these studies is probably the result of aggregation or self-association of the recombinant MAbs upon heating in crowded solutions and not due to a decrease in the intrinsic structural stability of the MAbs

Ionescu R. M., Vlasak J., Price C. and Kirchmeier M. (2008) Contribution of variable domains to the stability of humanized IgG1 monoclonal antibodies. *J Pharm. Sci* **97**, 1414-1426.

Abstract: Temperature-induced unfolding of three humanized IgG1 monoclonal antibodies and their Fab and Fc fragments was monitored by differential scanning calorimetry at neutral pH. With some exceptions, the thermogram of the intact antibody presents two peaks and the transition with the larger experimental enthalpy contains the contribution from the Fab fragments. Although the measured enthalpy was similar for all three Fab fragments studied, the apparent melting temperatures were found to vary significantly, even for Fab fragments originating from the same human germline. Therefore, we propose to use the measured enthalpy of unfolding as the key parameter to recognize the unfolding events in the melting profile of an intact IgG1 antibody. If the variable domain sequences, resulting from complementarity determining regions (CDRs) grafting and humanization, destabilize the Fab fragment with respect to the CH3 domain, the first transition represents the unfolding of the Fab fragment and the CH2 domain, while the second transition represents CH3 domain unfolding. Otherwise, the first transition represents CH2 domain unfolding, and the second transition represents the unfolding of the Fab fragment and the CH3 domain. In some cases, the DSC profile may present three transitions, with the Fab unfolding occurring at distinct temperatures compared to the melting of the CH2 and CH3 domains. If the DSC profile of a humanized IgG1 monoclonal antibody cannot be described by the model above, the result may be an indication of significant structural heterogeneity and/or of disruption of the Fab cooperative unfolding. Low stability or heterogeneity of the Fab fragment may prove problematic for long-term storage or consistency of production. Therefore, understanding the features of a DSC profile is important for clone selection and process maturation in the early stages of development of therapeutic monoclonal antibodies

Kameoka D., Masuzaki E., Ueda T. and Imoto T. (2007) Effect of buffer species on the unfolding and the aggregation of humanized IgG. *J Biochem (Tokyo)* **142**, 383-391.

Abstract: The aggregation propensity of humanized antibody after heat treatment is evaluated in the presence of six buffer species. The comparison under equivalent pH showed high aggregation propensity on phosphate and citrate buffer. In contrast, 2-(N-Morpholino) ethane sulfonate (MES), 3-(N-Morpholino) propane sulfonate (MOPS), acetate and imidazole buffer showed lower aggregation propensity than the above two buffers. Meanwhile, unfolding temperature evaluated by differential scanning calorimetry measurement was not altered among these buffer species. The light scattering analysis suggested that heat-denatured intermediate was aggregated slightly on MES and acetate buffer. Therefore, it was found that the different aggregation propensity among buffer species was caused from the aggregation propensity of heat-denatured intermediate rather than the unfolding temperature. Furthermore, it was revealed that the aggregation dependency on buffer species is accounted for by the specific molecular interaction between buffer and IgG, rather than the ionic strength. On the contrary, on the analyses of unfolding and aggregation propensity by molecular dissection of IgG into Fab and Fc fragments, aggregation propensity of Fc fragment on MES, acetate and phosphate buffer was almost the same as whole IgG. From the above results, it was suggested that the specific interaction between buffer molecule and Fc domain of IgG was involved in the aggregation propensity of heat-denatured IgG

Kamerzell T. J., Ramsey J. D. and Middaugh C. R. (2008) Immunoglobulin dynamics, conformational fluctuations, and nonlinear elasticity and their effects on stability. *J Phys. Chem B* **112**, 3240-3250.

Abstract: The relationships between protein dynamics, function, and stability are incompletely understood. Two external perturbations (temperature and pH) were used to modulate the flexibility and stability of an IgG1kappa monoclonal antibody (mAb) in an attempt to better understand the possible correlations between flexibility and stability. Ultrasonic velocimetry, densitometry, differential scanning calorimetry (DSC), and pressure perturbation calorimetry (PPC) were used to experimentally determine the adiabatic

and isothermal compressibility, expansibility, fractional volumes of unfolding, and various nonlinear thermoacoustical parameters as a function of pH and temperature. By combining these results, state parameter fluctuations were calculated from fundamental statistical mechanical relationships. The most dynamic and rigid mAb ensemble is measured at pH 4 and 6, respectively, based on state parameter fluctuations and compressibility. The effect of pH appears to couple mAb dynamics to solvent fluctuations, which control its dynamics and stability. A nonlinear response to mechanical perturbation, comparable to that seen with many polymers, is observed for this monoclonal antibody at pH 4-8. This behavior is characterized as strongly anisotropic and anharmonic, especially at pH 4. The midpoint of thermal unfolding as measured by DSC does not necessarily correlate with flexibility

Kravchuk Z. I., Vlasov A. P., Liakhnovich G. V., and Martsev S. P. (1994) [A stable conformer of IgG, prepared by an acidic influence: study by calorimetry, binding of the C1q complement component, and monospecific anti-IgG]. *Biokhimiia* **59**, 1458-1477.

Abstract: Thermal stability and functional activity of rabbit IgG in its native conformation and after incubation at pH 2.0 were studied using differential scanning calorimetry and binding of conformational probes, i.e., the C1q component of the complement and two monospecific anti-IgG antibodies. The latter reacted selectively with the "hinge" region joining the Fab and Fc fragments of IgG or with the CH2 domain in the Fc fragment. At pH 2.0 complete unfolding of rabbit IgG did not occur: the protein demonstrated the presence of secondary and compact tertiary structures but differed from the native conformation by decreased overall enthalpy and TM of thermal denaturation as well as by changed secondary structure parameters as could be evidenced from CD spectroscopy and scanning calorimetry data. Incubation at pH 2.0 followed by renaturation at neutral pH led to irreversible conformational changes in IgG. The most significant differences between the two IgG conformers were demonstrated by calorimetry at pH 3.5 which revealed that the acid-treated conformer differs from the native one by enhanced thermal stability of the CH2 domain. Using the combination of thermodynamic and functional studies, it was shown that the origin of stabilization was the increase in the extent of interaction between the CH2 domain in the Fc fragment and the CHI domain in the Fab fragment. This resulted in the increase of the functional link between the antigen-binding domain and the C1q binding site in the CH2 domain of the acid-induced IgG conformer. In parallel with the increase in stability of the CH2 domain, conformational changes in the "hinge" region were found, together with the absence of intrinsic conformational changes in the CH2 domain proper as could be judged from C1q and monospecific anti-IgG binding assays. The results obtained demonstrate one of possible mechanisms whereby functionally significant rearrangements in the IgG molecule can be induced by changes in the interactions between invariably folded domains rather than by intrinsic changes in the domain conformation.

Kravchuk Z. I., Chumanevich A. A., Vlasov A. P., and Martsev S. P. (1998) Two high-affinity monoclonal IgG2a antibodies with differing thermodynamic stability demonstrate distinct antigen-induced changes in protein A-binding affinity. *J Immunol Methods* **217**, 131-141.

Abstract: Two IgG2a monoclonal antibodies (G10 and F11) are described which have similar affinity for human spleen ferritin and identical protein A-binding affinity. The two mAbs display changes in protein A-binding affinity following binding of the antigen to its specific recognition site in the variable domains. However, while antigen-induced conformational changes in G10 enhance its affinity to protein A, interaction of F11 with ferritin results in a significant decrease in protein A-binding affinity. In contrast to the IgG2a antibodies, using a mouse IgG1 antiferritin antibody (C5) high-affinity binding of the antigen does not change an inherently low ability to bind protein A. Differential scanning calorimetry revealed that the enthalpy and Gibb's free energy of thermal unfolding for G10 was 19% and 23% higher, respectively, than the corresponding parameters for F11. The lower structural energetics of F11 are associated with the absence of a calorimetrically revealed folding unit, which may be responsible for interactions between the antigen-binding site and the protein A-binding site. This study provides the first demonstration that functionally significant interactions between two recognition sites in antibodies of the same subclass can be modulated by subclass-independent structural variations associated with different thermodynamic stability.

Liu D., Ren D., Huang H., Dankberg J., Rosenfeld R., Cocco M. J., Li L., Brems D. N. and Remmele R. L., Jr. (2008) Structure and stability changes of human IgG1 Fc as a consequence of methionine oxidation. *Biochemistry* **47**, 5088-5100.

Abstract: The Fc region has two highly conserved methionine residues, Met 33 (C(H)3 domain) and Met 209 (C(H)3 domain), which are important for the Fc's structure and biological function. To understand the effect of methionine oxidation on the structure and stability of the human IgG1 Fc expressed in *Escherichia coli*, we have characterized the fully oxidized Fc using biophysical (DSC, CD, and NMR) and bioanalytical (SEC and RP-HPLC-MS) methods. Methionine oxidation resulted in a detectable secondary and tertiary structural alteration measured by circular dichroism. This is further supported by the NMR data. The HSQC spectral changes indicate the structures of both C(H)2 and C(H)3 domains are affected by methionine oxidation. The melting temperature (T_m) of the C(H)2 domain of the human IgG1 Fc was significantly reduced upon methionine oxidation, while the melting temperature of the C(H)3 domain was only affected slightly. The change in the C(H)2 domain T_m depended on the extent of oxidation of both Met 33 and Met 209. This was confirmed by DSC analysis of methionine-oxidized samples of two site specific methionine mutants. When incubated at 45 degrees C, the oxidized Fc exhibited an increased aggregation rate. In addition, the oxidized Fc displayed an increased deamidation (at pH 7.4) rate at the Asn 67 and Asn 96 sites, both located on the C(H)2 domain, while the deamidation rates of the other residues were not affected. The methionine oxidation resulted in changes in the structure and stability of the Fc, which are primarily localized to the C(H)2 domain. These changes can impact the Fc's physical and covalent stability and potentially its biological functions; therefore, it is critical to monitor and control methionine oxidation during manufacturing and storage of protein therapeutics

Liu H., Bulseco G. G., and Sun J. (2006) Effect of posttranslational modifications on the thermal stability of a recombinant monoclonal antibody. *Immunol Lett* **106**, 144-153.

Abstract: The effect of oligosaccharides and C-terminal lysine residues on the thermal stability of a recombinant IgG(1) monoclonal antibody was investigated using differential scanning calorimetry (DSC). The C-terminal lysine did not appear to affect the thermal stability of this IgG(1) molecule. However, oligosaccharides, which are buried between the two CH(2) domains, provided significant stabilizing energy. Characterization of the Fab and Fc after papain digestion suggested that the stabilizing effect of oligosaccharides on this molecule was through stabilizing CH(2) domains. Oligosaccharides had little effect on the thermal stability of Fab region and CH(3) domains. It was also interesting to note that both intact IgG(1) antibody and its Fab, but not the Fc regions, appeared to form precipitate after thermal unfolding under the experimental conditions.

Maiorella B. L., Ferris R., Thomson J., White C., Brannon M., Hora M., Henriksson T., Triglia R., Kunitani M., Kresin L., and . (1993) Evaluation of product equivalence during process optimization for manufacture of a human IgM monoclonal antibody. *Biologicals* **21**, 197-205.

Abstract: We have developed a battery of tests to characterize monoclonal antibodies and assess the effect of potential manufacturing process changes. Tryptic peptide mapping, molecular weight determination by HPLC and classical light scattering, isoelectric focussing, oligosaccharide mapping by HPAE-PAD chromatography, circular dichroism spectra and differential scanning calorimetry were applied as sensitive assays of antibody structure. Biological activity was assessed by measurement of specific antigen binding activity, binding spectrum and opsonic activity. Pharmacokinetics was assessed by clearance rate studies in rats. The sensitivity of this battery of assays was demonstrated by the ability to readily detect differences between a human monoclonal antibody (IgM-2) produced by in vitro cell culture versus ascites culture. These same tests support equivalence of a second monoclonal antibody (IgM-1) produced before and after in vitro cell culture process improvements which resulted in a twofold increase in product titer.

Martsev S. P., Kravchuk Z. I., Vlasov A. P., and Lyakhovich G. V. (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.

Abstract: At pH2, rabbit IgG adopts a partially structured state that exhibits loss of thermal unfolding transition, tentatively assigned to the CH2 domain, whilst retaining a well-defined tertiary structure for the rest of the molecule and extensive secondary structure. Renaturation of IgG from this state yields a stable conformer that differs from native IgG by a lower degree of interaction between the CH2 and CH3 domains, and stronger interaction between the CH1 and CH2 domains, as judged by differential scanning calorimetry and probing the IgG conformation with specific ligands (C1q component of complement, protein A and monospecific antibodies to the CH2 domain and hinge region).

Martsev S. P., Chumanevich A. A., Vlasov A. P., Dubnovitsky A. P., Tsybovsky Y. I., Deyev S. M., Cozzi A., Arosio P., and Kravchuk Z. I. (2000) Antiferritin single-chain Fv fragment is a functional protein with properties of a partially structured state: comparison with the completely folded V(L) domain. *Biochemistry* **39**, 8047-8057.

Abstract: Differential scanning calorimetry and spectroscopic probes were applied to study folding and stability of the single-chain Fv fragment (scFv) of the anti-human ferritin antibody F11 and its isolated variable light-chain (V(L)) domain. The scFv fragment followed variable heavy-chain domain (V(H))-linker-V(L) orientation and contained (Gly(4)Ser(3)) linker peptide. The two proteins were produced in *Escherichia coli* and refolded from denaturant-solubilized inclusion bodies. The isolated V(L) domain demonstrated a typical immunoglobulin fold with well-defined secondary and tertiary structure and was capable of binding human ferritin with $K(a) = 1.8 \times 10^7 \text{ M}^{-1}$, approximately (1)/(30) of the affinity of the parent F11 antibody. Involvement of this V(L) domain into the two-domain scFv fragment yielded a distorted secondary and significantly destabilized tertiary structure in which neither of the two constituent domains attained complete folding. The thermal unfolding enthalpy of scFv F11 at pH 7.0 was as low as 5.0 J.g⁻¹(1) versus 16.3 J.g⁻¹(1) obtained for the V(L) domain and 24.7 J.g⁻¹(1) for the parent F11 antibody (mouse IgG2a subclass). Intrinsic fluorescence and near-ultraviolet circular dichroic (CD) spectra, and binding of the hydrophobic probe 8-anilino-1-naphthalene sulfonate, confirmed partial loss of tertiary interactions in scFv. The spectroscopic and calorimetric properties of scFv F11 under physiological conditions are consistent with a model of a partially structured state with a distorted beta-sheet as a secondary structure and partial loss of tertiary interactions, which closely resembles the alternatively folded A-state adopted by an immunoglobulin at pH 2-3 [Buchner, J., Renner, M., Lilie, H., Hinz, H.-J., Jaenicke, R., Kiefhaber, T., and Rudolph, R. (1991) *Biochemistry* 30, 6922-6929]. However, scFv F11 demonstrated only an approximately 4-fold decrease in the antigen-binding affinity ($K(a) = 1.3 \times 10^8 \text{ M}^{-1}$) versus the parent F11 antibody. The scFv fragment F11 provides the first description of a functional protein trapped under physiological conditions in a partially structured state. This state is either close to the native one in the antigen-binding affinity or, alternatively, initial weak binding of the antigenic epitope induces folding of scFv F11 into a more structured conformation that generates relatively high affinity.

Martsev S. P., Dubnovitsky A. P., Stremovsky O. A., Chumanevich A. A., Tsybovsky Y. I., Kravchuk Z. I., and Deyev S. M. (2002) Partially structured state of the functional VH domain of the mouse anti-ferritin antibody F11. *FEBS Lett* **518**, 177-182.

Abstract: An antibody combining site generally involves the two variable domains, VH from the heavy and VL from the light chain. We expressed the individual VH domain of the mouse anti-human ferritin monoclonal antibody F11. The loss of affinity was not dramatic ($K(a)=4.0 \times 10^7 \text{ M}^{-1}$ versus $8.6 \times 10^8 \text{ M}^{-1}$ for the parent antibody) and comparable to that previously observed for other VHs. However, the functional VH domain adopted a partially structured state with a significant amount of distorted secondary and compact yet greatly destabilized tertiary structures, as demonstrated by spectroscopic and calorimetric probes. These data provide the first description for a functional antibody domain that meets all the criteria of a partially structured state.

Martsev S. P., Dubnovitsky A. P., Vlasov A. P., Hoshino M., Hasegawa K., Naiki H., and Goto Y. (2002) Amyloid fibril formation of the mouse V(L) domain at acidic pH. *Biochemistry* **41**, 3389-3395.

Abstract: The recombinant V(L) domain that represents the variable part of the light chain (type kappa) of mouse monoclonal antibody F11 directed against human spleen ferritin was found to form amyloid fibrils at acidic pH as evidenced by electron microscopy, thioflavin T binding, and apple-green birefringence after Congo red staining. This is the first demonstration of amyloid fibril formation of the mouse V(L) domain. To understand the mechanism of acidic pH-induced amyloid fibril formation, conformational changes of the V(L) domain were studied by one-dimensional NMR, differential scanning calorimetry, analytical ultracentrifugation, hydrophobic dye binding, far-UV circular dichroism, and tryptophan fluorescence. The results indicated accumulation of two intermediate states during acid unfolding, which might be responsible for amyloid fibril formation. The more structured intermediate that exhibited maximal accumulation at pH 3 retained the natively-like secondary structure and a hydrophobic core, but exposed hydrophobic surfaces that bind 8-anilino-1-naphthalenesulfonate. Below pH 2, a more disordered intermediate with quenched tryptophan fluorescence but still retaining the beta-sheet structure accumulated. The optimal pH of amyloid fibril formation (i.e., pH 4) was close to the optimal pH of the accumulation of the natively-like intermediate, suggesting that the amyloid fibrils might be formed through this intermediate.

Matheus S., Friess W., and Mahler H. C. (2006) FTIR and nDSC as Analytical Tools for High-Concentration Protein Formulations. *Pharm Res* **23**, 1350-1363.

Abstract: PURPOSE: The aim of the study is to evaluate Fourier-transform infrared spectroscopy (FTIR) as an analytical tool for high-concentrated protein formulations. METHODS: FTIR is used to determine the melting temperature ($T(m)$ (FTIR)) of various proteins, such as bovine serum albumin (BSA), immunoglobulin (IgG(1)), beta-lactoglobulin (beta-LG), and lysozyme (HEWL), at different protein concentrations (5-100 mg/mL), where four data interpretation methods are discussed. The obtained $T(m)$ (FTIR) values are further compared to the $T(m)$ measured by the nanodifferential scanning calorimetry (nDSC) technique. RESULTS: The $T(m)$ (FTIR) values of IgG(1) and beta-LG showed strong consistency and corresponded to the nDSC results irrespective of the method of data interpretation and the protein concentration applied. In contrast, the $T(m)$ (FTIR) of BSA and HEWL is characterized by significant deviations. Only the midpoint of the second-derivative intensity-temperature curve of the intermolecular beta-sheet mode measured at a concentration of 100 mg/mL is consistent with the nDSC results. CONCLUSIONS: Determination of a $T(m)$ (FTIR) is feasible by the midpoint of the intensity-temperature plot of the arising intermolecular beta-sheet band. More significant results are obtained for proteins, which are predominantly composed of intramolecular beta-sheet elements as well as at higher protein concentrations. A further study was started to assess the predictability of long-term protein stability by $T(m)$ (FTIR).

Mimura Y., Church S., Ghirlando R., Ashton P. R., Dong S., Goodall M., Lund J., and Jefferis R. (2000) The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms. *Mol Immunol* **37**, 697-706.

Abstract: Antibodies are multifunctional molecules that following the formation of antibody antigen complexes, may activate mechanisms to effect the clearance and destruction of the antigen (pathogen). The IgG molecule is comprised of three globular protein moieties (2Fab+Fc) linked through a flexible hinge region. While the Fabs bind antigens, the Fc triggers effector mechanisms through interactions with specific ligands, e.g. cellular receptors (Fc γ R), and the C1 component of complement. Glycosylation of IgG-Fc has been shown to be essential for efficient activation of Fc γ R and C1. We report the generation of a series of truncated glycoforms of IgG-Fc, and the analysis of the contribution of the residual oligosaccharide to IgG-Fc function and thermal stability. Differential scanning microcalorimetry has been used to compare the stabilities of the homogeneous glycoforms of IgG1-Fc. The results show that all truncated oligosaccharides confer a degree of functional activity, and thermodynamic stability to the IgG1-Fc, in comparison with deglycosylated IgG1-Fc. The same truncated glycoforms of an intact IgG1 anti-MHC Class II antibody are shown to exhibit differential functional activity for Fc γ R1 and C1 ligands, relative to deglycosylated IgG1. The minimal glycoform investigated had a trisaccharide attached to each heavy chain and can be expected to influence protein structure primarily in the proximity of the N-terminal region of the C(H)2 domain, implicated as a binding site for multiple effector ligands. These data provide a thermodynamic rationale for the modulation of antibody effector functions by different glycoforms.

Mummert M. E. and Voss E. W., Jr. (1997) Effects of secondary forces on the ligand binding and conformational state of antifuorescein monoclonal antibody 9-40. *Biochemistry* **36**, 11918-11922.

Abstract: Biochemical interactions that occur external to the antibody active site have been termed secondary forces. Secondary forces are supplemental to interactions within the antibody active site (i.e., primary interactions) and can affect ligand binding efficiency as well as variable domain conformation. The antifuorescein antibody system has been determined to be a superior method for delineating primary from secondary interactive components due to the active site-filling properties of the fluorescein ligand. To date, all studies of secondary forces within the context of the antifuorescein system have been with the high-affinity monoclonal antibody 4-4-20 (mAb 4-4-20) (Mummert & Voss, 1995, 1996, 1997). In order to determine the generality of experimental observations and proposed models, we investigated the effects of secondary forces on the antifuorescein mAb 9-40. In addition to assessing the results of former studies, mAb 9-40 possesses properties unique from those of mAb 4-4-20, namely, a decreased affinity for fluorescein and increased conformational dynamics relative to mAb 4-4-20 (Carrero & Voss, 1996). Results of fluorescein and intrinsic mAb 9-40 tryptophan quenching as well as differential scanning calorimetric (DSC) studies indicated that secondary forces modulated the conformational (metatypic) state in accordance with previous investigations with mAb 4-4-20. Unlike mAb 4-4-20, mAb 9-40 did not exhibit altered ligand binding efficiency due to the inclusion of secondary interactive components. Models

were developed that proposed that the increased malleability of mAb 9-40 variable domains could account for functional differences in properties between mAb 9-40 and mAb 4-4-20.

Nellis D. F., Giardina S. L., Janini G. M., Shenoy S. R., Marks J. D., Tsai R., Drummond D. C., Hong K., Park J. W., Ouellette T. F., Perkins S. C., and Kirpotin D. B. (2005) Preclinical manufacture of anti-HER2 liposome-inserting, scFv-PEG-lipid conjugate. 2. Conjugate micelle identity, purity, stability, and potency analysis. *Biotechnol Prog* **21**, 221-232.

Abstract: Analytical methods optimized for micellar F5cys-MP-PEG(2000)-DPSE protein-lipopolymer conjugate are presented. The apparent micelle molecular weight, determined by size exclusion chromatography, ranged from 330 to 960 kDa. The F5cys antibody and conjugate melting points, determined by differential scanning calorimetry, were near 82 degrees C. Traditional methods for characterizing monodisperse protein species were inapplicable to conjugate analysis. The isoelectric point of F5cys (9.2) and the conjugate (8.9) were determined by capillary isoelectric focusing (cIEF) after addition of the zwitterionic detergent CHAPS to the buffer. Conjugate incubation with phospholipase B selectively removed DSPE lipid groups and dispersed the conjugate prior to separation by chromatographic methods. Alternatively, adding 2-propanol (29.4 vol %) and n-butanol (4.5 vol %) to buffers for salt-gradient cation exchange chromatography provided gentler, nonenzymatic dispersion, resulting in well-resolved peaks. This method was used to assess stability, identify contaminants, establish lot-to-lot comparability, and determine the average chromatographic purity (93%) for conjugate lots, described previously. The F5cys amino acid content was confirmed after conjugation. The expected conjugate avidity for immobilized HER-2/neu was measured by bimolecular interaction analysis (BIAcore). Mock therapeutic assemblies were made by conjugate insertion into preformed doxorubicin-encapsulating liposomes for antibody-directed uptake of doxorubicin by HER2-overexpressing cancer cells in vitro. Together these developed assays established that the manufacturing method as described in the first part of this study consistently produced F5cys-MP-PEG(2000)-DSPE having sufficient purity, stability, and functionality for use in preclinical toxicology investigations.

Oda M., Ito N., Tsumuraya T., Suzuki K., Sakakura M. and Fujii I. (2007) Thermodynamic and structural basis for transition-state stabilization in antibody-catalyzed hydrolysis. *J Mol Biol* **369**, 198-209.

Abstract: Catalytic antibodies 6D9 and 9C10, which were induced by immunization with a haptenic transition-state analog (TSA), catalyze the hydrolysis of a nonbioactive chloramphenicol monoester derivative to generate a bioactive chloramphenicol. These antibodies stabilize the transition state to catalyze the hydrolysis reaction, strictly according to the theoretical relationship: for 6D9, $k(\text{cat})/k(\text{uncat})=895$ and $K(\text{S})/K(\text{TSA})=900$, and for 9C10, $k(\text{cat})/k(\text{uncat})=56$ and $K(\text{S})/K(\text{TSA})=60$. To elucidate the molecular basis of the antibody-catalyzed reaction, the crystal structure of 6D9 was determined, and the binding thermodynamics of 6D9 and 9C10 with both the substrate and the TSA were analyzed using isothermal titration calorimetry. The crystal structure of the unliganded 6D9 Fab was determined at 2.25 Å resolution and compared with that of the TSA-liganded 6D9 Fab reported previously, showing that the TSA is bound into the hydrophobic pocket of the antigen-combining site in an "induced fit" manner, especially at the L1 and H3 CDR loops. Thermodynamic analyses showed that 6D9 binds the substrate of the TSA with a positive ΔS , differing from general thermodynamic characteristics of antigen-antibody interactions. This positive ΔS could be due to the hydrophobic interactions between 6D9 and the substrate or the TSA mediated by Trp H100i. The difference in ΔG between substrate and TSA-binding to 6D9 was larger than that to 9C10, which is in good correlation with the larger $k(\text{cat})$ value of 6D9. Interestingly, the $\Delta\Delta G$ was mainly because of the $\Delta\Delta H$. The correlation between $k(\text{cat})$ and $\Delta\Delta H$ is suggestive of "enthalpic strain" leading to destabilization of antibody-substrate complexes. Together with X-ray structural analyses, the thermodynamic analyses suggest that upon binding the substrate, the antibody alters the conformation of the ester moiety in the substrate from the planar Z form to a thermodynamically unstable twisted conformation, followed by conversion into the transition state. Enthalpic strain also contributes to the transition-state stabilization by destabilizing the ground state, and its degree is much larger for the more efficient catalytic antibody, 6D9.

Oganesyan V., Damschroder M. M., Leach W., Wu H. and Dall'Acqua W. F. (2008) Structural characterization of a mutated, ADCC-enhanced human Fc fragment. *Mol Immunol.* **45**, 1872-1882.

Abstract: We report here the three-dimensional structure of a human Fc fragment engineered for enhanced antibody dependent cell mediated cytotoxicity (ADCC). The triple mutation S239D/A330L/I332E ('3M')

was introduced into the C(H)2 portion of a human immunoglobulin G1 (IgG1) Fc. These three substitutions typically result in an about 10-100-fold increase in human IgG1 binding to human Fc gamma RIIIA (CD16). The recombinantly produced Fc/3M fragment was crystallized and its structure solved at a resolution of 2.5Å using molecular replacement. No dramatic structural changes were observed in Fc/3M when compared with unmutated human Fc fragments. However, we found that the relative positions of its C(H)2 domains allowed for an unusually 'open' conformation of the entire fragment. Although this particular structural feature could be due to crystallization artifacts or intrinsic variability, we propose that molecular mechanisms at the basis of the enhanced interaction between Fc/3M and CD16 could include enhanced Fc openness as well as the introduction of additional hydrophobic contacts, hydrogen bonds and/or electrostatic interactions at the corresponding interface. The existence of a more pronounced cleft between the two Fc chains as well as of repulsive, electrostatic intra-chain interactions may also account in part for the decreased thermostability of both Fc/3M and a 3M-modified humanized anti-human EphA2 IgG1 when compared with their respective unmutated counterparts

Parker M.H., Chen Y., Danehy F., Dufu K., Ekstrom J., Getmanova E., Gokemeijer J., Xu L., and Lipovsek D. (2005) Antibody mimics based on human fibronectin type three domain engineered for thermostability and high-affinity binding to vascular endothelial growth factor receptor two. *Protein Eng Des Sel.* **18**, 435-44.

Abstract: The tenth human fibronectin type three domain ((10)Fn3) is a small (10 kDa), extremely stable and soluble protein with an immunoglobulin-like fold, but without cysteine residues. Selections from (10)Fn3-based libraries of proteins with randomized loops have yielded high-affinity, target-specific antibody mimics. However, little is known about the biophysical properties of such antibody mimics, which will determine their suitability for in vitro and medical applications. We characterized target binding and biophysical properties of two related (10)Fn3-based antibody mimics that bind vascular endothelial growth factor receptor two (VEGF-R2). The first antibody mimic, which has a dissociation constant ($K(d)$) of 13 nM, is highly stable [melting temperature ($T(m)$)=62 degrees C] and soluble, whereas the second, which binds VEGF-R2 with 40 x higher affinity, is less stable ($T(m)$ < 40 degrees C) and relatively insoluble. We used our understanding of these two (10)Fn3 derivatives and of wild-type (10)Fn3 structure to engineer the next generation of antibody mimics, which have an improved combination of high affinity ($K(d)$ =0.59 nM), stability ($T(m)$ =53 degrees C) and solubility. Our findings illustrate that (10)Fn3-based antibody mimics can be engineered for favorable biophysical properties even when 20% of the wild-type (10)Fn3 sequence is mutated in order to satisfy target-binding requirements.

Perico N., Purtell J., Dillon T. M. and Ricci M. S. (2008) Conformational implications of an inversed pH-dependent antibody aggregation. *J Pharm. Sci (publication)*.

Abstract: Antibody formulation development relies on accelerated stability data at elevated temperatures to optimize formulation parameters. However, the pH- and temperature-dependence of aggregation is complicated for antibody formulations. In this study, a human monoclonal IgG2 antibody exhibited typical pH-dependent dimer formation under normal storage conditions (4 and/or 29 degrees C). However, an inversed pH-dependence was discovered for high molecular weight aggregate formation at elevated temperatures (37 degrees C). The different stability profiles exhibited at the various storage conditions resulted in nonlinearity of the Arrhenius kinetics. Thermal unfolding at or below 37 degrees C was not evident by differential scanning calorimetry. Enriched populations of the structural isoforms of the IgG2 subclass were tested for their unique temperature and pH-dependence of aggregation. The Arrhenius kinetics of aggregation for each of the individual IgG2 isoforms was also nonlinear. However, the temperature-dependence of clipping suggested that clip-mediated aggregation was responsible for the increased higher order aggregates at low pH and elevated temperatures. Unique clip species resulting from the conformational differences between the IgG2 isoforms lead to increased aggregation. These results have implications on the mechanisms of antibody aggregation and on the validity of accelerated data to predict shelf-life accurately. (c) 2008 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci*

Protasevich I. I., Ranjbar B., Varlamova E. Y., Cherkasov I. A., and Lapuk V. A. (1997) Comparative study of monoclonal immunoglobulin M and rheumatoid immunoglobulin M by differential scanning microcalorimetry. *Biochemistry (Mosc)* **62**, 914-918.

Abstract: Thermal denaturation of monoclonal immunoglobulin M (IgM) and rheumatoid immunoglobulin M (IgM-RF) and their Fab- and (Fc)₅-fragments was studied by differential scanning microcalorimetry. The melting of IgM-RF started at a higher temperature than that of IgM and the maximum temperature of its main asymmetric peak of heat absorption was higher by 4 degrees C. At equal values of enthalpy, the thermal denaturation of IgM-RF and IgM consisted of four and five individual transitions, respectively, between pairs of states. The comparison of thermal denaturation parameters of Fab- and (Fc)₅-fragments of IgM-RF and IgM showed a thermodynamic similarity of (Fc)₅-fragments of both proteins, while their Fab-fragments differed in the interaction between VL-CL and VH-CH domains.

Thies M. J., Kammermeier R., Richter K., and Buchner J. (2001) The alternatively folded state of the antibody C(H)3 domain. *J Mol Biol* **309**, 1077-1085.

Abstract: The C(H)3 domain of antibodies is characterized by two antiparallel beta-sheets forming a disulfide-linked sandwich-like structure. At acidic pH values and low ionic strength, C(H)3 becomes completely unfolded. The addition of salt transforms the acid-unfolded protein into an alternatively folded state exhibiting a characteristic secondary structure. The transition from native to alternatively folded C(H)3 is a fast reaction. Interestingly, this reaction involves the formation of a defined oligomer consisting of 12-14 subunits. Association is completely reversible and the native dimer is quantitatively reformed at neutral pH. This alternatively folded protein is remarkably stable against thermal and chemical denaturation and the unfolding transitions are highly cooperative. With a T_m of 80 degrees C, the stability of the alternatively folded state is comparable to that of the native state of C(H)3. The defined oligomeric structure of C(H)3 at pH 2 seems to be a prerequisite for the cooperative unfolding transitions.

Tischenko V. M., Abramov V. M., and Zav'yalov V. P. (1998) Investigation of the cooperative structure of Fc fragments from myeloma immunoglobulin G. *Biochemistry* **37**, 5576-5581.

Abstract: The cooperative structure of Fc fragments prepared from myeloma human IgG1 was studied using scanning microcalorimetry and fluorescence at pH 4.2-8.0. It was shown that the first to be melted are CH₂ domains whose interaction with each other is rather weak, while that with CH₃ domains is strong. Then CH₃ domains which form a single cooperative block are melted. The data for the structure of the Fc fragment in solution agree with the X-ray data according to which the interaction between CH₂ domains is mediated by the carbohydrate moiety while the two CH₃ domains are strongly associated. The presence of intensive CH₂-CH₃ interaction is a distinctive feature of the state of the Fc fragment in the given pH region as compared to that at pH <4.1 [Tischenko, V. M., et al. (1982) *Eur. J. Biochem.* 126, 517-521; Ryazantsev, S., et al. (1990) *Eur. J. Biochem.* 190, 393-399]. First, cis interactions greatly increase the free energy of the native structure stabilization in CH₂ domains. Second, they decrease this energy for CH₃ domains when compared to the state of the latter at pH 3.8 or within the Fc' fragment (the dimer of CH₃ domains). The temperature and enthalpy of melting of CH₂ domains coincide in all the samples studied despite heterogeneity of the carbohydrate moiety. Thus, it may be postulated that the conservative part of CH₂ domains makes a cardinal contribution to the interaction of these domains with the carbohydrate moiety.

Tischenko V. M. and Zav'yalov V. P. (2003) Core hinge of human immunoglobulin G3 as a system of four independent co-operative blocks. *Immunol Lett* **86**, 281-285.

Abstract: On the heat absorption curves of human immunoglobulin G3 (hIgG3) Kuc melting the scanning calorimetry method reveals a high-temperature (high-T_m) peak of high intensity that is absent at the curves of other hIgG subclasses and IgG of other species. An analogous peak is observed also at the curves of melting of hIgG3 fragments containing the hinge segments. The high-T_m peak is accompanied by characteristic changes in circular dichroism (CD) spectra at 220-230 nm. This allows relating the peak to the melting of a poly-L-proline conformation of an extremely long hIgG3 core hinge. The comparison of ΔH(cal) and ΔH(eff) testifies that the core hinge can be considered as a system of four independent co-operative blocks connected by flexible sites. These sites may provide additional flexibility to the hIgG3 molecule and also permit a transition of the rod-like shape of the hinge to compact globule-like conformation.

Tishchenko V. M., Zav'yalova G. A., and Zav'yalov V. P. (2003) Folding of the human immunoglobulin G3 Kus core hinge into the thirteenth globular domain. *Immunol Lett* **90**, 43-47.

Abstract: Earlier, the electron microscopy and hydrodynamic studies revealed the transformation of the globule-like form of the human (h) IgG3 Kus hinge into a rod-like shape under non-denaturing perturbations [Eur. J. Biochem. 190 (1990) 393]. In this work, it is shown with the differential scanning calorimetry (DSC) that the melting of a globule-like form of the hIgG3 Kus hinge is a co-operative process. The 'two-state' model accepted for small globular proteins well describes the transition. Thus, in the hIgG3 Kus molecule, the core hinge folds into the thirteenth globular domain. The model of folding of four double poly-L-proline (PLP) helices of the core hinge into the compact tertiary structure similar to 'a folded camp bed' is suggested.

Tishchenko V. M. (1999) [Interaction between CH2 and CH3-domains of human immunoglobulin G1 in glycosylated and aglycosylated Fc-fragments]. *Biofizika* **44**, 811-812.

Abstract: Heat denaturation of glycosylated and aglycosylated human immunoglobulin G1 Fc fragments was investigated by differential scanning microcalorimetry. The enthalpy of the interaction between aglycosylated CH2 and CH3 domains is significantly reduced at 37 degrees C (but not at 0 degree C) as compared to the glycosylated form. The temperature dependence is consistent with the data on restricted proteolysis by trypsin.

Tsybovsky Y. I., Kedrov A. A., and Martsev S. P. (2004) Independent folding and conformational changes of the barnase module in the VL-barnase immunofusion: calorimetric evidence. *FEBS Lett* **557**, 248-252.

Abstract: Although stability is critical for in vivo application of immunotoxins, a thermodynamic description of their folding/stability is still lacking. We applied differential scanning calorimetry (DSC) to RNase-based immunofusion comprising barnase, cytotoxic RNase from *Bacillus amyloliquefaciens*, fused to the light chain variable domain (VL) of anti-human ferritin antibody F11. By analyzing DSC curves recorded with or without preheating and addition of the barnase-stabilizing ligand guanosine 3'-monophosphate, we (i). assigned two well-resolved thermal transitions to the VL and barnase modules of VL-barnase, (ii). demonstrated independent folding of these two modules, and (iii). showed altered stability of the barnase module, which resulted from the dimeric state of VL-barnase.

Tsybovsky Y. I., Shubenok D. V., Stremovskiy O. A., Deyev S. M., and Martsev S. P. (2004) Folding and stability of chimeric immunofusion VL-barstar. *Biochemistry (Mosc)* **69**, 939-948.

Abstract: A chimeric protein VL-barstar that comprises the VL domain of anti-human ferritin monoclonal antibody F11 and barstar, the naturally occurring inhibitor of bacterial RNase barnase, has been constructed for study of structure-function characteristics of chimeric immunoglobulin fused proteins. Such chimeric constructs may be potentially employed for development of bivalent/bispecific antibodies on the basis of the high affinity interaction between barstar and barnase (the association constant is about 10^{14} M^{-1}). We have developed a protocol for VL-barstar expression in *E. coli* and purification and refolding from inclusion bodies that yields a homogeneous and soluble form of this protein. Differential scanning calorimetry in combination with fluorescence and CD spectroscopy revealed that the VL-barstar formed well-resolved ordered secondary and compact tertiary structures. However, partial loss of tertiary interactions resulted in low stability of the recombinant protein and the lack of functional activity of the two constituent modules. These conformational features suggest that the protein might be referred to the class of native molten globules, which comprises partially unfolded conformations stabilized under physiological conditions. Since individually expressed VL domain and barstar retain completely folded conformation and stable spatial structure, the incomplete folding of the chimeric protein may be attributed to interaction between heterologous domains, which appears at the folding stage preceding formation of a system of tertiary interactions in both structural modules. The results provide evidence for non-native interactions between heterologous modules that may occur in chimeric proteins composed of taxonomically distinct fusion partners.

Tsybovsky Y., Shubenok D. V., Kravchuk Z. I. and Martsev S. P. (2007) Folding of an antibody variable domain in two functional conformations in vitro: calorimetric and spectroscopic study of the anti-ferritin antibody VL domain. *Protein Eng Des Sel* **20**, 481-490.

Abstract: Understanding refolding pathways of recombinant antibody fragments is essential for efficient production of these proteins of high biomedical significance. The recombinant VL domain of mouse anti-

human ferritin antibody F11 formed two distinct functional conformations obtained by refolding from bacterial inclusion bodies using two different procedures. Involvement of a dialysis step at pH 2-3 resulted in the VL-1 conformation with fluorescence of the highly conserved Trp-35 residue quenched by the spatially proximal disulfide bond. This conformation was identical to the 'native' VL domain folded in host cells and purified from the cytoplasm. In the absence of the acidic dialysis step, the VL domain adopted a previously unreported conformation, VL-2, that demonstrated prominent fluorescence due to a local structural disorder around Trp-35. Furthermore, VL-2 showed changes in secondary structure and significantly lower stability as determined by differential scanning calorimetry and denaturant-induced unfolding. While more flexible VL-2 binds human ferritin both in solution and after surface adsorption of the antibody domain, the VL-1 conformer needs an adsorption-induced conformational change to allow the access of ferritin to the antigen-binding site. Noteworthy, the two macroscopic conformations constitute kinetically trapped dimers and do not interconvert at elevated temperatures (3 weeks at 37 degrees C or 15 min at 60 degrees C), which indicates a high energetic barrier between them. As a major finding, this paper provides the first description for two stable and functional conformations of an antibody domain.

Vermeer A. W., Bremer M. G., and Norde W. (1998) Structural changes of IgG induced by heat treatment and by adsorption onto a hydrophobic Teflon surface studied by circular dichroism spectroscopy. *Biochim Biophys Acta* **1425**, 1-12.

Abstract: Thermal denaturation of mouse monoclonal immunoglobulin G (isotype 1), as well as structural rearrangements resulting from adsorption on a hydrophobic Teflon surface, are studied by circular dichroism spectroscopy. Both heat-induced and adsorption-induced denaturation do not lead to complete unfolding into an extended polypeptide chain, but leave a significant part of the IgG molecule in a globular or corpuscular form. Heating dissolved IgG causes a decrease of the fractions of beta-sheet and beta-turn conformations, whereas those of random coil and, to a lesser extent, alpha-helix increase. Adsorption enhances the formation of alpha-helices and random coils, but the beta-sheet content is strongly reduced. Heating adsorbed IgG results in a gradual break-down of the alpha-helix and beta-turn contents, and a concomitant formation of beta-sheet structures. Thus, the structural changes in IgG caused by heating and by adsorption, respectively, are very different. However, after heating, the structure of adsorbed IgG approaches the structure of thermally denatured IgG in solution.

Vermeer A. W., Norde W., and van Amerongen A. (2000) The unfolding/denaturation of immunoglobulin of isotype 2b and its F(ab) and F(c) fragments. *Biophys J* **79**, 2150-2154.

Abstract: The unfolding and further denaturation of IgG and its F(ab) and F(c) fragments were studied both on a macroscopic and molecular level, using differential scanning calorimetry and circular dichroism spectroscopy, respectively. It was shown that the structural integrity of the F(ab) and F(c) units was retained after fragmentation of the IgG. The F(ab) fragment denatured at approximately 61 degrees C and the F(c) fragment at 71 degrees C. The structural transitions observed in the whole IgG is the sum effect of those determined for the isolated F(ab) and F(c) fragments.

Vermeer A. W. and Norde W. (2000) The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein. *Biophys J* **78**, 394-404.

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.

Vermeer A. W., Giacomelli C. E., and Norde W. (2001) Adsorption of IgG onto hydrophobic teflon. Differences between the F(ab) and F(c) domains. *Biochim Biophys Acta* **1526**, 61-69.

Abstract: The effect of differences in the degree of hydrophobicity of protein patches/fragments on the adsorption behaviour of the protein is investigated. The adsorption isotherm of a monoclonal mouse anti-human immunoglobulin G (isotype 2b) onto hydrophobic Teflon particles is measured using a depletion method. The adsorption-induced denaturation of the immunoglobulin as a function of the adsorbed amount is studied by differential scanning calorimetry, and the corresponding rearrangements in the secondary structure of the whole IgG molecule and its F(ab) and F(c) fragments are determined by circular dichroism spectroscopy. The effects of adsorption on the F(ab) and F(c) fragments in the intact IgG molecule occur independently. Adsorption of the whole IgG molecule leads to denaturation of the F(ab) fragments, whereas the F(c) fragment remains unperturbed; adsorption of the isolated fragments results in structural changes in both F(ab) and F(c). The surface hydrophobicity of the isolated fragments was studied by HPLC. These experiments support the hypothesis that differences in the degree of denaturation between F(ab) and F(c) are due to the higher degree of hydrophobicity of the F(ab) fragment. The adsorption-induced changes in the secondary structure are more prominent for the isolated fragments as compared to intact IgG. This is ascribed to the higher flexibility of the isolated fragment, as compared to the fragment in the whole molecule.

Vidanovic D., Milic A. J., Stankovic M., and Poprzen V. (2003) Effects of nonionic surfactants on the physical stability of immunoglobulin G in aqueous solution during mechanical agitation. *Pharmazie* **58**, 399-404.

Abstract: The objective of this study was to evaluate the influence of nonionic surfactants in the presence of glycine and sodium chloride on the physical stability of immunoglobulin G (IgG) in aqueous solution. Among surfactants suitable for parenteral preparation, Polysorbate 80 (Tween 80) and Polyoxyl 35 Castor Oil (Cremophor EL) were selected. The physical stability of IgG in the absence and in the presence of excipients was investigated in aqueous solution during mechanical agitation (concentration of IgG 15%; pH 7.1; temperature 6 +/- 2 degrees C). Suitable concentrations of Tween 80 and Cremophor EL were experimentally determined by surface tension measurements at 6 +/- 2 degrees C. Glycine and sodium chloride were used in different concentrations. The influence of the excipients on the physical stability of IgG in solution has been examined by surface tension measurements, protein content assay (Kjeldahl and HPLC) and differential scanning calorimetry (DSC). Based on the results of the investigations, it was found that Tween 80 and Cremophor EL, used in experimentally determined critical micelle concentration (cmc), decreased the physical stability of IgG in solution. Tween 80 and Cremophor EL in the presence of glycine (1.5 g/l) could stabilize the IgG in solution during mechanical agitation. The comparison of the effects of Tween 80 and Cremophor EL on the physical stability of IgG, showed that Tween 80 had better stabilization effects on IgG in solution under the experimental conditions selected.

Vlasov A. P., Kravchuk Z. I., and Martsev S. P. (1996) [Non-native conformational states of immunoglobulins: thermodynamic and functional analysis of rabbit IgG]. *Biokhimiia* **61**, 212-235.

Abstract: By changing pH within the pH range 2-7, the processes of intramolecular rearrangement in the multidomain structure of rabbit IgG were induced which resulted in the formation of four IgG conformers - N, NA, I and NI, differing in the amount of structure in the CH2 domain and as well as in its interaction with the neighboring domains. The stability of the IgG conformers was studied by differential scanning calorimetry and expressed in terms of thermodynamic parameters, ΔH , T_m , and ΔG . The pH-dependent changes in thermal stability of IgG and the range of stability for the conformers were described by conformational (phase) diagrams. The native N-conformer exists at pH 7.0-5.5. At pH < 5.5 the formation of the NA conformer showing significant decooperation (lower extent of interaction) of the domains occurs. Decooperation permits the CH2 domain in the NA conformer to undergo conformational transitions independently of the rest of the molecule-the property which cannot be observed for the more cooperative N-conformer of IgG. The formation of the intermediate I-state occurs at pH < 3 as a consequence of unfolding of the tertiary structure of the CH2 domain, while its secondary structure and compact tertiary structure of the remainder of the IgG domains remain unchanged. Such partially structured and stable states of immunoglobulins have not yet been described. Reversibility of pH-induced transitions in IgG was studied using calorimetry and ligand-binding assays involving the C1q component of the complement, protein A, antigen and monospecific anti-CH2 domain antibodies as conformational probes reporting local conformational changes in the CH2 domain and its interdomain interfaces. Refolding of IgG from the I-

state by increasing pH results in the formation of a functionally active N1 conformer differing from native IgG by a changed balance of interdomain interactions. In addition to previously reported data suggesting a greater extent of the CH2 and CH1 domain interactions in the NI conformer, this conformer was shown to possess a lower extent of interaction of the CH2 and CH3 domains. The changed stability and a balance of domain interactions in the NI conformer results in changes in some of the effector functions including enhanced two-site binding to protein A and antigen-dependent binding to C1q with a concomitant decrease in the affinity of protein A binding site and reduced antigen binding capacity of the high affinity IgG. Low affinity IgG does not change the antigen-binding parameters after refolding to the N1 conformer.

Wakankar A. A., Borchardt R. T., Eigenbrot C., Shia S., Wang Y. J., Shire S. J. and Liu J. L. (2007) Aspartate isomerization in the complementarity-determining regions of two closely related monoclonal antibodies. *Biochemistry* **46**, 1534-1544.

Abstract: The aspartic acid residues (Asp) present in the complementarity-determining regions (CDRs) of the light chains of two recombinant monoclonal antibodies (MAbs), MAb I and MAb II, are highly susceptible to isomerization due to the presence of glycine residues (Gly) on their C-terminal ends. Asp isomerization in these MAbs leads to formation of the isoaspartate (IsoAsp) and the cyclic imide (Asu) variants of these MAbs. Both MAb I and MAb II, employed in this study, elicit their pharmacological responses through binding human IgE. The formation of the MAb variants as a result of Asp isomerization significantly reduces the binding affinities of these antibodies to IgE, thereby reducing their potencies. Here we report on significant differences in the susceptibility of the MAb I and the MAb II to Asp isomerization. The molecular basis for these differences in rates of Asp isomerization was elucidated. The effect of primary sequence on Asp isomerization was evaluated using pentapeptide models of the MAbs, which included the labile Asp residues and their neighboring amino acid residues. The separation of the parent MAbs and pentapeptides from their isomerization products was achieved using hydrophobic interaction chromatography (HIC) and rp-HPLC, respectively. Structural characterization of the MAbs was performed using differential scanning calorimetry (DSC), circular dichroism (CD), and X-ray crystallography. Our investigations demonstrate that the differences in the Asp isomerization rates between MAb I and MAb II can be attributed to structural factors including the conformational flexibility and the extent of solvent exposure of the labile Asp residue.

Wakankar A. A., Liu J., Vandervelde D., Wang Y. J., Shire S. J. and Borchardt R. T. (2007) The effect of cosolutes on the isomerization of aspartic acid residues and conformational stability in a monoclonal antibody. *J Pharm. Sci* **96**, 1708-1718.

Abstract: The aspartate residue (Asp 32) located in the complementarity-determining region (CDR) of a recombinant humanized monoclonal antibody (MAb I) is highly susceptible to the isomerization reaction. The modification of Asp 32 residue due to the isomerization reaction results in a significant reduction in the binding affinity of MAb I to IgE. The binding of a MAb I therapeutic to IgE is important for its desired pharmacological effect. In earlier investigations, we demonstrated that the conformational flexibility and residue exposure are factors that are responsible for the observed reactivity of Asp 32 in MAb I. This report explores the role of cosolutes such as glycerol and sucrose in the modulation of Asp 32 reactivity in MAb I. These cosolutes are routinely incorporated in injectable pharmaceutical formulations. The reactivity of the Asp residue in MAb I in these different cosolute-based formulations was compared to its reactivity in a peptide model VDYDG comprising residues 29-33 of MAb I. The formulations of MAb I and VDYDG containing varying concentrations of glycerol and sucrose were incubated at 50 degrees C for a period of 5-7 days. The isomerization of the Asp residue in VDYDG and MAb I was monitored using rp-HPLC and hydrophobic interaction chromatography (HIC), respectively. Structural analysis of MAb I using differential scanning calorimetry (DSC) demonstrated that the structural stability of MAb I was increased in formulations containing glycerol and sucrose. However, the stability of Asp 32 in MAb I was significantly decreased in these formulations. This research suggests that a formulation approach that relies purely on enhancing the structural stability of proteins through addition of these cosolutes could result in problems associated with the chemical stability of these biomolecules.

Weber-Bornhauser S., Eggenberger J., Jelesarov I., Bernard A., Berger C., and Bosshard H. R. (1998) Thermodynamics and kinetics of the reaction of a single-chain antibody fragment (scFv) with the leucine zipper domain of transcription factor GCN4. *Biochemistry* **37**, 13011-13020.

Abstract: Single-chain Fv (scFv) fragments of antibodies have become important analytical and

therapeutic tools in biology and medicine. The reaction of scFv fragments has not been well-characterized with respect to the energetics and kinetics of antigen binding. This paper describes the thermodynamic and kinetic behavior of the high-affinity scFv fragment SW1 directed against the dimeric leucine zipper domain of the yeast transcription factor GCN4. The scFv fragment was selected by the phage display technique from the immune repertoire of a mouse that had been immunized with the leucine zipper domain of GCN4. The scFv fragment was produced in high yield in *Escherichia coli* inclusion bodies and refolded from the denatured state. Differential scanning calorimetry showed that SW1 was stable up to about 50 degreesC, but the subsequent thermal denaturation was irreversible (T_m approximately 68 degreesC). The scFv fragment specifically recognized the dimeric leucine zipper conformation. Two scFv fragments bound to the GCN4 dimer to form the complex (scFv)₂-GCN4. Because of its repetitive structure, the rod-shaped GCN4 leucine zipper may present two similar epitopes for the scFv fragment. Surprisingly, the binding reaction was highly cooperative, that is, the species (scFv)₂-GCN4 dominated over scFv-GCN4 even in the presence of a large excess of the antigen GCN4. It is speculated that cooperativity resulted from direct interaction between the two GCN4-bound scFv fragments. At 25 degreesC, the average binding enthalpy for a scFv fragment was favorable (-61 kJ mol⁻¹), the entropy change was unfavorable, and the change in heat capacity was -1.27 +/- 0.14 kJ mol⁻¹ K⁻¹. As a result of enthalpy-entropy compensation, the free binding energy was virtually independent of temperature in the physiological temperature range. Antigen binding in solution could be described by a single-exponential reaction with an apparent rate constant of 1×10^6 M⁻¹ s⁻¹. Binding followed in a biosensor with the dimeric GCN4 coupled to the surface of the metal oxide sensor chip was 20 times slower.

Welfle K., Misselwitz R., Hausdorf G., Hohne W., and Welfle H. (1999) 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* **1431**, 120-131.

Abstract: Conformation, acid-induced conformational changes and stability of the murine monoclonal antibody CB4-1 directed against the human immunodeficiency virus type 1 capsid protein p24, and its Fab and Fc fragments, were analysed by circular dichroism (CD), fluorescence, and differential scanning calorimetry (DSC) measurements. CD spectra show the characteristics expected for beta-proteins. Lowering the pH to 3.5 reduces the stability, but does not change the conformation. Between pH 3.5 and 2.0 conformational changes and the formation of new structures are indicated. Deconvolution of the bimodal DSC curves of CB4-1 reveals five 'two-state' transitions at pH 7.5. At pH 5 and below, only four transitions are found. Half transition temperatures T_m and molar enthalpy changes ΔH_m gradually decrease at pH 4 and 3.4. At pH 2.1, two low-temperature ($T_m=36.9$ and 44.1 degrees C) and two high-temperature ($T_m=74.6$ and 76.8 degrees C) transitions are identified. The Fab and Fc fragments behave similarly. Deconvolution of their monophasic DSC curves yields two 'two-state' transitions for each fragment. T_m and ΔH_m values gradually decrease at pH 4.0 and 3.4; and at pH 2.1 and 2.8 for Fab and Fc, respectively, one of the transitions is found at high temperature ($T_m=67.2$ and 75.9 degrees C for Fab and Fc, respectively).

Zhu L., van de Lavoie M.C., Albanese J., Beenhouwer D.O., Cardarelli P.M., Cuisson S., Deng D.F., Deshpande S., Diamond J.H., Green L., Halk E.L., Heyer B.S., Kay R.M., Kerchner A., Leighton P.A., Mather C.M., Morrison S.L., Nikolov Z.L., Passmore D.B., Pradas-Monne A., Preston B.T., Rangan V.S., Shi M., Srinivasan M., White S.G., Winters-Digiaccinto P., Wong S., Zhou W., and Etches R.J. (2005) Production of human monoclonal antibody in eggs of chimeric chickens. *Nat Biotechnol.* **23**,1159-69.

Abstract: The tubular gland of the chicken oviduct is an attractive system for protein expression as large quantities of proteins are deposited in the egg, the production of eggs is easily scalable and good manufacturing practices for therapeutics from eggs have been established. Here we examined the ability of upstream and downstream DNA sequences of ovalbumin, a protein produced exclusively in very high quantities in chicken egg white, to drive tissue-specific expression of human mAb in chicken eggs. To accommodate these large regulatory regions, we established and transfected lines of chicken embryonic stem (cES) cells and formed chimeras that express mAb from cES cell-derived tubular gland cells. Eggs from high-grade chimeras contained up to 3 mg of mAb that possesses enhanced antibody-dependent cellular cytotoxicity (ADCC), nonantigenic glycosylation, acceptable half-life, excellent antigen recognition and good rates of internalization.