

ITC IX - Antibody Studies

Anand N. N., Mandal S., MacKenzie C. R., Sadowska J., Sigurskjold B., Young N. M., Bundle D. R., and Narang S. A. (1991) Bacterial expression and secretion of various single-chain Fv genes encoding proteins specific for a Salmonella serotype B O-antigen. *J Biol Chem* **266**, 21874-21879.

Abstract: Active single-chain Fv molecules encoded by synthetic genes have been expressed and secreted to the periplasm of *Escherichia coli* using the *ompA* secretory signal. Four different constructs were developed to investigate the effects of peptide linker design and VL-VH orientation on expression, secretion, and binding to a Salmonella O-polysaccharide antigen. Peptide linker sequences derived from the elbow regions of the Fab molecule were used alone or in combination with the flexible (GGGS)₂ sequence. VL and VH domain order in the single chain molecules had a profound effect on the level of secretion but hardly influenced total expression levels, which were approximately 50 mg/liter, chiefly in the form of inclusion bodies. With VL in the NH₂-terminal position, the amount of secreted product obtained was 2.4 mg/liter, but when VH occupied this position the yield was less than 5% of this value. Enzyme immunoassays of the four products showed domain order and linker sequence affected antigen binding by less than an order of magnitude. Attempts to express active Fv from dicistronic DNA were unsuccessful, but active Fv was obtained from single-chain Fv by enzymic cleavage at a site in the elbow linker peptide. The thermodynamic binding parameters of intact and cleaved single-chain Fvs determined by titration microcalorimetry were similar to those of bacterially produced Fab and mouse IgG.

Arouri A., Garidel P., Kliche W. and Blume A. (2007) Hydrophobic interactions are the driving force for the binding of peptide mimotopes and Staphylococcal protein A to recombinant human IgG1. *Eur Biophys J* **36**, 647-660.

Abstract: We studied the interaction of several nona-peptide mimotopes of different sequence and Staphylococcal protein A (SpA) with a recombinant human IgG1 antibody using isothermal titration calorimetry (ITC). The amino acid primary structure of the peptides was varied in order to identify the specific antibody-peptide binding sites. Additionally, the influence of temperature and salt concentration was investigated. An attempt was made to elucidate the structural changes upon complex formation using the determined thermodynamic parameters. The amino acid composition of the mimotopes determined their binding affinity. The binding constant K (a) of the mimotopes was in the range 1×10^4 to 1×10^6 M⁻¹. The binding constant of SpA was on the average about three orders of magnitude higher than that of the peptides. The binding constant of the peptides and of SpA decreased with temperature and the binding process was connected with negative changes in enthalpy, entropy, and heat capacity. The binding of the mimotopes to the Fab part of the IgG1 antibody and binding of SpA to the Fc part of the IgG1 antibody were mainly driven by hydrophobic effects and associated with a relatively large change in water-accessible surface area. Determinants for a strong/reduced antibody-peptide binding were identified.

Battistel E., Lazzarini G., Manca F., and Rialdi G. (1985) Physico-chemical and thermodynamic properties of monomeric concanavalin A. *Eur Biophys J* **13**, 1-9.

Abstract: An alkylated monomer of Concanavalin A was prepared photochemically according to the method of Tanaka et al. (1981). Its affinities for methyl- α -D-gluco, methyl- α -D-manno and p-nitrophenyl- α -D-manno pyranoside were calculated. The enthalpies of these binding reactions were measured calorimetrically and the thermodynamic parameters were calculated. The values obtained suggest that the structure of the monomer differs from that of the dimeric and tetrameric molecules. Calorimetric studies also showed that the monomeric derivative reacts with IgM but not IgG. The enthalpy per binding site in the monomer-IgM reaction is equal to that of the monomer-mannose derivative reaction; mannose is the terminal residue of the saccharide chains of the IgM molecule. The stoichiometry of the reaction is ten ConA-m per IgM molecule.

Bhat T. N., Bentley G. A., Boulot G., Greene M. I., Tello D., Dall'Acqua W., Souchon H., Schwarz F. P., Mariuzza R. A., and Poljak R. J. (1994) Bound water molecules and conformational stabilization help mediate an antigen-antibody association. *Proc Natl Acad Sci U S A* **91**, 1089-1093.

Abstract: We report the three-dimensional structures, at 1.8-Å resolution, of the Fv fragment of the anti-hen egg white lysozyme antibody D1.3 in its free and antigen-bound forms. These structures reveal a role for solvent molecules in stabilizing the complex and provide a molecular basis for understanding the

thermodynamic forces which drive the association reaction. Four water molecules are buried and others form a hydrogen-bonded network around the interface, bridging antigen and antibody. Comparison of the structures of free and bound Fv fragment of D1.3 reveals that several of the ordered water molecules in the free antibody combining site are retained and that additional water molecules link antigen and antibody upon complex formation. This solvation of the complex should weaken the hydrophobic effect, and the resulting large number of solvent-mediated hydrogen bonds, in conjunction with direct protein-protein interactions, should generate a significant enthalpic component. Furthermore, a stabilization of the relative mobilities of the antibody heavy- and light-chain variable domains and of that of the third complementarity-determining loop of the heavy chain seen in the complex should generate a negative entropic contribution opposing the enthalpic and the hydrophobic (solvent entropy) effects. This structural analysis is consistent with measurements of enthalpy and entropy changes by titration calorimetry, which show that enthalpy drives the antigen-antibody reaction. Thus, the main forces stabilizing the complex arise from antigen-antibody hydrogen bonding, van der Waals interactions, enthalpy of hydration, and conformational stabilization rather than solvent entropy (hydrophobic) effects.

Braden B. C. and Poljak R. J. (1995) Structural features of the reactions between antibodies and protein antigens. *FASEB J* **9**, 9-16.

Abstract: Antibodies bind protein antigens over large sterically and electrostatically complementary surfaces. Van der Waals forces, hydrogen bonds, and occasionally ion pairs provide stability to antibody-antigen complexes. In addition, water molecules contribute hydrogen bonds linking antigen and antibody, and increase the complementarity of antigen-antibody interfaces. In qualification to a strict 'lock and key' mechanism, evidence of conformational changes between free and complexed antibodies indicate some accommodation to the antigen. Antibody-protein antigen reactions are enthalpically driven with varying degrees of entropic compensation, often dependent on the magnitude of the enthalpy of the reaction. In the case of two antibody-combining sites studied by X-ray diffraction, the relative arrangements of the variable domains of the light and heavy chains of the antibody change slightly from the free to the antigen-bound state. Furthermore, the contacting residues of both antibodies exhibit similar reduced mobilities when complexed to antigen, suggesting that differences in 'solvent entropy' rather than in conformational freedom may be the source of different entropic compensation factors. In concert, data from structural studies, reaction rates, calorimetric measurements, molecular dynamics simulations, and site-directed mutagenesis are beginning to detail the nature of antibody-protein antigen interactions.

Braden B. C., Goldman E. R., Mariuzza R. A., and Poljak R. J. (1998) Anatomy of an antibody molecule: structure, kinetics, thermodynamics and mutational studies of the antilysozyme antibody D1.3. *Immunol Rev* **163**, 45-57.

Abstract: Using site-directed mutagenesis, x-ray crystallography, microcalorimetric, equilibrium sedimentation and surface plasmon resonance detection techniques, we have examined the structure of an antibody-antigen complex and the structural and thermodynamic consequences of removing specific hydrogen bonds and van der Waals interactions in the antibody-antigen interface. These observations show that the complex is considerably tolerant, both structurally and thermodynamically, to the truncation of antibody and antigen side chains that form contacts. Alterations in interface solvent structure for two of the mutant complexes appear to compensate for the unfavorable enthalpy changes when antibody-antigen interactions are removed. These changes in solvent structure, along with the increased mobility of side chains near the mutation site, probably contribute to the observed entropy compensation. In concert, data from structural studies, reaction rates, calorimetric measurements and site directed mutations are beginning to detail the nature of antibody-protein antigen interactions.

Brockhaus M., Ganz P., Huber W., Bohrmann B., Loetscher H. R. and Seelig J. (2007) Thermodynamic studies on the interaction of antibodies with beta-amyloid peptide. *J Phys. Chem B* **111**, 1238-1243.

Abstract: Antibodies against beta-amyloid peptides (Abetas) are considered an important therapeutic opportunity in Alzheimer's disease. Despite the vast interest in Abeta no thermodynamic data on the interaction of antibodies with Abeta are available as yet. In the present study we use isothermal titration calorimetry (ITC) and surface plasmon resonance to provide a quantitative thermodynamic analysis of the interaction between soluble monomeric Abeta(1-40) and mouse monoclonal antibodies (mAb). Using four different antibodies directed against the N-terminal, middle, and C-terminal Abeta epitopes, we measured the thermodynamic parameters for the binding to Abeta. Each antibody species was found to have two

independent and equal binding sites for Abeta with binding constants in the range of $10(7)$ to $10(8)$ M⁻¹. The binding reaction was essentially enthalpy driven with a reaction enthalpy of $\Delta H(0)(\text{Abeta})$ approximately -19 to -8 kcal/mol, indicating the formation of tight complexes. The loss in conformational freedom was supported by negative values for the reaction entropy $\Delta S(0)(\text{Abeta})$. We also measured the heat capacity change of the 1mAb:2Abeta reaction. $\Delta C(0)(p, \text{abeta})$ was large and negative but could not be explained exclusively by the hydrophobic effect. The free energy of binding was found to be linearly correlated with the size of the epitope.

Brummell D. A., Sharma V. P., Anand N. N., Bilous D., Dubuc G., Michniewicz J., MacKenzie C. R., Sadowska J., Sigurskjold B. W., Sinnott B., and . (1993) Probing the combining site of an anti-carbohydrate antibody by saturation-mutagenesis: role of the heavy-chain CDR3 residues. *Biochemistry* **32**, 1180-1187.

Abstract: The carbohydrate-binding site in Fab fragments of an antibody specific for Salmonella serogroup B O-polysaccharide has been probed by site-directed mutagenesis using an Escherichia coli expression system. Of the six hypervariable loops, the CDR3 of the heavy chain was selected for exhaustive study because of its significant contribution to binding-site topography. A total of 90 mutants were produced and screened by an affinity electrophoresis/Western blotting method. Those of particular interest were further characterized by enzyme immunoassay, and on this basis seven of the mutant Fabs were selected for thermodynamic characterization by titration microcalorimetry. With regard to residues that hydrogen bond to ligand through backbone interactions, Gly102H could not be substituted, while several side chains could be introduced at Gly100H and Tyr103H with relatively little effect on antigen binding. There was, however, a preference for nonpolar side chains at position 103H. Substitution of His101H with carboxylate and amide side chains gave mutants with binding affinities approaching that of the wild type; complete side-chain removal by mutation to Gly was tolerated with a 10-fold reduction in binding constant. Analysis of binding by titration microcalorimetry revealed some dramatic thermodynamic changes hidden by the similarity of the binding constants. Similar effects were observed with residue changes in an Arg-Asp salt-bridge at the base of the loop. These results indicate that alterations to higher affinity anti-carbohydrate antibodies are characterized by an enthalpy-entropy compensation factor which allows for fundamental changes in the nature of the binding interactions but impedes engineering for increases in affinity.

Bundle D. R., Eichler E., Gidney M. A., Meldal M., Ragauskas A., Sigurskjold B. W., Sinnott B., Watson D. C., Yaguchi M., and Young N. M. (1994) Molecular recognition of a Salmonella trisaccharide epitope by monoclonal antibody Se155-4. *Biochemistry* **33**, 5172-5182.

Abstract: The binding site of monoclonal antibody Se155-4, which has been the object of successful crystallographic and antibody-engineering studies, is shown by solid-phase immunoassays to be complementary to a branched trisaccharide, $\alpha\text{-D-Galp}(1\rightarrow 2)[\alpha\text{-D-Abep}(1\rightarrow 3)]\text{-}\alpha\text{-D-Manp}(1, \text{ rather than to the tetrasaccharide repeating unit } \alpha\text{-D-Galp}(1\rightarrow 2)[\alpha\text{-D-Abep}(1\rightarrow 3)]\text{-}\alpha\text{-D-Manp}(1\rightarrow 4)\alpha\text{-L-Rhap}(1\text{- of the bacterial antigen. Specificity for the 3,6-dideoxy-D-xylo-hexose (3,6-dideoxy-D-galactose) epitope present in Salmonella paratyphi B O-antigens was ensured by screening hybridoma experiments with glycoconjugates derived from synthetic oligosaccharides. Detailed epitope mapping of the molecular recognition by modified and monodeoxy oligosaccharide derivatives showed that complementary surfaces and three antibody-saccharide hydrogen bonds are essential for full binding activity. Both hydroxyl groups of the 3,6-dideoxy-D-galactose residue were obligatory for binding and consistent with the directional nature of their involvement in carbohydrate-protein hydrogen bonds; related tetrasaccharides built from the isomeric 3,6-dideoxyhexoses, 3,6-dideoxy-D-glucose, paratose, and 3,6-dideoxy-D-mannose, tyvelose were not bound by the antibody. Titration microcalorimetry measurements were consistent with the hydrogen-bonding map inferred from the crystal structure and suggest that the displacement of water molecules from the binding site accounts for the favorable entropy that accompanies binding of the native trisaccharide determinant. The protein sequences determined for the antibody VL and VH domains reveal somatic mutation of the VL germ line gene, implying that this antibody-binding site results from a mature antibody response.$

Chrnyk B. A., Rosner M. H., Cong Y., McColl A. S., Otterness I. G., and Daumy G. O. (2000) Inhibiting protein-protein interactions: a model for antagonist design. *Biochemistry* **39**, 7092-7099.

Abstract: Protein-protein interactions (PPI) are a ubiquitous mode of transmitting signals in cells and tissues. We are testing a stepwise, generic, structure-driven approach for finding low molecular weight

inhibitors of protein-protein interactions. The approach requires development of a high-affinity, single chain antibody directed specifically against the interaction surface of one of the proteins to obtain structural information on the interface. To this end, we developed a single chain antibody (sc1E3) against hIL-1beta that exhibited the equivalent affinity of the soluble IL-1 receptor type I (sIL-1R) for hIL-1beta and competitively blocked the sIL-1R from binding to the cytokine. The antibody proved to be more specific for hIL-1beta than the sIL-1R in that it failed to bind to either murine IL-1beta or human/murine IL-1alpha proteins. Additionally, failure of sc1E3 to bind to several hIL-1beta mutant proteins, altered at receptor site B, indicated that the antibody interacted preferentially with this site. This, coupled with other surface plasmon resonance and isothermal titration calorimetry measurements, shows that sc1E3 can achieve comparable affinity of binding hIL-1beta as the receptor through interactions at a smaller interface. This stable single chain antibody based heterodimer has simplified the complexity of the IL-1/IL-1R PPI system and will facilitate the design of the low molecular weight inhibitors of this interaction.

Cohen G. H., Silverton E. W., Padlan E. A., Dyda F., Wibbenmeyer J. A., Willson R. C., and Davies D. R. (2005) Water molecules in the antibody-antigen interface of the structure of the Fab HyHEL-5-lysozyme complex at 1.7 Å resolution: comparison with results from isothermal titration calorimetry. *Acta Crystallogr D Biol Crystallogr* **61**, 628-633.

Abstract: The structure of the complex between hen egg-white lysozyme and the Fab HyHEL-5 at 2.7 Å resolution has previously been reported [Cohen et al. (1996), *Acta Cryst. D* **52**, 315-326]. With the availability of recombinant Fab, the X-ray structure of the complex has been re-evaluated at 1.7 Å resolution. The refined structure has yielded a detailed picture of the Fab-lysozyme interface, showing the high complementarity of the protein surfaces as well as several water molecules within the interface that complete the good fit. The model of the full complex has improved significantly, yielding an R(work) of 19.5%. With this model, the structural results can be compared with the results of isothermal titration calorimetry. An attempt has been made to estimate the changes in bound waters that accompany complex formation and the difficulties inherent in using the crystal structures to provide the information necessary to make this calculation are discussed.

Cunto-Amesty G., Dam T. K., Luo P., Monzavi-Karbassi B., Brewer C. F., Van Cott T. C., and Kieber-Emmons T. (2001) Directing the immune response to carbohydrate antigens. *J Biol Chem* **276**, 30490-30498.

Abstract: Peptide mimetics may substitute for carbohydrate antigens in vaccine design applications. At present, the structural and immunological aspects of antigenic mimicry, which translate into immunologic mimicry, as well as the functional correlates of each, are unknown. In contrast to screening peptide display libraries, we demonstrate the feasibility of a structure-assisted vaccine design approach to identify functional mimitopes. By using concanavalin A (ConA), as a recognition template, peptide mimetics reactive with ConA were identified. Designed peptides were observed to compete with synthetic carbohydrate probes for ConA binding, as demonstrated by enzyme-linked immunosorbent assay and isothermal titration calorimetry (ITC) analysis. ITC measurements indicate that a multivalent form of one particular mimetic binds to ConA with similar affinity as does trimannoside. Splenocytes from mimitope-immunized mice display a peptide-specific cellular response, confirming a T-cell-dependent nature for the mimetic. As ConA binds to the Envelope protein of the human immunodeficiency virus, type 1 (HIV-1), we observed that mimitope-induced serum also binds to HIV-1-infected cells, as assessed by flow cytometry, and could neutralize T-cell line adapted HIV-1 isolates in vitro, albeit at low titers. These studies emphasize that mimicry is based more upon functional rather than structural determinants that regulate mimitope-induced T-dependent antibody responses to polysaccharide and emphasize that rational approaches can be employed to develop further vaccine candidates.

D'Agostino B., Bellofiore P., De M. T., Punzo C., Rivieccio V. and Verdoliva A. (2008) Affinity purification of IgG monoclonal antibodies using the D-PAM synthetic ligand: chromatographic comparison with protein A and thermodynamic investigation of the D-PAM/IgG interaction. *J Immunol. Methods* **333**, 126-138.

Abstract: This study investigates the applicability of D-PAM, the inverso form of the Protein A Mimetic synthetic peptide affinity ligand (PAM) obtained from the screening of a multimeric combinatorial peptide library, in monoclonal IgG isolation from ascitic fluids and cellular supernatants. D-PAM affinity columns, prepared by immobilizing the all-D peptide on the commercially available support Emphaze, were able to

capture monoclonal antibodies in a single chromatographic step, with a recovery yield and purity degree above 90% and full recovery of antibody activity. D-PAM/Emphaze resin showed a host cell protein (HCP) and DNA reduction similar to protein A sorbent. Indeed, column capacity, determined by applying a large excess of purified antibodies to 1 mL of column bed volume, was always higher than 50 mg/mL. D-PAM/IgG interaction was characterized by isothermal titration calorimetry (ITC) and an analysis of binding isotherms, obtained for titration of ST2146, ST1485 and 7H3 IgG monoclonal antibodies, suggested that two peptides bind simultaneously to the IgG molecule, with a $K(A)$ (equilibrium association constant) of 3.4, 6.2 and 3.4 x 10(4) M(-1), and a ΔH (change in enthalpy) of -1.3, -4.2 and -4.1 kcal mol(-1), respectively

Dam T. K., Torres M., Brewer C. F. and Casadevall A. (2008) Isothermal Titration Calorimetry Reveals Differential Binding Thermodynamics of Variable Region-identical Antibodies Differing in Constant Region for a Univalent Ligand. *J Biol Chem* **283**, 31366-31370.

Abstract: The classical view of immunoglobulin molecules posits two functional domains defined by the variable (V) and constant (C) regions, which are responsible for antigen binding and antibody effector functions, respectively. These two domains are thought to function independently. However, several lines of evidence strongly suggest that C region domains can affect the specificity and affinity of an antibody for its antigen (Ag), independent of avidity-type effects. In this study, we used isothermal titration calorimetry to investigate the thermodynamic properties of the interactions of four V region-identical monoclonal antibodies with a univalent peptide antigen. Comparison of the binding of IgG1, IgG2a, IgG2b, and IgG3 with a 12-mer peptide mimetic of *Cryptococcus neoformans* polysaccharide revealed a stoichiometry of 1.9-2.0 with significant differences in thermodynamic binding parameters. Binding of this peptide to the antibodies was dominated by favorable entropy. The interaction of these antibodies with biotinylated peptides manifested greater enthalpy than for native peptides indicating that biotin labeling affected the types of Ag-Ab complexes formed. Our results provide unambiguous thermodynamic evidence for the notion that the C region can affect the interaction of the V region with an Ag

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 ($KD < 10^{-12}$ 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.

Deng S. J., MacKenzie C. R., Sadowska J., Michniewicz J., Young N. M., Bundle D. R., and Narang S. A. (1994) Selection of antibody single-chain variable fragments with improved carbohydrate binding by phage display. *J Biol Chem* **269**, 9533-9538.

Abstract: A single-chain variable fragment (Fv) version of a murine monoclonal antibody, Se155-4, specific for *Salmonella* serogroup B O-polysaccharide, was used as a model system for testing monovalent phage display as a route for enhancing the relatively low affinities that typify anti-carbohydrate antibodies. Random single-chain Fv mutant libraries generated by chemical and error-prone polymerase chain reaction

methods were panned against the serogroup B lipopolysaccharide. Panning of a randomly mutated heavy chain variable domain library indicated selection for improved serogroup B binders and yielded six mutants, five of which showed wild type activity by enzyme immunoassay. Two of these were apparently selected on the basis of better functional single-chain Fv yield in *Escherichia coli*. A heavy chain mutation (Ile77-->Thr) in one mutant, 3B1, appeared to have a particularly dramatic effect, resulting in yields of approximately 120 mg/liter of functional periplasmic product. The sixth mutant, 4B2, had complementarity determining region 1 (CDR1) and CDR2 mutations and demonstrated 10-fold improved binding, by enzyme immunoassay, relative to the wild type. Extensive analysis of antigen-antibody interactions indicated that the improved binding properties of 4B2 were attributable to a higher association rate constant and interaction with an epitope that is larger than the trisaccharide recognized by the wild type. None of the mutations involved known trisaccharide contact residues; this was consistent with analysis of wild type and mutant single-chain Fvs by titration microcalorimetry. Examination of the structure indicated that two mutations in the heavy chain CDR2 provided improved surface complementarity between the protein and the extended epitope encompassing 2 additional hexose residues. However, introduction of only the CDR2 mutations into the wild type structure failed to confer the improved binding properties of 4B2, indicating an indirect effect by the more distant mutations. Panning of randomly mutated light chain variable domain and full-length single-chain Fv mutant libraries did not yield mutants with improved assembly or binding properties.

Dey B., Pancera M., Svehla K., Shu Y., Xiang S. H., Vainshtein J., Li Y., Sodroski J., Kwong P. D., Mascola J. R. and Wyatt R. (2007) Characterization of human immunodeficiency virus type 1 monomeric and trimeric gp120 glycoproteins stabilized in the CD4-bound state: antigenicity, biophysics, and immunogenicity. *J Virol.* **81**, 5579-5593.

Abstract: The human immunodeficiency virus type 1 exterior gp120 envelope glycoprotein is highly flexible, and this flexibility may contribute to the inability of monomeric gp120 immunogens to elicit broadly neutralizing antibodies. We previously showed that an S375W modification of a critical interfacial cavity central to the primary receptor binding site, the Phe43 cavity, stabilizes gp120 into the CD4-bound state. However, the immunological effects of this cavity-altering replacement were never tested. Subsequently, we screened other mutations that, along with the S375W alteration, might further stabilize the CD4-bound state. Here, we define a selected second cavity-altering replacement, T257S, and analyze the double mutations in several gp120 envelope glycoprotein contexts. The gp120 glycoproteins with the T257S-plus-S375W double mutation (T257S+S375W) have a superior antigenic profile compared to the originally identified single S375W replacement in terms of enhanced recognition by the broadly neutralizing CD4 binding-site antibody b12. Isothermal titration calorimetry measuring the entropy of the gp120 interaction with CD4 indicated that the double mutant was also stabilized into the CD4-bound state, with increasing relative fixation between core, full-length monomeric, and full-length trimeric versions of gp120. A significant increase in gp120 affinity for CD4 was also observed for the cavity-filling mutants relative to wild-type gp120. The most conformationally constrained T257S+S375W trimeric gp120 proteins were selected for immunogenicity analysis in rabbits and displayed a trend of improvement relative to their wild-type counterparts in terms of eliciting neutralizing antibodies. Together, the results suggest that conformational stabilization may improve the ability of gp120 to elicit neutralizing antibodies.

Dieterle M., Blaschke T. and Hasse H. (2008) Microcalorimetric study of adsorption of human monoclonal antibodies on cation exchange chromatographic materials. *J Chromatogr. A* **1205**, 1-9.

Abstract: Adsorption of two human monoclonal antibodies on two different strong cation exchange resins is studied by isothermal titration microcalorimetry and independent adsorption isotherm measurements. The pH value is varied between 4.5 and 7.0, using different buffer systems, the temperature is always 25 degrees C. The adsorption isotherm data is fitted using two different Langmuir type models. Combining the calorimetric and the adsorption data, the specific enthalpy of adsorption of the protein $\Delta h(p)(ads)$ is determined. At pH values near 7.0, where the antibodies are only weakly charged, the adsorption is exothermal. At small loadings the absolute number of $\Delta h(p)(ads)$ is then large and almost constant but it significantly decreases at higher loadings. This shows that the arrangement of antibody molecules on the adsorbent material depends on the loading and is less favourable at higher loadings. Despite the high positive charge of the antibody at pH values of about 5 the value of $\Delta h(p)(ads)$ is almost zero along the entire isotherm. Furthermore, at pH 4.5 even endothermal effects are observed, although high binding capacities are found. At these conditions the adsorption process seems to be strongly influenced by the ions

bound to the antibody. Their release upon adsorption explains the endothermal caloric effect. The adsorption equilibrium constant $K_{(eq)}$ is calculated from the isotherms. From $\Delta G_{(p)}(ads)$ and the calorimetric results for $\Delta H_{(p)}(ads)$, $\Delta S_{(p)}(ads)$, the entropy change upon adsorption of the protein is found for the different studied conditions

Dolk E., van Vliet C., Perez J. M., Vriend G., Darbon H., Ferrat G., Cambillau C., Frenken L. G., and Verrips T. (2005) Induced refolding of a temperature denatured llama heavy-chain antibody fragment by its antigen. *Proteins* **59**, 555-564.

Abstract: In a previous study we have shown that llama VHH antibody fragments are able to bind their antigen after a heat shock of 90 degrees C, in contrast to the murine monoclonal antibodies. However, the molecular mechanism by which antibody:antigen interaction occurs under these extreme conditions remains unclear. To examine in more detail the structural and thermodynamic aspects of the binding mechanism, an extensive CD, ITC, and NMR study was initiated. In this study the interaction between the llama VHH -R2 fragment and its antigen, the dye Reactive Red-6 (RR6) has been explored. The data show clearly that most of the VHH-R2 population at 80 degrees C is in an unfolded conformation. In contrast, CD spectra representing the complex between VHH-R2 and the dye remained the same up to 80 degrees C. Interestingly, addition of the dye to the denatured VHH-R2 at 80 degrees C yielded the spectrum of the native complex. These results suggest an induced refolding of denatured VHH-R2 by its antigen under these extreme conditions. This induced refolding showed some similarities with the well established "induced fit" mechanism of antibody-antigen interactions at ambient temperature. However, the main difference with the "induced fit" mechanism is that at the start of the addition of the antigen most of the VHH molecules are in an unfolded conformation. The refolding capability under these extreme conditions and the stable complex formation make VHHs useful in a wide variety of applications.

Doyle M. L., Brigham-Burke M., Blackburn M. N., Brooks I. S., Smith T. M., Newman R., Reff M., Stafford W. F., III, Sweet R. W., Truneh A., Hensley P., and O'Shannessy D. J. (2000) Measurement of protein interaction bioenergetics: application to structural variants of anti-sCD4 antibody. *Methods Enzymol* **323**, 207-230.

Abstract: This chapter has described a bioenergetic analysis of the interaction of sCD4 with an IgG1 and two IgG4 derivatives of an anti-sCD4 MAbs. The MAbs have identical VH and VL domains but differ markedly in their CH and CL domains, raising the question of whether their antigen-binding chemistries are altered. We find the sCD4-binding kinetics and thermodynamics of the MAbs are indistinguishable, which indicates rigorously that the molecular details of the binding interactions are the same. We also showed the importance of using multiple biophysical methods to define the binding model before the bioenergetics can be appropriately interpreted. Analysis of the binding thermodynamics and kinetics suggests conformational changes that might be coupled to sCD4 binding by these MAbs are small or absent.

Duschl C., Sevin-Landais A. F., and Vogel H. (1996) Surface engineering: optimization of antigen presentation in self-assembled monolayers. *Biophys J* **70**, 1985-1995.

Abstract: The formation of self-assembled monolayers (SAMs) on gold surfaces containing an antigenic peptide (NANP)₆ and HS(CH₂)₁₁OH, and the specific binding of a monoclonal antibody to these layers were investigated by surface plasmon resonance (SPR). Peptides were synthesized by solid-state phase synthesis and were linked either to cysteine or to an alkyl-thiol to allow covalent attachment to gold. The content of the peptide in the SAMs was systematically varied, and the binding properties of the monoclonal antibody were compared with those measured by microcalorimetry in solution. At a critical peptide concentration in the SAM an optimal antibody binding and complete surface coverage was attained. At lower peptide concentrations, the amount of adsorbed antibody decreased; at higher peptide concentrations, the binding constant decreased. These effects can be explained if the accessibility of the antigenic epitopes depends on the peptide density. Addition of free antigen induced the desorption of bound antibodies and allowed accurate measurements of the dissociation rate constant. Binding constants obtained from steady-state measurements and from measurements of the kinetic rate constants were compared.

Evans, E.J., Castro, M.A.A., O'Brien, R., Kearney, A., Walsh, H., Sparks, L.M., Tucknott, M.G., Davies, E.A., Carmo, A.M., van der Merwe, P.A., Stuart, D.I., Jones, E.Y., Ladbury, J.E., Shinji Ikemizu, S., and

Simon J. Davis, S.J. (2006) Crystal Structure and Binding Properties of the CD2 and CD244 (2B4)-binding Protein, CD48. *J. Biol. Chem.* **281**, 29309-29320.

Abstract: The structural analysis of surface proteins belonging to the CD2 subset of the immunoglobulin superfamily has yielded important insights into transient cellular interactions. In mice and rats, CD2 and CD244 (2B4), which are expressed predominantly on T cells and natural killer cells, respectively, bind the same, broadly expressed ligand, CD48. Structures of CD2 and CD244 have been solved previously, and we now present the structure of the receptor-binding domain of rat CD48. The receptor-binding surface of CD48 is unusually flat, as in the case of rat CD2, and shares a high degree of electrostatic complementarity with the equivalent surface of CD2. The relatively simple arrangement of charged residues and this flat topology explain why CD48 cross-reacts with CD2 and CD244 and, in rats, with the CD244-related protein, 2B4R. Comparisons of modeled complexes of CD2 and CD48 with the complex of human CD2 and CD58 are suggestive of there being substantial plasticity in the topology of ligand binding by CD2. Thermodynamic analysis of the native CD48-CD2 interaction indicates that binding is driven by equivalent, weak enthalpic and entropic effects, in contrast to the human CD2-CD58 interaction, for which there is a large entropic barrier. Overall, the structural and biophysical comparisons of the CD2 homologues suggest that the evolutionary diversification of interacting cell surface proteins is rapid and constrained only by the requirement that binding remains weak and specific.

Evans S. V., Sigurskjold B. W., Jennings H. J., Brisson J. R., To R., Tse W. C., Altman E., Frosch M., Weisgerber C., Kratzin H. D., and . (1995) Evidence for the extended helical nature of polysaccharide epitopes. The 2.8 Å resolution structure and thermodynamics of ligand binding of an antigen binding fragment specific for alpha-(2-->8)-polysialic acid. *Biochemistry* **34**, 6737-6744.

Abstract: The antigen binding fragment from an IgG2a kappa murine monoclonal antibody with specificity for alpha-(2-->8)-linked sialic acid polymers has been prepared and crystallized in the absence of hapten. Crystals were grown by vapor diffusion equilibrium with 16-18% polyethylene glycol 4000 solutions. The structure was solved by molecular replacement methods and refined to a conventional R factor of 0.164 for data to 2.8 Å. The binding site is observed to display a shape and distribution of charges that is complementary to that of the predicted conformation of the oligosaccharide epitope. A thermodynamic description of ligand binding has been compiled for oligosaccharides ranging in length from 9 to 41 residues, and the data for the largest ligand has been used in a novel way to estimate the size of the antigen binding site. A model of antigen binding is presented that satisfies this thermodynamic data, as well as a previously reported requirement of conformational specificity of the oligosaccharide. X-ray crystallographic and thermodynamic evidence are consistent with a binding site that accommodates at least eight sialic acid residues.

Faiman G. A. and Horovitz A. (1997) Thermodynamic analysis of the interaction between the 0.5beta Fv fragment and the RP135 peptide antigen derived from the V3 loop of HIV-1 gp120. *J Biol Chem* **272**, 31407-31411.

Abstract: The Fv fragment of the 0.5beta monoclonal antibody has recently been constructed, expressed, and purified. It binds with nanomolar affinity to the immunogenic RP135 peptide that is derived from the principal neutralizing determinant of HIV-1 in the third hypervariable region of gp120. Here, we analyzed the temperature-dependence of binding of the 0.5beta Fv fragment to the RP135 peptide and a series of mutants thereof. Our results show that there is almost complete enthalpy-entropy compensation in the effects of mutations in the peptide on binding to the Fv, indicating that the mutations do not change the binding mechanism. There is good correlation, for residues within the antigenic epitope, between mutational effects on ΔC_p and calculated values of $\Delta\Delta C_p$ based on the extent of burial of polar and non-polar surface areas of amino acids. The value of ΔC_p for the binding of the 0.5beta Fv fragment to the wild-type RP135 peptide is found to be -5.0 (± 0.9) kcal K⁻¹ mol⁻¹ in the presence of 0.1% Tween-20 but only -0.1 (± 0.9) kcal K⁻¹ mol⁻¹ in its absence. This result has important implications for the successful application of the structural parameterization approach to predicting changes in heat capacity that accompany binding reactions carried out in the presence of detergent or protein-stabilizing agents.

Fields B. A., Goldbaum F. A., Dall'Acqua W., Malchiodi E. L., Cauerhff A., Schwarz F. P., Ysern X., Poljak R. J., and Mariuzza R. A. (1996) Hydrogen bonding and solvent structure in an antigen-antibody interface. Crystal structures and thermodynamic characterization of three Fv mutants complexed with

lysozyme. *Biochemistry* **35**, 15494-15503.

Abstract: Using site-directed mutagenesis, X-ray crystallography, and titration calorimetry, we have examined the structural and thermodynamic consequences of removing specific hydrogen bonds in an antigen-antibody interface. Crystal structures of three antibody FvD1.3 mutants, VLTyr50Ser (VLY50S), VHTyr32Ala (VHY32A), and VHTyr101Phe (VHY101F), bound to hen egg white lysozyme (HEL) have been determined at resolutions ranging from 1.85 to 2.10 Å. In the wild-type (WT) FvD1.3-HEL complex, the hydroxyl groups of VLTyr50, VHTyr32, and VHTyr101 each form at least one hydrogen bond with the lysozyme antigen. Thermodynamic parameters for antibody-antigen association have been measured using isothermal titration calorimetry, giving equilibrium binding constants K_b (M^{-1}) of 2.6×10^7 (VLY50S), 7.0×10^7 (VHY32A), and 4.0×10^6 (VHY101F). For the WT complex, K_b is $2.7 \times 10^8 M^{-1}$; thus, the affinities of the mutant Fv fragments for HEL are 10-, 4-, and 70-fold lower than that of the original antibody, respectively. In all three cases entropy compensation results in an affinity loss that would otherwise be larger. Comparison of the three mutant crystal structures with the WT structure demonstrates that the removal of direct antigen-antibody hydrogen bonds results in minimal shifts in the positions of the remaining protein atoms. These observations show that this complex is considerably tolerant, both structurally and thermodynamically, to the truncation of antibody side chains that form hydrogen bonds with the antigen. Alterations in interface solvent structure for two of the mutant complexes (VLY50S and VHY32A) appear to compensate for the unfavorable enthalpy changes when protein-protein interactions are removed. These changes in solvent structure, along with the increased mobility of side chains near the mutation site, probably contribute to the observed entropy compensation. For the VHY101F complex, the nature of the large entropy compensation is not evident from a structural comparison of the WT and mutant complexes. Differences in the local structure and dynamics of the uncomplexed Fv molecules may account for the entropic discrepancy in this case.

Fellouse F. A., Barthelemy P. A., Kelley R. F., and Sidhu S. S. (2006) Tyrosine plays a dominant functional role in the paratope of a synthetic antibody derived from a four amino acid code. *J Mol Biol* **357**, 100-114.

Abstract: The antigen-binding fragment Fab-YADS2 recognizes vascular endothelial growth factor (VEGF) and was derived from a library with chemical diversity restricted to only four amino acids (Tyr, Ser, Ala and Asp). The structure of the Fab:antigen complex revealed that the structural paratope is dominated by Tyr side-chains. Isothermal titration calorimetry and cell-based assays show that restricted chemical diversity does not limit the affinity or specificity of Fab-YADS2, which behaves in a manner comparable to natural antibodies. Mutagenesis experiments reveal that the functional paratope is dominated by Tyr, which represents 11 of the 15 functionally important residues. However, mutagenesis experiments also indicate that substitution of any of these tyrosine residues by Phe does not significantly affect binding to VEGF. Furthermore, saturation mutagenesis shows that replacement of three functionally important tyrosine residues by combinations of other hydrophobic residues is not only tolerated, but can actually improve affinity. The results support a model for naive antigen recognition in which large Tyr side-chains establish binding contacts with antigen, and small Ser and Ala side-chains serve as auxiliaries that help to position Tyr in favorable binding conformations. While Tyr may not be optimal for any particular antigen contact, it is nonetheless capable of mediating favorable interactions with a diverse array of surfaces. Furthermore, the side-chain hydroxyl group makes Tyr significantly more hydrophilic than Phe and other hydrophobic amino acids. Increased hydrophilicity may reduce non-specific binding in the unbound state, and this may be critical for a naive repertoire that is exposed to a diverse range of potential antigenic surfaces. The results show that the chemical nature of Tyr endows the amino acid with a privileged role in antigen recognition, and this likely explains the high abundance of Tyr in natural antigen-binding sites.

Furukawa K., Shimizu T., Murakami A., Kono R., Nakagawa M., Sagawa T., Yamato I. and Azuma T. (2007) Strategy for affinity maturation of an antibody with high evolvability to (4-hydroxy-3-nitrophenyl) acetyl hapten. *Mol Immunol.* **44**, 2436-2445.

Abstract: In order to quantitate the contribution of amino acid replacements to an increase in affinity during affinity maturation, we measured thermodynamic parameters of the antigen-antibody interaction for a group of anti-(4-hydroxy-3-nitrophenyl) acetyl monoclonal antibodies whose differences in amino acid sequences had arisen only from somatic hypermutation. We prepared a common ancestor and hypothetical intermediate clones that might occur on the affinity maturation pathway, by employing site-directed mutagenesis. Isothermal calorimetric titration of the antigen-antibody reaction revealed that antibody

evolution proceeds in two steps. The first step is driven by a decrease in enthalpy, in which two amino acid replacements in the VL region play an essential role. Further accumulation of amino acid replacements in VH and VL regions during the second step induce a progressive increase in affinity, which is driven by an increase in entropy, which has a cooperative mutational effect.

Ghirlando R., Keown M. B., Mackay G. A., Lewis M. S., Unkeless J. C., and Gould H. J. (1995) Stoichiometry and thermodynamics of the interaction between the Fc fragment of human IgG1 and its low-affinity receptor Fc gamma RIII. *Biochemistry* **34**, 13320-13327.

Abstract: IgG-Fc receptors, cell surface glycoproteins binding the Fc region of antibodies, play a crucial role in the immune system. To better understand the nature of the recognition process, we have examined the interaction between huIgG1-Fc and a soluble fragment of huFc gamma RIII (sCD16). Analytical ultracentrifugation experiments clearly demonstrate that IgG1-Fc and sCD16 interact weakly to form a 1:1 complex with an association constant of $1.7 \times 10^5 \text{ M}^{-1}$ in PBS at 22.0 degrees C. The thermodynamic parameters, obtained from the temperature dependence of the equilibrium binding constants, exhibit an enthalpy-entropy compensation with a favorable enthalpy at physiological temperatures. The value of $-360 \text{ cal mol}^{-1} \text{ K}^{-1}$ for ΔC_p zero possibly identifies the process as one in which local folding/rearrangement is coupled to complex formation. The 1:1 stoichiometry and thermodynamic parameters provide a basis for understanding the nature of the Fc gamma R-IgG interactions.

Ghose S., Allen M., Hubbard B., Brooks C., and Cramer S.M. (2005) Antibody variable region interactions with Protein A: implications for the development of generic purification processes. *Biotechnol Bioeng.* **92**, 665-73.

Abstract: In this paper, a wide range of antibodies from various subclasses and subfamilies are employed to evaluate the creation of generic separation processes using Protein A chromatography. The reasons for elution pH differences amongst several IgG1s, IgG2s, antibody fragments, and Fc-fusion proteins during Protein A chromatography are investigated using several complimentary techniques. The results indicate that variable region interactions play a major role in determining elution pH for VH3 subfamily antibodies while using traditional protein A chromatographic materials. On the other hand, experiments with a resin which employs a ligand consisting solely of B domain of Protein A indicate that variable region interactions can be mitigated, enabling the use of a single elution pH for a range of antibodies. Finally, the moderation of elution conditions associated with this engineered ligand are shown to minimize problems associated with low pH induced aggregation. It is expected that the findings reported in this paper will facilitate faster process development cycle times for this important class of human therapeutics. (c) 2005 Wiley Periodicals, Inc.

Goldbaum F. A., Schwarz F. P., Eisenstein E., Cauerhff A., Mariuzza R. A., and Poljak R. J. (1996) The effect of water activity on the association constant and the enthalpy of reaction between lysozyme and the specific antibodies D1.3 and D44.1. *J Mol Recognit* **9**, 6-12.

Abstract: The reactions of lysozyme with the specific monoclonal antibody D1.3, its Fv fragment and a mutant of the Fv, were studied under conditions of reduced water activity through the addition of the cosolutes glycerol, ethanol, dioxane and methanol. Titration calorimetry, BIAcoreTM and ultracentrifugal analyses were used to determine enthalpy of reactions and affinity constants. There was a decrease in the values of the enthalpies of reactions as well as in the association constants which was proportional to the decrease in water activity. These results are consistent with a structural model in which water molecules bound to the antigen and the antibody are conserved upon complex formation and provide bonds which are important for the stability of the complex. In contrast, the reaction of lysozyme with the specific monoclonal antibody D44.1, or its Fab, showed the inverse effect: a small increase in the value of the association constant with decreasing water molarities. This is in agreement with a model in which binding of antigen to antibody D44.1 is accompanied by the release of a very small number of water molecules.

Hahn M., Winkler D., Welfle K., Misselwitz R., Welfle H., Wessner H., Zahn G., Scholz C., Seifert M., Harkins R., Schneider-Mergener J., and Hohne W. (2001) Cross-reactive binding of cyclic peptides to an anti-TGFalpha antibody Fab fragment: an X-ray structural and thermodynamic analysis. *J Mol Biol* **314**, 293-309.

Abstract: The monoclonal antibody tAb2 binds the N-terminal sequence of transforming growth factor alpha, VVSHFND. With the help of combinatorial peptide libraries it is possible to find homologous

peptides that bind tAb2 with an affinity similar to that of the epitope. The conformational flexibility of short peptides can be constrained by cyclization in order to improve their affinity to the antibody and their stability towards proteolysis. Two cyclic peptides which are cross-reactive binders for tAb2 were selected earlier using combinatorial peptide libraries. One is cyclized by an amide bond between the N-alpha group and the side-chain of the last residue (cyclo-SHFNEYE), and the other by a disulfide bridge (cyclo-CSHFNDYC). The complex structures of tAb2 with the linear epitope peptide VVSHFND and with cyclo-SHFNEYE were determined by X-ray diffraction. Both peptides show a similar conformation and binding pattern in the complex. The linear peptide SHFNEYE does not bind tAb2, but cyclo-SHFNEYE is stabilized in a loop conformation suitable for binding. Hence the cyclization counteracts the exchange of aspartate in the epitope sequence to glutamate. Isothermal titration calorimetry was used to characterize the binding energetics of tAb2 with the two cyclic peptides and the epitope peptide. The binding reactions are enthalpically driven with an unfavorable entropic contribution under all measured conditions. The association reactions are characterized by negative ΔC_p changes and by the uptake of one proton per binding site. A putative candidate for proton uptake during binding is the histidine residue in each of the peptides. Hydrogen bonds and the putative formation of an electrostatic pair between the protonated histidine and a carboxy group may contribute markedly to the favorable enthalpy of complex formation. Implications to cyclization of peptides for stabilization are discussed.

Halsey J. F., Cebra J. J., and Biltonen R. L. (1975) Structural and thermodynamic basis of affinity in anti-dinitrophenyl antibody. *Biochemistry* **14**, 5221-5224.

Abstract: The thermodynamic quantities of the anti-dinitrophenyl antibody-hapten interaction are reported for rabbit, goat, and guinea pig antibodies. Rabbit and goat antibodies had similar exothermic enthalpy changes for their reaction with 2,4-dinitrophenyl-L-lysine (-13.9 and -14.8 kcal/mol, respectively). The enthalpy change with guinea pig antibody was much less exothermic (-8.7 kcal/mol), and this value was the same for two guinea pig antibody preparations that differed in affinity by almost two orders of magnitude. A heterogeneous goat anti-dinitrophenyl antibody preparation was fractionated on a molecular sieve column in the presence of a bivalent ligand, a procedure that has been reported to separate antibodies according to differences in the depth of interaction with the ligand. The relationship of these differences in apparent site depth to changes in interactions with the hapten tail was examined by comparing the affinities of various fractions for two haptens. The results show that the presumed deeper sites have stronger interactions with the hapten tail. These studies suggest that the heterogeneity of anti-dinitrophenyl antibodies with respect to affinity results from differences in entropy driven lysyl side-chain interactions which arise from a heterogeneity in antigen binding site depth.

Halsey J. F. and Biltonen R. L. (1975) The thermodynamics of hapten and antigen binding by rabbit anti-dinitrophenyl antibody. *Biochemistry* **14**, 800-804.

Abstract: Rabbit anti-dinitrophenyl antibody from a serum pool was obtained as five fractions of purified specific antibody by a limiting antigen precipitation method. Each fraction had a different binding affinity for epsilon-N-2,4-dinitrophenyl-L-lysine. The free energy changes for hapten binding to the five antibody fractions varied from -8.35 to -10.0 kcal/mol. An average ΔH of -13.9 kcal/mol was measured for the fractions with a batch calorimeter. The results indicate no significant correlation between enthalpy changes and free energy changes. However, a statistically significant correlation between the free energy changes and the entropy changes was found. The enthalpy of the anti-dinitrophenyl antibody interaction with multivalent dinitrophenyl human serum albumin was determined. These are the first enthalpy measurements of an antibody antigen reaction in which the intrinsic binding enthalpy between the antibody and the determinant group is known. The ΔH for the antigen binding reaction was -10.1 kcal/mol which is 3.8 kcal/mol less exothermic than the ΔH for the hapten binding reaction. The interactions that could lead to such a difference in enthalpy are discussed.

Harris S. L. and Fernsten P. (2008) Thermodynamics and Binding Density of Binding of a Panel of Anti-Polysaccharide Antibodies to High Molecular Weight Capsular Polysaccharides. *Clin. Vaccine Immunol* (epublication).

Abstract: The interaction between anti-polysaccharide (PS) antibodies and their antigens was investigated via isothermal titration calorimetry (ITC) to determine the thermodynamic binding constant (K), the enthalpy of binding (ΔH) and the binding density (N) to high molecular weight PS. From these values, the entropy of binding (ΔS) was calculated. The thermodynamic parameters of binding to high

molecular weight capsular PS are reported for two monoclonal antibodies (mAbs) with different specificities for meningococcal Serogroup C PS, five mAbs specific for different pneumococcal serotypes, and Fab fragments of two anti-pneumococcal mAbs. The K values were in the $10(6)$ to $10(7)$ $M(-1)$ range, which are one to two orders of magnitude greater than previously reported K values derived from antibody-oligosaccharide interactions. The enthalpy change, ΔH , associated with binding was favorable for each mAb and Fab fragment. The entropy change, ΔS , associated with binding was also generally favorable for both the mAbs and Fab fragments, with the exception of the anti-serotype 14 mAb and its Fab fragment. N provides information regarding how densely mAbs or Fabs can bind along PS chains and, expressed in terms of monosaccharides, was very similar for the seven mAbs, with an average of twelve monosaccharides per bound mAb. The N for each Fab was smaller, with five or seven monosaccharides per bound Fab. These results suggest that steric interactions between antibody molecules are a major influence on N or binding density of high affinity mAbs to capsular PS

Harrison S. L., Housden N. G., Bottomley S. P., Cossins A. J. and Gore M. G. (2008) Generation and expression of a minimal hybrid Ig-receptor formed between single domains from proteins L and G. *Protein Expr Purif* **58**, 12-22.

Abstract: The Ig-binding properties of protein L from *Peptostreptococcus magnus* and protein G from *Streptococcus* have been successfully combined through the construction of a novel hybrid protein, consisting of a single Ig-binding domain from each protein. The biophysical and biochemical properties of this construct have been characterized through equilibrium and pre-equilibrium fluorescence spectroscopy, circular dichroism, isothermal titration calorimetry, affinity chromatography, and conformational stability studies using a chemical denaturant in order to examine the structure and availability of ligand binding sites in each domain. These studies show that despite the small size of the protein ($M_w=16.5$ kDa) each domain behaves in an independent manner with respect to the binding characteristics of the same domain in isolation

Helg A., Mueller M. S., Joss A., Poltl-Frank F., Stuart F., Robinson J. A., and Pluschke G. (2003) Comparison of analytical methods for the evaluation of antibody responses against epitopes of polymorphic protein antigens. *J Immunol Methods* **276**, 19-31.

Abstract: Surface exposed protein antigens of the malaria parasite *Plasmodium falciparum* frequently harbor multiple dimorphic amino acid positions. These are associated with parasite immune evasion and represent a major obstacle for subunit vaccine design. Here, we have analyzed the flexibility of the humoral immune response against a semiconserved sequence (YX(44)LFX(47)KEKMX(52)L) of the key malaria blood stage vaccine candidate merozoite surface protein-1 (MSP-1). Monoclonal antibodies (mAbs) raised against one of the six described natural sequence variants of MSP-1(43-53) were analyzed for cross-reactivity with the other allelic forms, which differ in one to three positions from the immunizing sequence. Enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) spectroscopy demonstrated marked differences in mAb binding avidity to the variant sequences and isothermal titration calorimetry (ITC) provided evidence for a very low affinity of some of the interactions. In immunofluorescence analysis (IFA) and Western blotting analysis, the mAbs nevertheless stained all analyzed parasite clones expressing MSP-1(43-53) variant sequences. When used for the evaluation of humoral immune responses in clinical malaria vaccine trials, these two commonly used methods may thus not be suitable to distinguish biologically functional high affinity antibody responses from irrelevant low-affinity cross-reactivities.

Hibbits K. A., Gill D. S., and Willson R. C. (1994) Isothermal titration calorimetric study of the association of hen egg lysozyme and the anti-lysozyme antibody HyHEL-5. *Biochemistry* **33**, 3584-3590.

Abstract: The thermodynamics of association of hen egg lysozyme and the antibody HyHEL-5 was characterized by isothermal titration calorimetry. The structure of this complex has been determined to 2.8-Å resolution by Sheriff et al. [Sheriff, S., Silverton, E. W., Padlan, E. A., Cohen, G. H., Smith-Gill, S. J., Finzel, B. C., & Davies, D. R. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8075-8079]. The calorimetric enthalpy of association is negative and declines linearly with temperature in the range 10-37 degrees C ($\Delta C_p = -340 \pm 40 \text{ cal mol}^{-1} \text{ K}^{-1}$). Entropic contributions calculated using previously determined values of the affinity of association are negative (unfavorable) in this temperature range. This result is consistent with the loss of mobility upon association of the unusually mobile segments of HEL which form the HyHEL-5 epitope. As the affinity of association is approximately constant in this temperature range, an enthalpy-

entropy compensation effect is implied. The hydrophobic and vibrational contributions to ΔS and ΔC_p are estimated using the method of Sturtevant [Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 2236-2240]. The experimental value of ΔC_p is in rather close agreement with the ΔC_p estimated from the polar and nonpolar surface areas buried upon association.

Hillig R. C., Urlinger S., Fanghanel J., Brocks B., Haenel C., Stark Y., Sulzle D., Svergun D. I., Baesler S., Malawski G., Moosmayer D., Menrad A., Schirner M. and Licha K. (2008) Fab MOR03268 triggers absorption shift of a diagnostic dye via packaging in a solvent-shielded Fab dimer interface. *J Mol Biol* **377**, 206-219.

Abstract: Molecular interactions between near-IR fluorescent probes and specific antibodies may be exploited to generate novel smart probes for diagnostic imaging. Using a new phage display technology, we developed such antibody Fab fragments with subnanomolar binding affinity for tetrasulfocyanine, a near-IR in vivo imaging agent. Unexpectedly, some Fabs induced redshifts of the dye absorption peak of up to 44 nm. This is the largest shift reported for a biological system so far. Crystal structure determination and absorption spectroscopy in the crystal in combination with microcalorimetry and small-angle X-ray scattering in solution revealed that the redshift is triggered by formation of a Fab dimer, with tetrasulfocyanine being buried in a fully closed protein cavity within the dimer interface. The derived principle of shifting the absorption peak of a symmetric dye via packaging within a Fab dimer interface may be transferred to other diagnostic fluorophores, opening the way towards smart imaging probes that change their wavelength upon interaction with an antibody

Housden N. G., Harrison S., Housden H. R., Thomas K. A., Beckingham J. A., Roberts S. E., Bottomley S. P., Graille M., Stura E., and Gore M. G. (2004) Observation and characterization of the interaction between a single immunoglobulin binding domain of protein L and two equivalents of human kappa light chains. *J Biol Chem* **279**, 9370-9378.

Abstract: Detailed stopped-flow studies in combination with site-directed mutagenesis, isothermal titration calorimetry data and x-ray crystallographic knowledge have revealed that the biphasic pre-equilibrium fluorescence changes reported for a single Ig-binding domain of protein L from *Peptostreptococcus magnus* binding to kappa light chain are due to the binding of the kappa light chain at two separate sites on the protein L molecule. Elimination of binding site 2 through the mutation A66W has allowed the K_d for kappa light chain binding at site 1 to be measured by stopped-flow fluorescence and isothermal titration calorimetry techniques, giving values of 48.0 +/- 8.0 nM and 37.5 +/- 7.3 nM respectively. Conversely, a double mutation Y53F/L57H eliminates binding at site 1 and has allowed the K_d for binding at site 2 to be determined. Stopped-flow fluorimetry suggests this to be 3.4 +/- 0.8 μ M in good agreement with the value of 4.6 +/- 0.8 μ M determined by isothermal titration calorimetry. The mutation Y53F reduces the affinity of site 1 to approximately that of site 2.

James L. C., Keeble A. H., Khan Z., Rhodes D. A. and Trowsdale J. (2007) Structural basis for PRYSPRY-mediated tripartite motif (TRIM) protein function. *Proc. Natl. Acad. Sci U. S. A* **104**, 6200-6205.

Abstract: The human tripartite motif (TRIM) family comprises 70 members, including HIV restriction factor TRIM5alpha and disease-associated proteins TRIM20 (pyrin) and TRIM21. TRIM proteins have conserved domain architecture but diverse cellular roles. Here, we describe how the C-terminal PRYSPRY domain mediates diverse TRIM functions. The crystal structure of TRIM21 PRYSPRY in complex with its target IgG Fc reveals a canonical binding interface comprised of two discrete pockets formed by antibody-like variable loops. Alanine scanning of this interface has identified the hot-spot residues that control TRIM21 binding to Fc; the same hot-spots control HIV/murine leukemia virus restriction by TRIM5alpha and mediate severe familial Mediterranean fever in TRIM20/pyrin. Characterization of the IgG binding site for TRIM21 PRYSPRY reveals TRIM21 as a superantigen analogous to bacterial protein A and suggests that an antibody bipolar bridging mechanism may contribute to the pathogenic accumulation of anti-TRIM21 autoantibody immune complex in autoimmune disease.

Jelesarov I., Leder L., and Bosshard H. R. (1996) Probing the Energetics of Antigen-Antibody Recognition by Titration Microcalorimetry. *Methods* **9**, 533-541.

Abstract: Our understanding of the energetics that govern antigen-antibody recognition lags behind the increasingly rapid accumulation of structural information on antigen-antibody complexes. Thanks to the development of highly sensitive microcalorimeters, the thermodynamic parameters of antigen-antibody

interactions can now be measured with precision and using only nanomole quantities of protein. The method of choice is isothermal titration calorimetry, in which a solution of the antibody (or antigen) is titrated with small aliquots of the antigen (or antibody) and the heat change accompanying the formation of the antigen-antibody complex is measured with a sensitivity as high as $0.1 \mu\text{cal s}^{-1}$. The free energy of binding (ΔG), the binding enthalpy (ΔH), and the binding entropy (ΔS) are usually obtained from a single experiment, and no spectroscopic or radioactive label must be introduced into the antigen or antibody. The often large and negative change in heat capacity (ΔC_p) accompanying the formation of an antigen-antibody complex is obtained from ΔH measured at different temperatures. The basic theory and the principle of the measurements are reviewed and illustrated by examples. The thermodynamic parameters relate to the dynamic physical forces that govern the association of the freely moving antigen and antibody into a well-structured and unique complex. This information complements the static picture of the antigen-antibody complex that results from X-ray diffraction analysis. Attempts to correlate dynamic and static aspects are discussed briefly.

Joss L., Morton T. A., Doyle M. L., and Myszka D. G. (1998) Interpreting kinetic rate constants from optical biosensor data recorded on a decaying surface. *Anal Biochem* **261**, 203-210.

Abstract: A capturing assay was used to monitor a Fab-antigen interaction using a BIACORE optical biosensor. The antigen, a truncated single-site mutant (F43V) version of the CD4 receptor, was captured onto the sensor surface using an immobilized nonneutralizing monoclonal antibody. While this assay design created an oriented antigen surface, the antigen slowly dissociated during subsequent binding of the Fab, thus complicating the binding responses. In this paper, we illustrate how binding events occurring on a decaying surface can be accurately described by globally fitting the response data to a model that accounts for the background surface decay. Support for the method was obtained by showing the equilibrium dissociation constant calculated from the kinetic rate constants ($K_d = 2.20 \pm 0.01 \text{ nM}$) was similar to the value measured in solution using titration calorimetry ($K_d = 2.6 \pm 0.5 \text{ nM}$). The ability to interpret rate constants from decaying surfaces significantly extends the types of experimental systems that can be quantitatively studied on optical biosensors.

Kang J. and Warren A. S. (2008) Thermodynamic analysis of additivity between the heavy and light chains in affinity maturation of an antibody. *Mol Immunol.* **45**, 304-305.

Abstract: In a recent article published in Molecular Immunology [Furukawa, K., Shimizu, T., Murakami, A., Kono, R., Nakagawa, M., Sagawa, T., Yamato, I., Azuma, T., 2007. Strategy for affinity maturation of an antibody with high evolvability to (4-hydroxy-3-nitrophenyl) acetyl hapten. *Mol. Immunol.* **44**, 2436-2445], the authors measure thermodynamic parameters of the antigen-antibody interaction for a set of antibodies using an isothermal titration calorimetry to quantitatively assess the contribution of amino acid replacements to an increase in affinity during antibody maturation. One of the findings in their study is that the binding free energy change elicited by mutations in the heavy and light chains is additive. In this letter, we report our analysis of their results in terms of equilibrium thermodynamics to show that enthalpy-entropy compensation is responsible for the additivity

Kelley R. F., O'Connell M. P., Carter P., Presta L., Eigenbrot C., Covarrubias M., Snedecor B., Bourell J. H., and Vetterlein D. (1992) Antigen binding thermodynamics and antiproliferative effects of chimeric and humanized anti-p185HER2 antibody Fab fragments. *Biochemistry* **31**, 5434-5441.

Abstract: The murine monoclonal antibody 4D5 (anti-p185HER2) inhibits the proliferation of human tumor cells overexpressing p185HER2 in vitro and has been "humanized" [Carter, P., Presta, L., Gorman, C. M., Ridgway, J. B. B., Henner, D., Wong, W.-L. T., Rowland, A. M., Kotts, C., Carver, M. E., & Shepard, H. M. (1992) *Proc. Natl. Acad. Sci. U.S.A.* (in press)] for use in human cancer therapy. We have determined the antigen binding thermodynamics and the antiproliferative activities of chimeric 4D5 Fab (ch4D5 Fab) fragment and a series of eight humanized Fab (hu4D5 Fab) fragments differing by amino acid substitutions in the framework regions of the variable domains. Fab fragments were expressed by secretion from *Escherichia coli* and purified from fermentation supernatants by using affinity chromatography on immobilized streptococcal protein G or staphylococcal protein A for ch4D5 and hu4D5, respectively. Circular dichroism spectroscopy indicates correct folding of the *E. coli* produced Fab, and scanning calorimetry shows a greater stability for hu4D5 ($T_m = 82 \text{ degrees C}$) as compared with ch4D5 Fab ($T_m = 72 \text{ degrees C}$). K_D values for binding to the extracellular domain (ECD) of p185HER2 were determined by using a radioimmunoassay; the ΔH and ΔC_p for binding were determined by using isothermal titration

calorimetry. ch4D5 Fab and one of the humanized variants (hu4D5-8 Fab) bind p185HER2-ECD with comparable affinity (ΔG degrees = -13.6 kcal mol⁻¹). (ABSTRACT TRUNCATED AT 250 WORDS).

Keown M. B., Henry A. J., Ghirlando R., Sutton B. J., and Gould H. J. (1998) Thermodynamics of the interaction of human immunoglobulin E with its high-affinity receptor Fc epsilon RI. *Biochemistry* **37**, 8863-8869.

Abstract: We have employed isothermal titration calorimetry (ITC) and circular dichroism (CD) spectroscopy to characterize the binding of soluble fragments of IgE (IgE-Fc and Fc epsilon 3-4) to a soluble fragment of the high-affinity receptor Fc epsilon RI alpha-chain (sFc epsilon RI alpha). The thermodynamic parameters for the interaction of IgE-Fc and Fc epsilon 3-4 with sFc epsilon RI alpha, determined using ITC, confirm the earlier conclusion that the C epsilon 2 domain is not involved in the interaction and that the stoichiometry of both complexes is 1:1. For both IgE-Fc and Fc epsilon 3-4, the value of ΔH degrees is -36.9 +/- 4.6 kcal mol⁻¹ at 37.3 degrees C and ΔC_p degrees is -820 +/- 120 cal mol⁻¹ K⁻¹. The temperature at which ΔS degrees is zero is 284 +/- 1 K, indicating that the entropy contribution to the thermodynamics of association is unfavorable at physiological temperature. Of particular interest is the large value of ΔC_p degrees. The large surface area of IgE and Fc epsilon RI alpha that is implicated in complex formation from previous mutagenesis studies on the two proteins may account in part for the magnitude of ΔC_p degrees. Additional contributions may arise from hydration within the binding site and changes in tertiary structure of the individual components of the complex. However, the CD spectra of IgE, IgE-Fc, and Fc epsilon 3-4 complexes with sFc epsilon RI alpha are merely the sum of the spectra of their individual components, indicating that the secondary structure of the immunoglobulin domain folds are preserved on complex formation. Thus, any change in tertiary structure must be limited to the relative disposition of the immunoglobulin domains C epsilon 3 and C epsilon 4 in IgE and the two immunoglobulin-like domains in the alpha-chain of Fc epsilon RI.

Kral V., Mader P., Collard R., Fabry M., Horejsi M., Rezacova P., Kozisek M., Zavada J., Sedlacek J., Rulisek L. and Brynda J. (2008) Stabilization of antibody structure upon association to a human carbonic anhydrase IX epitope studied by X-ray crystallography, microcalorimetry, and molecular dynamics simulations. *Proteins* **71**, 1275-1287.

Abstract: Specific antibodies interfere with the function of human tumor-associated carbonic anhydrase IX (CA IX), and show potential as tools for anticancer interventions. In this work, a correlation between structural elements and thermodynamic parameters of the association of antibody fragment Fab M75 to a peptide corresponding to its epitope in the proteoglycan-like domain of CA IX, is presented. Comparisons of the crystal structures of free Fab M75 and its complex with the epitope peptide reveal major readjustments of CDR-H1 and CDR-H3. In contrast, the overall conformations and positions of CDR-H2 and CDR-L2 remain unaltered, and their positively charged residues may thus present a fixed frame for epitope recognition. Adoption of the altered CDR-H3 conformation in the structure of the complex is accompanied by an apparent local stabilization. Analysis of domain mobility with translation-libration-screw (TLS) method shows that librations of the entire heavy chain variable domain (V(H)) decrease and reorient in the complex, which correlates well with participation of the heavy chain in ligand binding. Isothermal titration microcalorimetry (ITC) experiments revealed a highly unfavorable entropy term, which can be attributed mainly to the decrease in the degrees of freedom of the system, the loss of conformational freedom of peptide and partially to a local stabilization of CDR-H3. Moreover, it was observed that one proton is transferred from the environment to the protein-ligand complex upon binding. Molecular dynamics simulations followed by molecular mechanics/generalized Born surface area (MM-GBSA) calculations of the ligand (epitope peptide) binding energy yielded energy values that were in agreement with the ITC measurements and indicated that the charged residues play crucial role in the epitope binding. Theoretical arguments presented in this work indicate that two adjacent arginine residues (ArgH50 and ArgH52) are responsible for the observed proton transfer

Krauss N., Wessner H., Welfle K., Welfle H., Scholz C., Seifert M., Zubow K., Ay J., Hahn M., Scheerer P., Skerra A. and Hohne W. (2008) The structure of the anti-c-myc antibody 9E10 Fab fragment/epitope peptide complex reveals a novel binding mode dominated by the heavy chain hypervariable loops. *Proteins* **73**, 552-565.

Abstract: The X-ray structure of the Fab fragment from the anti-c-myc antibody 9E10 was determined both as complex with its epitope peptide and for the free Fab. In the complex, two Fab molecules adopt an

unusual head to head orientation with the epitope peptide arranged between them. In contrast, the free Fab forms a dimer with different orientation. In the Fab/peptide complex the peptide is bound to one of the two Fabs at the "back" of its extended CDR H3, in a cleft with CDR H1, thus forming a short, three-stranded antiparallel beta-sheet. The N- and C-terminal parts of the peptide are also in contact with the neighboring Fab fragment. Comparison between the CDR H3s of the two Fab molecules in complex with the peptide and those from the free Fab reveals high flexibility of this loop. This structural feature is in line with thermodynamic data from isothermic titration calorimetry

Kuba H., Furukawa A., Okajima T. and Furukawa K. (2008) Efficient bacterial production of functional antibody fragments using a phagemid vector. *Protein Expr Purif* **58**, 292-300.

Abstract: The so-called 'in vitro evolutionary method' using a phage display system has been applied for protein engineering of the antigen-binding fragment of antibodies (Fab) by conducting random mutagenesis at the antigen-binding site in combination with antigen-based biopanning. However, isolated phage clones displaying Fab cannot necessarily be used for efficient bacterial production of engineered Fab proteins, often due to deleterious defects in their proper folding abilities derived in compensation for the gain of high affinity for a particular antigen. We here report a new method of an efficient and direct bacterial expression system for the phagemid-coded Fab proteins without use of the helper phage. To overcome a low folding efficiency derived from somatic hypermutations, if any, we have established optimum conditions for bacterial cultivation and protein expression, utilizing unusually long cultivation time (>50 h) and very low temperature (25 degrees C) and thereby leading to the production and extracellular secretion of Fab proteins in a very high yield (3-15 mg/L of culture). The purified Fab folded correctly and could efficiently bind an antigen, as judged by circular dichroism and isothermal titration calorimetry, respectively

Leder L., Berger C., Bornhauser S., Wendt H., Ackermann F., Jelesarov I., and Bosshard H. R. (1995) Spectroscopic, calorimetric, and kinetic demonstration of conformational adaptation in peptide-antibody recognition. *Biochemistry* **34**, 16509-16518.

Abstract: Little is known about the extent to which protein flexibility contributes to antigen-antibody recognition and cross-reactivity. Using short coil peptides (leucine zippers) as model antigens, we demonstrate that a monoclonal antibody can force a noncognate peptide into a conformation that is similar to the conformation of the cognate peptide against which the monoclonal antibody is directed. Monoclonal antibodies 29AB and 13AD were raised against the 29-residue peptide LZ (Ac-EYEALEKKLAALAKLQALEKKLEALEHG-amide) that forms a very stable coiled coil. The two antibodies cross-reacted strongly with the random coil analogue LZ(7P14P) that contains Lys-->Pro and Ala-->Pro substitutions in positions 7 and 14, respectively. The antibody-bound peptide LZ(7P14P) adopted an altered conformation that possibly was coiled coil-like, as shown by CD difference spectroscopy and fluorescence quenching experiments on coumarin-labeled peptides. Isothermal titration calorimetry revealed that the cross-reaction of antibodies 13AD and 29AB with the random coil peptide LZ(7P14P) exhibited a large unfavorable entropy. This, however, was strongly compensated by a more favorable enthalpy, resulting in only a small difference between the association constants for peptide LZ and LZ(7P14P), respectively. To investigate the opposite type of cross-reaction, monoclonal antibody 42PF was raised against the random coil peptide LZ(7P14P). 42PF cross-reacted with coiled coil peptide LZ by forcing it to dissociate into single chains. Enthalpy/entropy compensation again enabled the cross-reaction, which now was entropically favored and enthalpically disfavored. The rate of reaction of antibody 42PF with peptide LZ was controlled by the rate of dissociation of LZ into single chains. This observation, as well as the generally much slower reaction rate with the noncognate peptides, indicated that the cross-reactivity occurred because the antibody selected the conformer of the antigen that binds the strongest, a mechanism we call "induced fit by conformational selection."

Livingstone J. R. (1996) Antibody characterization by isothermal titration calorimetry. *Nature* **384**, 491-492.

Lullau E., Heyse S., Vogel H., Marison I., von Stockar U., Kraehenbuhl J. P., and Corthesy B. (1996) Antigen binding properties of purified immunoglobulin A and reconstituted secretory immunoglobulin A antibodies. *J Biol Chem* **271**, 16300-16309.

Abstract: The hybridoma cell line ZAC3 expresses *Vibrio cholerae* lipopolysaccharide (LPS)-specific mouse IgA molecules as a heterogeneous population of monomeric (IgAm), dimeric (IgAd), and polymeric

(IgAp) forms. We describe a gentle method combining ultrafiltration, ion-exchange chromatography, and size exclusion chromatography for the simultaneous and qualitative separation of the three molecular forms. Milligram quantities of purified IgA molecules were recovered allowing for direct comparison of the biological properties of the three forms. LPS binding specificity was tested after purification; IgAd and IgAp were found to bind strongly to LPS whereas IgAm did not. Secretory IgA (sIgA) could be reconstituted in vitro by combining recombinant secretory component (rSC) and purified IgAd or IgAp, but not IgAm. Surface plasmon resonance-based binding experiments using LPS monolayers indicated that purified reconstituted sIgA and IgA molecules recognize LPS with identical affinity ($K_A 1.0 \times 10^8 M^{-1}$). Thus, this very sensitive assay provides the first evidence that the function of SC in sIgA complex is not to modify the affinity for the antigen. K_A falls to $6.6 \times 10^5 M^{-1}$ when measured by calorimetry using detergent-solubilized LPS and IgA, suggesting that the LPS environment is critical for recognition by the antibody.

McGavin R. S. and Bundle D. R. (2005) Developing high affinity oligosaccharide inhibitors: conformational pre-organization paired with functional group modification. *Org Biomol Chem* **3**, 2733-2740.

Abstract: Intramolecular tethering combined with functional group modification has been investigated as an approach to design high affinity oligosaccharide ligands. The preceding paper reported successful tethering to constrain a trisaccharide in the conformation of its bound state with an antibody and thereby achieved a 15-fold increase in association constant. Here we report the synthesis of two beta-alanyl tethered derivatives that employ monochlorination and monodeoxygenation strategies to create inhibitors that should enhance the binding affinity of the target molecules by an additional 10-25-fold, provided that free energy changes are additive when tethering is paired with functional group changes. The binding parameters of the new ligands were measured by isothermal titration calorimetry and the results rationalized with molecular dynamics calculations and a simple docking analysis. The data indicate that while the alanine tether is a reasonable method to constrain trisaccharide, free energy gains obtained by pairing it with functional group modification are not additive and in one case counter-productive.

McGavin R. S., Gagne R. A., Chervenak M. C., and Bundle D. R. (2005) The design, synthesis and evaluation of high affinity macrocyclic carbohydrate inhibitors. *Org Biomol Chem* **3**, 2723-2732.

Abstract: Carbohydrate-protein interactions have been investigated for a model system of a monoclonal antibody, SYA/J6, which binds a trisaccharide epitope of the O-polysaccharide of the *Shigella flexneri* variant Y lipopolysaccharide. The thermodynamics of binding for the methyl glycoside of the native trisaccharide epitope, Rha-Rha-GlcNAc () to SYA/J6 over a range of temperatures exhibits strong, linear enthalpy-entropy compensation and a negative heat capacity change ($\Delta C(p) = -152 \text{ cal mol}^{-1} \text{ degree}^{-1}$). At 293 K the free energy of association is the sum of favourable enthalpy and entropy contributions ($\Delta H = -3.9 \text{ kcal mol}^{-1}$ and $-\Delta S = -2.9 \text{ kcal mol}^{-1}$). Crystal structures for SYA/J6 Fab detailed the position of the native trisaccharide epitope, Rha-Rha-GlcNAc, and facilitated a strategy to design a tighter binding, low molecular weight ligand. This involved pre-organization of the native trisaccharide in its bound conformation by addition of intramolecular constraints (a beta-alanyl or glycinyl tether). ELISA measurements indicated that the glycinyl tethered trisaccharide was not an optimal candidate for further analysis, while microcalorimetry provided data showing that the beta-alanyl tethered trisaccharide displayed a 15-fold increase in affinity for SYA/J6. Tethering resulted in a favourable entropic contribution to binding, relative to the native trisaccharide ($-\Delta\Delta S = -1.2 \text{ kcal mol}^{-1}$). Potential energy and dynamics calculations using the AMBER Plus force fields indicated that trisaccharide adopted a rigid conformation similar to that of the bound conformation of the native trisaccharide epitope. While this strategy resulted in modest free energy gains by minimizing losses due to conformational entropy, thermodynamic data are consistent with significant contributions from solvent reorganization.

Midelfort K. S., Hernandez H. H., Lippow S. M., Tidor B., Drennan C. L., and Wittrup K. D. (2004) Substantial energetic improvement with minimal structural perturbation in a high affinity mutant antibody. *J Mol Biol* **343**, 685-701.

Abstract: Here, we compare an antibody with the highest known engineered affinity ($K_d = 270 \text{ fM}$) to its high affinity wild-type ($K_d = 700 \text{ pM}$) through thermodynamic, kinetic, structural, and theoretical analyses. The 4M5.3 anti-fluorescein single chain antibody fragment (scFv) contains 14 mutations from the wild-type 4-4-20 scFv and has a 1800-fold increase in fluorescein-binding affinity. The dissociation rate is

approximately 16,000 times slower in the mutant; however, this substantial improvement is offset somewhat by the association rate, which is ninefold slower in the mutant. Enthalpic contributions to binding were found by calorimetry to predominate in the differential binding free energy. The crystal structure of the 4M5.3 mutant complexed with antigen was solved to 1.5Å resolution and compared with a previously solved structure of an antigen-bound 4-4-20 Fab fragment. Strikingly, the structural comparison shows little difference between the two scFv molecules (backbone RMSD of 0.6Å), despite the large difference in affinity. Shape complementarity exhibits a small improvement between the variable light chain and variable heavy chain domains within the antibody, but no significant improvement in shape complementarity of the antibody with the antigen is observed in the mutant over the wild-type. Theoretical modeling calculations show electrostatic contributions to binding account for -1.2 kcal/mol to -3.5 kcal/mol of the binding free energy change, of which -1.1 kcal/mol is directly associated with the mutated residue side-chains. The electrostatic analysis reveals several mechanistic explanations for a portion of the improvement. Collectively, these data provide an example where very high binding affinity is achieved through the cumulative effect of many small structural alterations.

Morikis D. and Lambris J. D. (2004) Physical methods for structure, dynamics and binding in immunological research. *Trends Immunol* **25**, 700-707.

Abstract: We present four experimental physical methods--X-ray and neutron diffraction, nuclear magnetic resonance spectroscopy, mass spectrometry and calorimetry--and two computational methods--molecular dynamics simulations and electrostatics calculations--which are general and widely applicable in the study of protein structure, dynamics and binding. These methods are useful tools for biologists that lead to structure-function, dynamics-function and binding-function correlations, in efforts to understand biomolecular function. Standard and emerging technologies within these methods are discussed and representative examples of applications in immunology are presented, from antigen-antibody, complement and MHC-T-cell receptor research. The examples demonstrate the power of the reviewed methods in immunological studies at the molecular level.

Murphy K. P., Freire E., and Paterson Y. (1995) Configurational effects in antibody-antigen interactions studied by microcalorimetry. *Proteins* **21**, 83-90.

Abstract: In this paper we study the binding of two monoclonal antibodies, E3 and E8, to cytochrome c using high-sensitivity isothermal titration calorimetry. We combine the calorimetric results with empirical calculations which relate changes in heat capacity to changes in entropy which arise from the hydrophobic effect. The change in heat capacity for binding E3 is -350 +/- 60 cal K⁻¹ mol⁻¹ while for E8 it is -165 +/- 40 cal K⁻¹ mol⁻¹. This result indicates that the hydrophobic effect makes a much larger contribution for E3 than for E8. Since the total entropy change at 25 degrees C is very similar for both antibodies, it follows that the configurational entropy cost for binding E3 is much larger than for binding E8 (-77 +/- 15 vs. -34 +/- 11 cal K⁻¹ mol⁻¹). These results illustrate a case of entropy compensation in which the cost of restricting conformational degrees of freedom is to a large extent compensated by solvent release. We also show that the thermodynamic data can be used to make estimates of the surface area changes that occur upon binding. The results of the present study are consistent with previous hydrogen-deuterium exchange data, detected using 2D NMR, on the two antibody-antigen interactions. The NMR study indicated that protection from exchange is limited to the binding epitope for E8, but extends beyond the epitope for E3.(ABSTRACT TRUNCATED AT 250 WORDS).

Mutz M., Hawthorne T., Ferrone S., and Pluschke G. (1997) Titration calorimetry study of an anti-idiotypic antibody cascade in a human melanoma-associated antigen system. *Mol Immunol* **34**, 695-707.

Abstract: The thermodynamic parameters of interactions between six variants of the anti-idiotypic monoclonal antibody (mAb) CGP 60686 produced by the hybridoma MK2-23 with an idiotypic mAb and five different anti-anti-idiotypic mAb were studied with high sensitivity titration calorimetry. CGP 60686 recognizes an epitope in the antigen-combining region of the human high-molecular-weight-melanoma-associated antigen (HMW-MAA)-specific mouse mAb CGP 76873 produced by the hybridoma 763.74. The five HMW-MAA-specific anti-anti-idiotypic mAbs GH 464, GH 518, GH 149, GH 386 and GH 586 were generated from mice immunization with mAb CGP 60686. All interactions between the anti-idiotypic mAb and the idiotypic mAb or the anti-anti-idiotypic mAb showed large exothermic binding enthalpies between -15 and -23 kcal/mol and binding affinities larger than 6 x 10⁹ M⁻¹. Four of the five anti-anti-idiotypic mAbs tested exhibited significantly higher binding enthalpies for the interaction with the anti-

idiotypic than the idiotypic mAbs. Replacement of either the heavy or the light chain variable region of the anti-idiotypic mAbs with an unrelated variable region abolished the idiotype to anti-idiotype interaction. Thus, both the heavy and the light chain variable region of the anti-idiotypic mAbs are required for binding to the idiotype. The values of the binding enthalpy showed only small variations when binding of the idiotypic mAb CGP 76873 to four variants of the anti-idiotypic mAb CGP 60686 with different immunoglobulin constant regions, but identical variable regions were compared. Furthermore, Fab fragments of the idiotypic mAbs showed almost the same binding enthalpy per binding site as the whole IgG molecules. Immunoglobulin constant regions thus had little influence on the idiotype to anti-idiotype interactions. Taken together, the observed thermodynamic parameters suggest that the idiotype to anti-idiotype interactions studied here are enthalpy-driven processes with only minor entropic contributions. High sensitivity titration calorimetry was used to monitor protein-protein interactions within an anti-idiotypic antibody cascade. It was found that the direct measurement of the interaction enthalpy allowed a quantitative characterization of the binding processes studied.

Myszka D. G., Morton T. A., Doyle M. L., and Chaiken I. M. (1997) Kinetic analysis of a protein antigen-antibody interaction limited by mass transport on an optical biosensor. *Biophys Chem* **64**, 127-137.

Abstract: Using BIAcore technology, we determined the rate constants for a protein antigen-antibody interaction that was mass transport limited on the optical biosensor. The antigen consisted of a soluble form of the human T-cell receptor CD4 (two amino terminal domains, D1D2) and the antibody was an anti-CD4 monoclonal from monkey engineered with the constant domains from human IgG1. High quality response data were obtained for this interaction by orienting the attachment of the antibody on the sensor surface and correcting for instrument artifacts with control experiments. Using numerical integration and global fitting, we demonstrate that a mass transport limited reaction was the only model of those tested that described well D1D2 binding to three different surface densities of the antibody. Statistical profiling techniques showed that the error space and correlation for the parameters in the non-linear model were essentially linear, but only when the model was simultaneously fitted to data from multiple surface densities. The "on" and "off" rate constants ($1.2 \times 10^{-6} \text{ M}^{-1}\text{s}^{-1}$ and $2.9 \times 10^{-4} \text{ s}^{-1}$) determined from the kinetic analysis predict an equilibrium dissociation constant ($K_D = 0.24 \pm 0.01 \text{ nM}$) that agrees with the value measured in solution by titration calorimetry ($K_D = 0.2 \pm 0.1 \text{ nM}$). The results indicate that, although the D1D2-antibody reaction is partially controlled by mass transport on the optical biosensor, by optimizing the experimental design and analyzing data from multiple surface densities it is possible to determine accurate estimates of the intrinsic equilibrium and kinetic rate constants.

Nakanishi T., Tsumoto K., Yokota A., Kondo H. and Kumagai I. (2008) Critical contribution of VH-VL interaction to reshaping of an antibody: the case of humanization of anti-lysozyme antibody, HyHEL-10. *Protein Sci* **17**, 261-270.

Abstract: To clarify the effects of humanizing a murine antibody on its specificity and affinity for its target, we examined the interaction between hen egg white lysozyme (HEL) and its antibody, HyHEL-10 variable domain fragment (Fv). We selected a human antibody framework sequence with high homology, grafted sequences of six complementarity-determining regions of murine HyHEL-10 onto the framework, and investigated the interactions between the mutant Fvs and HEL. Isothermal titration calorimetry indicated that the humanization led to 10-fold reduced affinity of the antibody for its target, due to an unfavorable entropy change. Two mutations together into the interface of the variable domains, however, led to complete recovery of antibody affinity and specificity for the target, due to reduction of the unfavorable entropy change. X-ray crystallography of the complex of humanized antibodies, including two mutants, with HEL demonstrated that the complexes had almost identical structures and also paratope and epitope residues were almost conserved, except for complementary association of variable domains. We conclude that adjustment of the interfacial structures of variable domains can contribute to the reversal of losses of affinity or specificity caused by humanization of murine antibodies, suggesting that appropriate association of variable domains is critical for humanization of murine antibodies without loss of function

Nishimiya Y., Tsumoto K., Shiroishi M., Yutani K., and Kumagai I. (2000) Thermodynamic consequences of grafting enhanced affinity toward the mutated antigen onto an antibody. The case of anti-lysozyme antibody, HyHEL-10. *J Biol Chem* **275**, 12813-12820.

Abstract: In order to address the mechanism of enhancement of the affinity of an antibody toward an antigen from a thermodynamic viewpoint, anti-hen lysozyme (HEL) antibody HyHEL-10, which also

recognize the mutated antigen turkey lysozyme (TEL) with reduced affinity, was examined. Grafting high affinity toward TEL onto HyHEL-10 was performed by saturation mutagenesis into four residues (Tyr(53), Ser(54), Ser(56), and Tyr(58)) in complementarity-determining region 2 of the heavy chain (CDR-H2) followed by selection with affinity for TEL. Several clones enriched have a Phe residue at site 58. Thermodynamic analyses showed that the clones selected had experienced a greater than 3-fold affinity increase toward TEL in comparison with wild-type Fv, originating from an increase in negative enthalpy change. Substitution of HyHEL-10 HTyr(58) with Phe led to the increase in negative enthalpy change and to almost identical affinity for TEL in comparison with mutants selected, indicating that mutations at other sites decrease the entropy loss despite little contribution to the affinity for TEL. These results suggest that the affinity of an antibody toward the antigen is enhanced by the increase in enthalpy change by some limited mutation, and excess entropy loss due to the mutation is decreased by other energetically neutral mutations.

Oda M., Kozono H., Morii H., and Azuma T. (2003) Evidence of allosteric conformational changes in the antibody constant region upon antigen binding. *Int Immunol* **15**, 417-426.

Abstract: We have addressed the question of whether antigen binding induces a conformational change in the heavy chain constant (C(H)) domain of antibodies using staphylococcal protein A or streptococcal protein G as probes, since these proteins are known to bind to IgG domains such as C(H)1 and C(H)2-C(H)3 domains. Biosensor assays on interactions between these proteins and mouse IgG specific to (4-hydroxy-3-nitrophenyl)acetyl (NP) or their enzymatic fragments conducted in the presence or absence of the hapten, NP-epsilon-aminocaproic acid (NP-Cap), showed that the binding of IgG to these proteins was inhibited by the binding of NP-Cap. The results of isothermal titration calorimetry also revealed that the association constant for the interaction of protein A with IgG2b decreased by the addition of NP-Cap. These results suggested that antigen binding induced conformational changes in binding sites for protein G or protein A located at C(H)1 and C(H)2-C(H)3 domains, respectively.

Ou Z., Bottoms C. A., Henzl M. T. and Tanner J. J. (2007) Impact of DNA Hairpin Folding Energetics on Antibody-ssDNA Association. *J Mol Biol* **734**, 1029-1040.

Abstract: Deposition of anti-DNA antibodies in the kidney contributes to the pathogenesis of the autoimmune disease, systemic lupus erythematosus. Antibodies that bind to hairpin-forming DNA ligands may be particularly prone to deposition. Here we report the first structure of a Fab complexed with hairpin-forming DNA. The ligand used for co-crystallization is 5'-d [CTG(CCTT)CAG]-3', which has a predicted hairpin structure consisting of a four-nucleotide loop (CCTT) and a stem of three base-pairs. The 1.95 Å resolution crystal structure of Fab DNA-1 complexed with this ligand shows that the conformation of the bound ligand differs radically from the predicted hairpin conformation. The three base-pairs in the stem are absent in the bound form. The protein binds to the last six nucleotides at the 3' end of the ligand. These nucleotides form a loop (TTCA) closed by a G:C base-pair in the bound state. Stacking of aromatic side-chains against DNA bases is the dominant interaction in the complex. Interactions with the DNA backbone are conspicuously absent. Thermodynamics of binding are examined using isothermal titration calorimetry. The apparent dissociation constant is 4 µM, and binding is enthalpically favorable and entropically unfavorable. Increasing the number of base-pairs in the DNA stem from three to six decreases binding affinity. These data suggest a conformational selection binding mechanism in which the Fab binds preferentially to the unstructured state of the ligand. In this interpretation, the ligand binding and ligand folding equilibria are coupled, with lower hairpin stability leading to greater effective binding affinity. Thus, pre-organization of the DNA loop into the preferred binding conformation does not play a major role in complexation. Rather, it is argued that the stem of the hairpin serves to reduce the degrees of freedom in the free DNA ligand, thereby limiting the entropic cost attendant to complexation with the Fab.

Okazaki A., Shoji-Hosaka E., Nakamura K., Wakitani M., Uchida K., Kakita S., Tsumoto K., Kumagai I., and Shitara K. (2004) Fucose depletion from human IgG1 oligosaccharide enhances binding enthalpy and association rate between IgG1 and FcγRIIIa. *J Mol Biol* **336**, 1239-1249.

Abstract: Depletion of fucose from human IgG1 oligosaccharide improves its affinity for FcγRIIIa receptor IIIa (FcγRIIIa). This is the first case where a glycoform modification is shown to improve glycoprotein affinity for the receptors without carbohydrate-binding capacity, suggesting a novel glyco-engineering strategy to improve ligand-receptor binding. To address the mechanisms of affinity improvement by the fucose depletion, we used isothermal titration calorimetry (ITC) and biosensor analysis

with surface plasmon resonance. ITC demonstrated that IgG1-Fcγ3 binding was driven by favorable binding enthalpy (ΔH) but opposed by unfavorable binding entropy change (ΔS). Fucose depletion from IgG1 enhanced the favorable ΔH , leading to the increase in the binding constant of IgG1 for the receptor by a factor of 20-30. The increase in the affinity was mainly attributed to an enhanced association rate. A triple amino acid substitution in IgG1, S298A/E333A/K334A, is also known to improve IgG1 affinity for Fcγ3. ITC demonstrated that the amino acid substitution attenuated the unfavorable ΔS resulting in a three- to fourfold increase in the binding constant. The affinity enhancement by the amino acid substitution was due to a reduced dissociation rate. These results indicate that the mechanism of affinity improvement by the fucose depletion is quite distinct from that by the amino acid substitution. Defucosylated IgG1 exhibited higher antibody-dependent cellular cytotoxicity (ADCC) than S298A/E333A/K334A-IgG1, showing a correlation between IgG1 affinity for Fcγ3 and ADCC. We also examined the effect of Fcγ3 polymorphism (Val158/Phe158) on IgG1-Fcγ3 binding. The Phe to Val substitution increased Fcγ3 affinity for IgG1 in an enthalpy-driven manner with the reduced dissociation rate. These results together highlight the distinctive functional improvement of affinity by IgG1 defucosylation and suggest that engineering of non-interfacial monosaccharides can improve glycoprotein affinity for receptors via an enthalpy-driven and association rate-assisted mechanism.

Pitner J. B., Beyer W. F., Venetta T. M., Nycz C., Mitchell M. J., Harris S. L., Marino-Albernas J. R., Auzanneau F. I., Forooghian F., and Pinto B. M. (2000) Bivalency and epitope specificity of a high-affinity IgG3 monoclonal antibody to the Streptococcus group A carbohydrate antigen. Molecular modeling of a Fv fragment. *Carbohydr Res* **324**, 17-29.

Abstract: The binding of Strep 9, a mouse monoclonal antibody (mAb) of the IgG3 subclass directed against the cell-wall polysaccharide of Group A Streptococcus (GAS), has been characterized. The intact antibody and proteolytic fragments of Strep 9 bind differently to GAS: the intact mAb and F(ab)₂' have greater affinity for the carbohydrate epitope than the monomeric Fab or F(ab)'. A mode of binding in which Strep 9 binds bivalently to portions of the polysaccharide on adjacent chains on GAS is proposed. A competitive ELISA protocol using a panel of carbohydrate inhibitors shows that the branched trisaccharide, beta-D-GlcNAc-(1->3)-[alpha-L-Rhap-(1->2)]-alpha-L-Rhap, and an extended surface are key components of the epitope recognized by Strep 9. Microcalorimetry measurements with the mAb and two synthetic haptens, a tetrasaccharide and a hexasaccharide, show enthalpy-entropy compensation as seen in other oligosaccharide-protein interactions. Molecular modeling of the antibody variable region by homology modeling techniques indicates a groove-shaped combining site that can readily accommodate extended surfaces. Visual docking of an oligosaccharide corresponding to the cell-wall polysaccharide into the site provides a putative model for the complex, in which a heptasaccharide unit occupies the site and the GlcNAc residues of two adjacent branched trisaccharide units occupy binding pockets within the groove-shaped binding site.

Rademacher C., Shoemaker G. K., Kim H. S., Zheng R. B., Taha H., Liu C., Nacario R. C., Schriemer D. C., Klassen J. S., Peters T. and Lowary T. L. (2007) Ligand specificity of CS-35, a monoclonal antibody that recognizes mycobacterial lipoarabinomannan: a model system for oligofuranoside-protein recognition. *J Am. Chem Soc.* **129**, 10489-10502.

Abstract: The CS-35 antibody is widely used in the characterization of glycans containing D-arabinofuranose residues, in particular polysaccharides present in the mycobacterial cell wall. A detailed understanding of the combining site of this antibody and the measurement of its binding to different ligands is of interest as this knowledge will have implications in the characterization of arabinofuranose-containing glycoconjugates that are increasingly recognized as important biological molecules. Of even greater significance is that an in-depth study of this carbohydrate-protein interaction will provide insights into the mechanisms by which oligosaccharides containing furanose rings are bound by proteins, an area that has, to date, received little attention. This system has been refractory to X-ray crystallography, and thus we report here a study of the interaction of CS-35 with its ligands using a combination of chemical synthesis, mass spectrometry, titration microcalorimetry, and NMR spectroscopy. Through these investigations we have established that the binding pocket recognizes, as a minimum epitope, a linear tetrasaccharide motif and that the residues at the reducing and non-reducing end of the oligosaccharide are essential for tight binding. The residue at the non-reducing end appears to be bound in an aliphatic pocket, whereas the rest of the tetrasaccharide interacts more strongly with aromatic amino acids.

Raman C. S., Allen M. J., and Nall B. T. (1995) Enthalpy of antibody--cytochrome c binding. *Biochemistry* **34**, 5831-5838.

Abstract: High-sensitivity titration calorimetry is used to measure changes in enthalpy, heat capacity, and protonation for binding of two monoclonal antibodies (MAbs) to topologically distinct surfaces of cytochrome c. MAb 2B5 binds near the exposed heme crevice in a reaction involving proton uptake, while there is no change in protonation for MAb 5F8 binding to the opposite side of the molecule. Both antibodies have association rate constants with the activation enthalpy and viscosity dependence expected of diffusion-limited reactions [Raman et al. (1992) *Biochemistry* 31, 10370-10379], and bind with high affinity ($\Delta G_{\text{zerob}} = -12.6 \text{ kcal mol}^{-1}$ for MAb 2B5 and $-13.9 \text{ kcal mol}^{-1}$ for MAb 5F8, at pH 7, 25 degrees C). At 25 degrees C, the equilibrium enthalpy and entropy contributions to the free energy of binding are negative for both antibodies ($\Delta H_{\text{zerob}} = -21.0 \text{ kcal mol}^{-1}$, $\Delta S_{\text{zerob}} = -28.2 \text{ cal mol}^{-1} \text{ K}^{-1}$ for MAb 2B5; and $\Delta H_{\text{zerob}} = -21.7 \text{ kcal mol}^{-1}$, $\Delta S_{\text{zerob}} = 26.3 \text{ cal mol}^{-1} \text{ K}^{-1}$ for MAb 5F8). The enthalpy of MAb 2B5-cytochrome c association exhibits a marked temperature dependence ($\Delta C_p = -580 \text{ cal mol}^{-1} \text{ K}^{-1}$), but the enthalpy for MAb 5F8 binding is much less dependent on temperature ($\Delta C_p = -172 \text{ cal mol}^{-1} \text{ K}^{-1}$). The large differences in ΔC_p for binding of the two antibodies suggest corresponding differences in the mode of binding, or in the molecular surfaces buried in the binding reactions. In particular, factors other than hydrophobic effects may be significant contributors to the thermodynamics of antibody-cytochrome c binding, especially when ΔC_p is small (MAb 5F8).

Rispens T., Lakemond C. M., Derksen N. I. and Aalberse R. C. (2008) Detection of conformational changes in immunoglobulin G using isothermal titration calorimetry with low-molecular-weight probes. *Anal Biochem* **380**, 303-309.

Abstract: Proteins for therapeutic use may contain small amounts of partially misfolded monomeric precursors to postproduction aggregation. To detect these misfolded proteins in the presence of an excess of properly folded protein, fluorescent probes such as 8-anilino-1-naphthalene sulfonate (ANS) are commonly used. We investigated the possibility of using isothermal titration calorimetry (ITC) to improve the detection of this type of conformational change using hydrophobic probes. As a case study, conformational changes in human polyclonal immunoglobulin G (IgG) were monitored by measuring the enthalpies of binding of ANS using ITC. Results were compared with those using fluorescence spectroscopy. IgG heated at 63 degrees C was used as a model system for "damaged" IgG. Heat-treated IgG can be detected already at levels below 5% with both ITC and fluorescence. However, ITC allows a much wider molar probe-to-protein ratio to be sampled. In particular, using reverse titration experiments (allowing high probe-to-protein ratios not available to fluorescence spectroscopy), an increase in the number of binding sites with a $K(d) > 10 \text{ mM}$ was observed for heat-treated IgG, reflecting subtle changes in structure. Both ITC and fluorescence spectroscopy showed low background signals for native IgG. The nature of the background signals was not clear from the fluorescence measurements. However, further analysis of the ITC background signals shows that a fraction (8%) binds ANS with a dissociation constant of approximately 0.2 mM. Measurements were also carried out at pH 4.5. Precipitation of IgG was induced by ANS at concentrations above 0.5 mM, interfering with the ITC measurements. Instead, with the nonfluorescent probes 4-amino-1-naphthalene sulfonate and 1-naphthalene sulfonate, no precipitation is observed. These probes yield differences in the enthalpies of binding to heated and nonheated IgG similar to ANS. The data illustrate that ITC with low-molecular-weight probes is a versatile tool to monitor conformational changes in proteins with a wider application potential than fluorescence measurements

Sagawa T., Oda M., Morii H., Takizawa H., Kozono H., and Azuma T. (2005) Conformational changes in the antibody constant domains upon hapten-binding. *Mol Immunol* **42**, 9-18.

Abstract: Bacterial proteins A and G (SpA and SpG) are immunoglobulin receptors that can be used as probes for monitoring change in the conformation of heavy chain constant (C(H)) domains. Interaction of anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibody (Ab) with SpA and SpG were measured by isothermal titration calorimetry and surface plasmon resonance in order to address the question of whether hapten-binding induces a conformational change in the C(H) domain. The interactions of IgG2a or its enzymatic fragments with SpA were measured in the presence or absence of the hapten. Although binding of Fab and F(ab')₂ fragments were not observed to free SpA, they did bind to immobilized SpA. In addition, the association constant (K_a) for interaction of IgG2a with immobilized SpA was approximately 20-fold higher than that with free SpA. This was explained in terms of high avidity resulting from multivalent interaction between IgG2a and immobilized SpA on the chip. Interestingly, the hapten-binding weakened the

interaction between the F(ab')₂ fragment and SpA. Furthermore, approximately half of the IgG_{2a} was incapable of binding to immobilized SpA in the presence of hapten. These results were explained using a model which assumed the formation of two kinds of SpA/IgG complexes; one through sites on F(ab')₂ arms and the other through sites on the Fc region. The former type dissociated as a result of hapten-binding, as did the F(ab')₂ fragment and suggested that a conformational change had occurred around the Fab arms, while the latter type did not dissociate because of the higher avidity of the Fc region. However, using a mutant SpA with a lower K_a value for the interaction with IgG_{2a}, it was shown that hapten-binding induced long range conformational changes in the Fc region of IgG_{2a}. Similar evidence of conformational change upon hapten-binding was also obtained using SpG as a probe.

Saito M., Okazaki I., Oda M., and Fujii I. (2005) A free energy calculation study of the effect of H→F substitution on binding affinity in ligand-antibody interactions. *J Comput Chem* **26**, 272-282.

Abstract: Changes in binding affinity to catalytic antibody 6D9 of chloramphenicol phosphonate derivatives (CPDs) containing H or F were investigated by performing free energy calculations based on molecular dynamics simulations. We calculated the binding free energy, enthalpy, and entropy changes ($\Delta\Delta G$, $\Delta\Delta H$, and $-\Delta\Delta S$) attributable to H→F substitution by comparing results for CPDs containing a trifluoroacetyl amino group (CPD-F) or an acetyl amino group (CPD-H). The calculated $\Delta\Delta G$, $\Delta\Delta H$, and $-\Delta\Delta S$ values were -2.9, -6.3, and 3.5 kcal mol⁻¹ and close to experimental values observed for a series of similar ligands, chloramphenicol phosphonates with F and H (-1.4, -3.5, and 2.1 kcal mol⁻¹). Therefore, CPD-F binds more strongly to 6D9 than does CPD-H. To clarify the origin of the large difference in $\Delta\Delta G$, we apportioned the calculated values of $\Delta\Delta G$ and $\Delta\Delta G$ for the associated and dissociated states into contributions from various atomic interactions. We found that the H→F substitution increased the binding affinity mainly by decreasing the hydration free energy and not by increasing favorable interactions with the antibody. The decreased hydration free energy of the ligand was mainly due to unfavorable coulombic interactions between the trifluoroacetyl amino group and solvent waters, which increased the free energy of the dissociated state (by about 3.7 kcal mol⁻¹). Also, the trifluoroacetyl amino group slightly increased the free energy level of the associated state (about 0.8 kcal mol⁻¹) because favorable van der Waals interactions compensated for unfavorable coulombic interactions with antibody atoms. In addition, the enthalpy and entropy changes, $\Delta\Delta H$ and $-\Delta\Delta S$ (computationally -6.3 and 3.5 kcal mol⁻¹), originated mainly from a decrease in hydration free energy in the dissociated state. The CPD-F and CPD-H ligands had substantially different structures in the dissociated and complexed states.

Schwarz F. P., Tello D., Goldbaum F. A., Mariuzza R. A., and Poljak R. J. (1995) Thermodynamics of antigen-antibody binding using specific anti-lysozyme antibodies. *Eur J Biochem* **228**, 388-394.

Abstract: Titration calorimetry measurements on the binding of hen lysozyme to the specific monoclonal IgG antibodies D1.3, D11.15, D44.1, F9.13.7, F10.6.6, their papain-cleaved antigen binding fragments (Fab) and their protein-engineered fragments consisting of non-covalently linked heavy variable chain and light variable chain domains (Fv) were performed between 6-50 degrees C in 0.15 M NaCl, 0.01 M sodium phosphate pH 7.1. The binding thermodynamic free energy change (ΔG degrees b), enthalpy change (ΔH_b), and entropy change (ΔS_b) were the same for the whole IgG and its Fv and Fab fragments. With the exception of F9.13.7 at 13 degrees C, all the binding reactions were enthalpically driven with enthalpy changes ranging from -129 +/- 7 kJ mol⁻¹ (D1.3 at 49.8 degrees C) to -26.2 +/- 0.6 kJ mol⁻¹ (D44.1 at 8.0 degrees C). The heat capacity changes for the binding reaction (ΔC_p) ranged from -2.72 +/- 0.16 kJ mol⁻¹K⁻¹ (F9.13.7) to -0.95 +/- 0.06 kJ mol⁻¹K⁻¹ (F10.6.6). The apolar surface areas buried at the binding sites estimated from the heat capacity changes indicate that the binding reactions are primarily hydrophobic, contrary to the mainly observed enthalpy-driven nature of the reactions. Conformational stabilization and the presence of water at the antigen-antibody interface may account for this discrepancy.

Schuermann J. P., Henzl M. T., Deutscher S. L., and Tanner J. J. (2004) Structure of an anti-DNA fab complexed with a non-DNA ligand provides insights into cross-reactivity and molecular mimicry. *Proteins* **57**, 269-278.

Abstract: Antibodies that recognize DNA (anti-DNA) are part of the autoimmune response underlying systemic lupus erythematosus. To better understand molecular recognition by anti-DNA antibodies, crystallographic studies have been performed using an anti-ssDNA antigen-binding fragment (Fab) known as DNA-1. The previously determined structure of a DNA-1/dT5 complex revealed that thymine bases

insert into a narrow groove, and that ligand recognition primarily involves the bases of DNA. We now report the 1.75-Å resolution structure of DNA-1 complexed with the biological buffer HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid). All three light chain complementarity-determining regions (CDRs) and HCDR3 contribute to binding. The HEPES sulfonate hydrogen bonds to His L91, Asn L50, and to the backbone of Tyr H100 and Tyr H100A. The Tyr side-chains of L32, L92, H100, and H100A form nonpolar contacts with the HEPES ethylene and piperazine groups. Comparison to the DNA-1/dT5 structure reveals that the dual recognition of dT5 and HEPES requires a 13-Å movement of HCDR3. This dramatic structural change converts the combining site from a narrow groove, appropriate for the edge-on insertion of thymine bases, to one sufficiently wide to accommodate the HEPES sulfonate and piperazine. Isothermal titration calorimetry verified the association of HEPES with DNA-1 under conditions similar those used for crystallization (2 M ammonium sulfate). Interestingly, the presence of 2 M ammonium sulfate increases the affinities of DNA-1 for both HEPES and dT5, suggesting that non-polar Fab-ligand interactions are important for molecular recognition in highly ionic solvent conditions. The structural and thermodynamic data suggest a molecular mimicry mechanism based on structural plasticity and hydrophobic interactions.

Shick K. A., Xavier K. A., Rajpal A., Smith-Gill S. J., and Willson R. C. (1997) Association of the anti-hen egg lysozyme antibody HyHEL-5 with avian species variant and mutant lysozymes. *Biochim Biophys Acta* **1340**, 205-214.

Abstract: The energetics of association of the murine anti-hen egg lysozyme antibody HyHEL-5 with bobwhite quail lysozyme, California quail lysozyme, and the Arg45→Lys mutant of hen egg lysozyme was characterized by isothermal titration calorimetry. The association of each lysozyme with HyHEL-5 is enthalpically driven in the temperature range 10 degrees C to 37 degrees C. The calorimetric results indicate that the salt-links between Arg45 and Arg68 of hen egg lysozyme and GluH50 on the HyHEL-5 paratope are energetically important in HyHEL-5/HEL association. In contrast to previous studies, the results suggest that the three characteristic 'quail' mutations affect the energetics of antibody/antigen association, even though they are buried and not in direct contact with the antibody.

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

Abstract: In order to investigate the role of the constant domains on the antigen-binding property of the variable domains, we have carried out a comparative thermodynamic study of the anti-dansyl Fv, Fab* and Fab fragments that possess the identical amino acid sequence of the variable domains. The thermodynamic analyses have shown that binding constants, enthalpy changes and entropy changes are similar for the three antigen-binding fragments, whereas the thermal stability of Fab is much higher than that of Fv and Fab*. We have concluded that (i) the variable domains of the three antigen-binding fragments possess identical intrinsic capability for antigen binding and (ii) the two constant domains serve to improve the stability of the variable domains.

Sigurskjold B. W., Altman E., and Bundle D. R. (1991) Sensitive titration microcalorimetric study of the binding of Salmonella O-antigenic oligosaccharides by a monoclonal antibody. *Eur J Biochem* **197**, 239-246.

Abstract: The binding of several oligosaccharide haptens by a monoclonal antibody, Se155-4, specific for Salmonella serogroup B O-antigen was studied by titration microcalorimetry. In the software developed by Wiseman et al. [Wiseman, T., Williston, S. & Brandts, J.F. (1989) *Anal. Biochem.* **17**, 131-137] the number of binding sites/macromolecule is one of the optional regression parameters in the non-linear least-squares analysis of the calorimetric data. Instead, an approach was adopted in which the concentration of binding sites was treated as a regression parameter, obviating the requirement for precise values of antibody absorption coefficients and minimizing effects due to partially inactive antibody preparations. Furthermore, performing the least-squares analysis in two steps, first using a differential heat mode and then an integral heat mode, was shown to yield the most accurate results. The technique gave accurate results using not more than 1-2 μmol ligand and less than 7 mg antibody. Haptens 2-5 were oligomers of the O-antigenic repeating unit varying in chain length by 2-5 repeating units and a trisaccharide glycoside 1, which filled the binding site. The latter hapten exhibited a favourable entropy contribution to binding ($\Delta G_o = -31 \text{ kJ.mol}^{-1}$; $\Delta H_o = -21 \text{ kJ.mol}^{-1}$ and $-\Delta S_o = -10 \text{ kJ.mol}^{-1}$), while all four oligomers 2-5 showed a

constant binding energy $\Delta G_0 = -33 \text{ kJ.mol}^{-1}$, composed of increasingly stronger enthalpy forces compensated by an increasingly unfavourable entropy contribution. These observations are compared with results from enzyme immunoassays and a high-resolution crystal structure for the dodecasaccharide 3 bound to the Fab derived from Se155-4.

Sigurskjold B. W. and Bundle D. R. (1992) Thermodynamics of oligosaccharide binding to a monoclonal antibody specific for a Salmonella O-antigen point to hydrophobic interactions in the binding site. *J Biol Chem* **267**, 8371-8376.

Abstract: The thermodynamic characteristics of oligosaccharide binding to an antibody binding site that is dominated by aromatic amino acids suggest that the hydrophobic effect contributes substantially to complex formation as well as hydrogen bonding and van der Waals interactions. A detailed titration microcalorimetric study on the temperature dependence of the binding of a trisaccharide, representing the epitope of a Salmonella O-antigen, showed that its maximum binding to the monoclonal antibody Se155-4 occurs just below room temperature and both enthalpy and entropy changes are strongly dependent on temperature in a mutually compensating manner. The heat capacity change also shows an unusually strong temperature dependence being large and negative above room temperature and positive below. van't Hoff analysis of the temperature dependence of the binding constant yielded a biphasic curve with two apparent intrinsic enthalpy estimations (approximately -100 kJ mol^{-1} above 18 degrees C and approximately $+100 \text{ kJ mol}^{-1}$ below), each very different from the calorimetrically determined enthalpies (ranging from about -60 kJ mol^{-1} to -20 kJ mol^{-1}). This was interpreted as being due to large enthalpy contributions from concomitant reactions, most notably changes in solvation. Linear plots, $-\Delta H_0$ versus $-T\Delta S_0$, observed for temperature-dependent measurements mirror the behavior seen for a series of functional group replacements, suggesting that the molecular and physical origin of these phenomena are closely related and linked to the role of water in complex formation. The thermodynamic results are compared to the mode of binding determined from a 2.05-Å resolution structure of the Fab-oligosaccharide complex, and with literature data for the heat capacities of sugars in aqueous solution and for the thermodynamics of carbohydrate binding to transport proteins and lectins.

Starovasnik M. A., O'Connell M. P., Fairbrother W. J., and Kelley R. F. (1999) Antibody variable region binding by Staphylococcal protein A: thermodynamic analysis and location of the Fv binding site on E-domain. *Protein Sci* **8**, 1423-1431.

Abstract: Immunoglobulins of human heavy chain subgroup III have a binding site for Staphylococcal protein A on the heavy chain variable domain (V(H)), in addition to the well-known binding site on the Fc portion of the antibody. Thermodynamic characterization of this binding event and localization of the Fv-binding site on a domain of protein A is described. Isothermal titration calorimetry (ITC) was used to characterize the interaction between protein A or fragments of protein A and variants of the hu4D5 antibody Fab fragment. Analysis of binding isotherms obtained for titration of hu4D5 Fab with intact protein A suggests that 3-4 of the five immunoglobulin binding domains of full length protein A can bind simultaneously to Fab with a K_a of $5.5 \pm 0.5 \times 10^5 \text{ M}^{-1}$. A synthetic single immunoglobulin binding domain, Z-domain, does not bind appreciably to hu4D5 Fab, but both the E and D domains are functional for hu4D5 Fab binding. Thermodynamic parameters for titration of the E-domain with hu4D5 Fab are $n = 1.0 \pm 0.1$, $K_a = 2.0 \pm 0.3 \times 10^5 \text{ M}^{-1}$, and $\Delta H = -7.1 \pm 0.4 \text{ kcal mol}^{-1}$. Similar binding thermodynamics are obtained for titration of the isolated V(H) domain with E-domain indicating that the E-domain binding site on Fab resides within V(H). E-domain binding to an IgG1 Fc yields a higher affinity interaction with thermodynamic parameters $n = 2.2 \pm 0.1$, $K_a > 1.0 \times 10^7 \text{ M}^{-1}$, and $\Delta H = -24.6 \pm 0.6 \text{ kcal mol}^{-1}$. Fc does not compete with Fab for binding to E-domain indicating that the two antibody fragments bind to different sites. Amide 1H and 15N resonances that undergo large changes in NMR chemical shift upon Fv binding map to a surface defined by helix-2 and helix-3 of E-domain, distinct from the Fc-binding site observed in the crystal structure of the B-domain/Fc complex. The Fv-binding region contains negatively charged residues and a small hydrophobic patch which complements the basic surface of the region of the V(H) domain implicated previously in protein A binding.

Sundberg E. J., Urrutia M., Braden B. C., Isern J., Tsuchiya D., Fields B. A., Malchiodi E. L., Tormo J., Schwarz F. P., and Mariuzza R. A. (2000) Estimation of the hydrophobic effect in an antigen-antibody protein-protein interface. *Biochemistry* **39**, 15375-15387.

Abstract: Antigen-antibody complexes provide useful models for analyzing the thermodynamics of

protein-protein association reactions. We have employed site-directed mutagenesis, X-ray crystallography, and isothermal titration calorimetry to investigate the role of hydrophobic interactions in stabilizing the complex between the Fv fragment of the anti-hen egg white lysozyme (HEL) antibody D1.3 and HEL. Crystal structures of six FvD1.3-HEL mutant complexes in which an interface tryptophan residue (V(L)W92) has been replaced by residues with smaller side chains (alanine, serine, valine, aspartate, histidine, and phenylalanine) were determined to resolutions between 1.75 and 2.00 Å. In the wild-type complex, V(L)W92 occupies a large hydrophobic pocket on the surface of HEL and constitutes an energetic "hot spot" for antigen binding. The losses in apolar buried surface area in the mutant complexes, relative to wild-type, range from 25 (V(L)F92) to 115 Å² (V(L)A92), with no significant shifts in the positions of protein atoms at the mutation site for any of the complexes except V(L)A92, where there is a peptide flip. The affinities of the mutant Fv fragments for HEL are 10-100-fold lower than that of the original antibody. Formation of all six mutant complexes is marked by a decrease in binding enthalpy that exceeds the decrease in binding free energy, such that the loss in enthalpy is partly offset by a compensating gain in entropy. No correlation was observed between decreases in apolar, polar, or aggregate (sum of the apolar and polar) buried surface area in the V(L)92 mutant series and changes in the enthalpy of formation. Conversely, there exist linear correlations between losses of apolar buried surface area and decreases in binding free energy ($R(2) = 0.937$) as well as increases in the solvent portion of the entropy of binding ($R(2) = 0.909$). The correlation between binding free energy and apolar buried surface area corresponds to 21 cal mol⁻¹ Å⁻² (1 cal = 4.185 J) for the effective hydrophobicity at the V(L)92 mutation site. Furthermore, the slope of the line defined by the correlation between changes in binding free energy and solvent entropy approaches unity, demonstrating that the exclusion of solvent from the binding interface is the predominant energetic factor in the formation of this protein complex. Our estimate of the hydrophobic contribution to binding at site V(L)92 in the D1.3-HEL interface is consistent with values for the hydrophobic effect derived from classical hydrocarbon solubility models. We also show how residue V(L)W92 can contribute significantly less to stabilization when buried in a more polar pocket, illustrating the dependence of the hydrophobic effect on local environment at different sites in a protein-protein interface.

Swaminathan C. P., Nandi A., Visweswariah S. S., and Surolia A. (1999) Thermodynamic analyses reveal role of water release in epitope recognition by a monoclonal antibody against the human guanylyl cyclase C receptor. *J Biol Chem* **274**, 31272-31278.

Abstract: The thermodynamics of a monoclonal antibody (mAb)-peptide interaction have been characterized by isothermal titration microcalorimetry. GCC:B10 mAb, generated against human guanylyl cyclase C, a membrane-associated receptor and a potential marker for metastatic colon cancer, recognizes the cognate peptide epitope HIPPENIFPLE and its two contiguous mimotopes, HIPPEN and ENIFPLE, specifically and reversibly. The exothermic binding reactions between 6.4 and 42 degrees C are driven by dominant favorable enthalpic contributions between 20 and 42 degrees C, with a large negative heat capacity (ΔC_p) of -421 +/- 27 cal mol⁻¹ K⁻¹. The unfavorable negative value of entropy ($\Delta S(b)(0)$) at 25 degrees C, an unusual feature among protein-protein interactions, becomes a positive one below an inversion temperature of 20.5 degrees C. Enthalpy-entropy compensation due to solvent reorganization accounts for an essentially unchanged free energy of interaction ($\Delta\Delta G(b)(0)$ congruent with 0). The role of water molecules in the recognition process was tested by coupling an osmotic stress technique with isothermal titration microcalorimetry. The results provide direct and compelling evidence that GCC:B10 mAb recognizes the peptides HIPPENIFPLE, HIPPEN, and ENIFPLE differentially, with a concomitant release of variable and nonadditive numbers of water molecules (15, 7, and 3, respectively) from the vicinity of the binding site.

Tello D., Goldbaum F. A., Mariuzza R. A., Ysern X., Schwarz F. P., and Poljak R. J. (1993) Three-dimensional structure and thermodynamics of antigen binding by anti-lysozyme antibodies. *Biochem Soc Trans* **21**, 943-946.

Tello D., Eisenstein E., Schwarz F. P., Goldbaum F. A., Fields B. A., Mariuzza R. A., and Poljak R. J. (1994) Structural and physicochemical analysis of the reaction between the anti-lysozyme antibody D1.3 and the anti-idiotopic antibodies E225 and E5.2. *J Mol Recognit* **7**, 57-62.

Abstract: The reaction between the mouse (BALB/c) anti-idiotopic monoclonal antibodies E225 and E5.2 and idiotopes on the (BALB/c) anti-lysozyme monoclonal antibody D1.3 has been characterized by

titration calorimetry, by equilibrium sedimentation and by the determination of binding association and dissociation rates. The reaction between E5.2 and D1.3 is driven by a large negative enthalpy and its rate and equilibrium association constants are comparable to those observed in other antigen-antibody reactions. In contrast, the reaction between E225 and D1.3 is entropically driven and characterized by slow association kinetics ($1 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$) and a resulting low equilibrium constant ($K_a = 2 \times 10^5 \text{ M}^{-1}$). A correlation of these properties with the three-dimensional structure of the Fab225-FabD1.3 complex, previously determined by X-ray diffraction methods to 2.5 Å resolution, indicates that conformational changes of several D1.3 contacting residues, located in its complementarity determining regions, may explain these features of the reaction.

Thielges M. C., Zimmermann J., Yu W., Oda M. and Romesberg F. E. (2008) Exploring the energy landscape of antibody-antigen complexes: protein dynamics, flexibility, and molecular recognition. *Biochemistry* **47**, 7237-7247.

Abstract: The production of antibodies that selectively bind virtually any foreign compound is the hallmark of the immune system. While much is understood about how sequence diversity contributes to this remarkable feat of molecular recognition, little is known about how sequence diversity impacts antibody dynamics, which is also expected to contribute to molecular recognition. Toward this goal, we examined a panel of antibodies elicited to the chromophoric antigen fluorescein. On the basis of isothermal titration calorimetry, we selected six antibodies that bind fluorescein with diverse binding entropies, suggestive of varying contributions of dynamics to molecular recognition. Sequencing revealed that two pairs of antibodies employ homologous heavy chains that were derived from common germline genes, while the other two heavy chains and all six of the light chains were derived from different germline genes and are not homologous. Interestingly, more than half of all the somatic mutations acquired during affinity maturation among the six antibodies are located in positions unlikely to contact fluorescein directly. To quantify and compare the dynamics of the antibody-fluorescein complexes, three-pulse photon echo peak shift and transient grating spectroscopy were employed. All of the antibodies exhibited motions on three distinct time scales, ultrafast motions on the <100 fs time scale, diffusive motions on the picosecond time scale, and motions that occur on time scales longer than nanoseconds and thus appear static. However, the exact frequency of the picosecond time scale motion and the relative contribution of the different motions vary significantly among the antibody-chromophore complexes, revealing a high level of dynamic diversity. Using a hierarchical model, we relate the data to features of the antibodies' energy landscapes as well as their flexibility in terms of elasticity and plasticity. In all, the data provide a consistent picture of antibody flexibility, which interestingly appears to be correlated with binding entropy as well as with germline gene use and the mutations introduced during affinity maturation. The data also provide a gauge of the dynamic diversity of the antibody repertoire and suggest that this diversity might contribute to molecular recognition by facilitating the recognition of the broadest range of foreign molecules

Torigoe H., Nakayama T., Imazato M., Shimada I., Arata Y., and Sarai A. (1995) The affinity maturation of anti-4-hydroxy-3-nitrophenylacetyl mouse monoclonal antibody. A calorimetric study of the antigen-antibody interaction. *J Biol Chem* **270**, 22218-22222.

Abstract: To understand the mechanism of affinity maturation, we examined the antigen-antibody interactions between 4-hydroxy-3-nitrophenylacetyl (NP) caproic acid and the Fab fragments of three anti-NP antibodies, N1G9, 3B44, and 3B62, by isothermal titration calorimetry. The analyses have revealed that all of these interactions are mainly driven by negative changes in enthalpy. The enthalpy changes decreased linearly with temperature in the range of 25-45 degrees C, producing negative changes in heat capacity. On the basis of the dependence of binding constants on the sodium chloride concentration, we have shown that, during the affinity maturation of the anti-NP antibody, the electrostatic effect does not significantly contribute to the increase in the binding affinity. We have found that, as the logarithm of the binding constants increases during the affinity maturation of the anti-NP antibody, the magnitudes of the corresponding enthalpy, heat capacity, and unitary entropy changes increase almost linearly. On the basis of this correlation, we have concluded that, during the affinity maturation of the anti-NP antibody, a better surface complementarity is attained in the specific complex in order to obtain a higher binding affinity.

Tsumoto K., Nakaoki Y., Ueda Y., Ogasahara K., Yutani K., Watanabe K., and Kumagai I. (1994) Effect of the order of antibody variable regions on the expression of the single-chain HyHEL10 Fv fragment in *E. coli* and the thermodynamic analysis of its antigen-binding properties. *Biochem Biophys Res Commun* **201**,

546-551.

Abstract: In order to physically stabilize the Fv fragment of anti-lysozyme monoclonal antibody, HyHEL10, the variable domains were linked covalently with a flexible linker. A marked difference in the level of expression in *E. coli* was observed between VH-linker-VL (scFvHL) and VL-linker-VH (scFvLH). The highly expressed scFvLH was purified by a single step of affinity chromatography from the culture supernatant with a typical yield of 3-5 mg per liter of culture. This HyHEL10 scFvLH showed reduced binding activity toward its antigen, HEL, in comparison with Fv. Thermodynamic study showed that this reduced activity was due to entropic loss upon binding to its antigen, although this interaction between scFvLH and its antigen was enthalpically favorable.

Tsumoto K., Ueda Y., Maenaka K., Watanabe K., Ogasahara K., Yutani K., and Kumagai I. (1994) Contribution to antibody-antigen interaction of structurally perturbed antigenic residues upon antibody binding. *J Biol Chem* **269**, 28777-28782.

Abstract: For elucidating the contribution of structurally perturbed antigenic residues upon antibody binding to antigen-antibody interaction, the interaction between hen egg white lysozyme (HEL) and HyHEL10 Fv fragment, which is one of several monoclonal antibodies against HEL and structurally well defined (Padlan, E.A., Silverton, E. W., Sheriff, S., Cohen, G. H., Smith-Gill, S. J., and Davies, D. R. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 5938-5942), was investigated. Asp-101 and Trp-62 of HEL, whose conformations are perturbed by the binding of antibody HyHEL10 in this interaction, were replaced with Gly, and the resulting interactions were studied by assay of the inhibition of the lysozyme activity with the Fv fragment and by titration calorimetry. The results can be summarized as follows. 1) It was possible to prepare the fully functional Fv fragment of HyHEL10 using a secretory expression system in *Escherichia coli*. Its inhibition profile for HEL activity was almost indistinguishable from that of HyHEL10 IgG, and the contribution of enthalpy to driving the interaction was shown to be significant. 2) A thermodynamic study of the interaction between the D101G mutant HEL and the Fv fragment revealed that, although the negative enthalpy change was smaller than that for the wild type, the Gibbs energy was almost identical to that of the wild type, which resulted from the smaller entropy loss. 3) Study of the interaction between the W62G mutant HEL and this Fv fragment indicated that the rotation of the Trp-62 indole ring upon binding of the antibody made an enthalpic contribution to antibody-antigen interaction, although Trp-62 of HEL was proposed not to be the direct contact residue in the HyHEL10.HEL complex. 4) From these results, it was confirmed experimentally that structural perturbations of antigenic residues upon antibody binding of antigen would contribute to the gain of enthalpic energy, in spite of partial offset by entropic loss, and to driving the interaction.

Tsumoto K., Ogasahara K., Ueda Y., Watanabe K., Yutani K., and Kumagai I. (1995) Role of Tyr residues in the contact region of anti-lysozyme monoclonal antibody HyHEL10 for antigen binding. *J Biol Chem* **270**, 18551-18557.

Abstract: It has been shown that Tyr residues are unusually localized in the regions of antibodies responsible for contact with antigens (Padlan, E. A. (1990) *Proteins Struct. Funct. Genet.* 7, 112-124). In order to clarify the role of these Tyr residues in antigen binding, the interaction between hen egg white lysozyme (HEL) and its monoclonal antibody HyHEL10, whose structure has been well studied in complex with its antigen, was investigated. Four Tyr residues in the VH chain (HTyr-33, HTyr-50, HTyr-53, and HTyr-58) were replaced with Ala, Leu, Phe, or Trp, and the interactions between these mutant Fv fragments and HEL were studied by inhibition assay of the enzymatic activity of HEL and isothermal titration calorimetry. Twelve mutant Fv fragments could be expressed, but two mutants (HY50W and HY58W) could not be obtained in the *Escherichia coli* expression system, and a further two mutants (HY33A and HY50A) could not be purified by affinity chromatography. It was shown by inhibition assay that Tyr residues at each mutated site made positive contributions to the interaction to different degrees. Thermodynamic studies showed that the role of Tyr residues in antigen binding was to obtain enthalpic energy. The roles of Tyr residues in antibody HyHEL10 for the association with antigen, HEL, can be summarized as follows: 1) formation of hydrogen bonds by the hydroxyl group, 2) creating more favorable interactions through the aromatic ring and decreasing the entropic loss upon binding, and 3) allowing hydrophobic interaction through the side chain. The four Tyr residues studied here were found to play significant roles in the association in various ways.

Tsumoto K., Ogasahara K., Ueda Y., Watanabe K., Yutani K., and Kumagai I. (1996) Role of salt bridge formation in antigen-antibody interaction. Entropic contribution to the complex between hen egg white lysozyme and its monoclonal antibody HyHEL10. *J Biol Chem* **271**, 32612-32616.

Abstract: For elucidation of the role of salt bridge formation in the antigen-antibody complex, the interaction between hen egg white lysozyme (HEL) and its monoclonal antibody HyHEL10, the structure of which has been well characterized and forms one salt bridge (Lys97 of HEL and Asp32 of HyHEL10 heavy chain variable region (VH)), was investigated. Asp32 of VH was substituted with Ala, Asn, or Glu by site-directed mutagenesis, and the interaction between HEL and the mutant fragments of the variable region of light chain was investigated by inhibition of the enzymatic activity of HEL and isothermal titration calorimetry. Inhibition assay indicated that these mutations lowered the inhibition only slightly. Thermodynamic study indicated that the negative enthalpic change in the interaction between each of the mutant variable regions of light chain and HEL was significantly increased, although the association constant was slightly decreased, suggesting that these mutations increased the entropy change upon antigen-antibody binding. These results indicate that the role of salt bridge formation in the HyHEL10-HEL interaction is to lower the entropic loss due to binding. In the mutant proteins, the numbers of residues that were perturbed structurally on binding increased, suggesting that the salt bridge suppresses excess structural movement of the antibody upon binding.

Tsumoto K., Nishimiya Y., Kasai N., Ueda H., Nagamune T., Ogasahara K., Yutani K., Tokuhisa K., Matsushima M., and Kumagai I. (1997) Novel selection method for engineered antibodies using the mechanism of Fv fragment stabilization in the presence of antigen. *Protein Eng* **10**, 1311-1318.

Abstract: Although the heavy and light chain domains of some antibody variable region fragments (Fvs) readily dissociate under physiological conditions, the Fvs are stable in the presence of antigen. This 'antigen-driven Fv stabilization mechanism' was applied to the selection of clones with specificity toward target antigens. The results can be summarized as follows. (i) Some of the residues in the heavy chain complementarity determining region 2 (HCDR2) of anti-hen egg white lysozyme (HEL) monoclonal antibody HyHEL10 heavy chain variable region (VH) were randomized. (ii) The randomized VH fragments of HyHEL10 were displayed on a filamentous bacteriophage and mixed with the target antigen, before being applied to a light chain variable region (VL) which was immobilized on microtiter plates and subjected to selection by panning. (iii) After four rounds of panning, four clones that showed significant binding to human lysozyme (hL), which HyHEL10 recognized poorly, were selected from the HCDR2 library. (iv) The soluble Fv fragments selected were expressed in *Escherichia coli*, purified, and subjected to an inhibition assay of lysozyme enzymatic activities and an isothermal titration calorimetry. These Fv fragments had increased affinity toward hL, and thermodynamic analysis suggested that the reduced entropy loss due to binding by the replacement of residues in HCDR2 resulted in the higher hL binding activity.

VanAntwerp J. J. and Wittrup K. D. (1998) Thermodynamic characterization of affinity maturation: the D1.3 antibody and a higher-affinity mutant. *J Mol Recognit* **11**, 10-13.

Abstract: Understanding the structural and dynamic determinants of binding free energy in the antigen-antibody bond is of great interest. Much work has focused on selective mutations in order to locate key interaction residues, but this generally results in reduced affinity. The present work instead examines a higher-affinity mutant to characterize the thermodynamic pathway of the affinity maturation process. We have compared the antigen binding energetics of scFv D1.3, an anti-hen egg lysozyme single chain antibody, with a higher-affinity mutant (Hawkins, R. E., Russell, S. J., Baier, M. and Winter, G. (1993). *J. Mol. Biol.* 234, 958-964). The mutant has five-fold higher affinity for lysozyme but nearly the same enthalpy and heat capacity change upon binding, as measured by isothermal titration calorimetry. Thus, much of the binding free energy difference can be attributed to entropic effects. Fluorescence quenching with acrylamide indicates that this more favorable entropy change may result from a more flexible mutant-lysozyme complex and thus be a configurational entropy effect.

van Roon A. M., Pannu N. S., de Vrind J. P., van der Marel G. A., van Boom J. H., Hokke C. H., Deelder A. M., and Abrahams J. P. (2004) Structure of an anti-Lewis X Fab fragment in complex with its Lewis X antigen. *Structure (Camb)* **12**, 1227-1236.

Abstract: The Lewis X trisaccharide is pivotal in mediating specific cell-cell interactions. Monoclonal antibody 291-2G3-A, which was generated from mice infected with schistosomes, has been shown to

recognize the Lewis X trisaccharide. Here we describe the structure of the Fab fragment of 291-2G3-A, with Lewis X, to 1.8 Å resolution. The crystallographic analysis revealed that the antigen binding site is a rather shallow binding pocket, and residues from all six complementary determining regions of the antibody contact all sugar residues. The high specificity of the binding pocket does not result in high affinity; the K_D determined by isothermal calorimetry is 11 μ M. However, this affinity is in the same range as for other sugar-antibody complexes. The detailed understanding of the antibody-Lewis X interaction revealed by the crystal structure may be helpful in the design of better diagnostic tools for schistosomiasis and for studying Lewis X-mediated cell-cell interactions by antibody interference.

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.

Vyas N. K., Vyas M. N., Chervenak M. C., Bundle D. R., Pinto B. M., and Quijcho F. A. (2003) Structural basis of peptide-carbohydrate mimicry in an antibody-combining site. *Proc Natl Acad Sci U S A* **100**, 15023-15028.

Abstract: The structure of a complex between the Fab fragment of the antibody (SYA/J6) specific for the cell surface O-antigen polysaccharide of the pathogen *Shigella flexneri* Y and an octapeptide (Met-Asp-Trp-Asn-Met-His-Ala-Ala), a functional mimic of the O-antigen, has been determined at 1.8-Å resolution. Comparison of the structure with that of the complex with the pentasaccharide antigen [\rightarrow 2]- α -l-Rha-(1 \rightarrow 2)- α -l-Rha-(1 \rightarrow 3)- α -l-Rha-(1 \rightarrow 3)- β -d-GlcNAc-(1 \rightarrow 2)- α -l-Rha-(1 \rightarrow) reveals the molecular recognition process by which a peptide mimics a carbohydrate in binding to an antibody. The binding modes of the two ligands differ considerably. Octapeptide binding complements the shape of the combining site groove much better than pentasaccharide binding. Moreover, the peptide makes a much greater number of contacts (126), which are mostly van der Waals interactions, with the Fab than the saccharide (74). An unusual feature is also the involvement of 12 water molecules in mediating hydrogen bonds between residues within the peptide or of the peptide and Fab. Despite better shape complementarity and greater number of contacts, the octapeptide binds with an affinity ($K_A = 2.5 \times 10^5 \text{ M}^{-1}$, measured by calorimetry) only approximately 2-fold tighter than the pentasaccharide. The structural results are relevant to the design of peptide mimetics with improved affinity for use as vaccines.

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 $-1.27 \pm 0.14 \text{ kJ mol}^{-1} \text{ K}^{-1}$. 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 $1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. 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., Sabat R., Volk H. D., Schneider-Mergener J., Reineke U., and Welfle H. (2001) Interaction of a designed interleukin-10 epitope mimic with an antibody studied by isothermal titration microcalorimetry. *J Mol Recognit* **14**, 89-98.

Abstract: The mechanism of recognition of proteins and peptides by antibodies and the factors determining binding affinity and specificity are mediated by essentially the same features. However, additional effects of the usually unfolded and flexible solution structure of peptide ligands have to be considered. In an earlier study we designed and optimized six peptides (pepI to pepVI) mimicking the discontinuous binding site of interleukin-10 for the anti-interleukin-10 monoclonal antibody (mab) CB/RS/1. Three of them were selected for analysis of their solution conformation by circular dichroism measurements. The peptides differ in the content of alpha-helices and in the inducibility of helical secondary structures by trifluoroethanol. These properties, however, do not correlate with the binding affinity. PepVI, a 32-mer cyclic epitope mimic, has the highest affinity to mab CB/RS/1 identified to date. CD difference spectroscopy suggests an increase of the alpha-helix content of pepVI with complex formation. Binding of pepVI to mab CB/RS/1 is characterized by a large negative, favorable binding enthalpy and a smaller unfavorable loss of entropy (ΔH degrees = $-16.4 \text{ kcal} \times \text{mol}^{-1}$, ΔS degrees = $-6.9 \text{ kcal} \times \text{mol}^{-1}$) resulting in ΔG degrees = $-9.5 \text{ kcal} \times \text{mol}^{-1}$ at 25 degrees C as determined by isothermal titration calorimetry. Binding of pepVI is enthalpically driven over the entire temperature range studied (10-35 degrees C). Complex formation is not accompanied by proton uptake or release. A negative heat capacity change ΔC_p of $-0.354 \text{ kcal} \times \text{mol}^{-1} \times \text{K}^{-1}$ was determined from the temperature dependence of ΔH degrees. The selection of protein mimics with the observed thermodynamic properties is promoted by the applied identification and iterative optimization procedure.

Welfle K., Misselwitz R., Hohne W., and Welfle H. (2003) Interaction of epitope-related and -unrelated peptides with anti-p24 (HIV-1) monoclonal antibody CB4-1 and its Fab fragment. *J Mol Recognit* **16**, 54-62.

Abstract: The binding of four epitope-related peptides and three library-derived, epitope-unrelated peptides of different lengths (10-14 amino acids) and sequence by anti-p24 (HIV-1) monoclonal antibody CB4-1 and its Fab fragment was studied by isothermal titration calorimetry. The binding constants $K(A)$ at 25 degrees C vary between $5.1 \times 10^7 \text{ M}^{-1}$ for the strongest and $1.4 \times 10^5 \text{ M}^{-1}$ for the weakest binder. For each of the peptides complex formation is enthalpically driven and connected with unfavorable entropic contributions; however, the ratio of enthalpy and entropy contributions to ΔG_0 differs markedly for the

individual peptides. A plot of $-\Delta H_0$ vs $-T\Delta S_0$ shows a linear correlation of the data for a wide variety of experimental conditions as expected for a process with ΔC_p much larger than ΔS_0 . The dissimilarity of ΔC_p and ΔS_0 also explains why ΔH_0 and $T\Delta S_0$ show similar temperature dependences resulting in relatively small changes of ΔG_0 with temperature. The heat capacity changes ΔC_p upon antibody-peptide complex formation determined for three selected peptides vary only in a small range, indicating basic thermodynamic similarity despite different key residues interacting in the complexes. Furthermore, the comparison of van't Hoff and calorimetric enthalpies point to a non-two-state binding mechanism. Protonation effects were excluded by measurements in buffers of different ionization enthalpies. Differences in the solution conformation of the peptides as demonstrated by circular dichroic measurements do not explain different binding affinities of the peptides; specifically a high helix content in solution is not essential for high binding affinity despite the helical epitope conformation in the crystal structure of p24.

Wibbenmeyer J. A., Xavier K. A., Smith-Gill S. J., and Willson R. C. (1999) Cloning, expression, and characterization of the Fab fragment of the anti-lysozyme antibody HyHEL-5. *Biochim Biophys Acta* **1430**, 191-202.

Abstract: Hybridoma cDNAs encoding the individual chains of the Fab fragment of the well characterized murine monoclonal antibody HyHEL-5 were cloned and sequenced. The recombinant Fab fragment was produced by expressing each chain in a separate *Escherichia coli* pET vector, denaturing inclusion bodies and co-refolding. Characterization of the purified Fab by MALDI-TOF mass spectrometry and N-terminal amino acid sequencing demonstrated proper processing of the individual chains. The association of the recombinant Fab fragment with hen egg lysozyme and the avian epitope variant bobwhite quail lysozyme was found by isothermal titration calorimetry to have energetics very similar to that of the HyHEL-5 IgG. Heterologous expression of the HyHEL-5 Fab fragment opens the way to structure/function studies in this well-known system.

Wibbenmeyer J. A., Schuck P., Smith-Gill S. J., and Willson R. C. (1999) Salt links dominate affinity of antibody HyHEL-5 for lysozyme through enthalpic contributions. *J Biol Chem* **274**, 26838-26842.

Abstract: The binding of murine monoclonal antibody HyHEL-5 to lysozyme has been the subject of extensive crystallographic, computational, and experimental investigations. The complex of HyHEL-5 with hen egg lysozyme (HEL) features salt bridges between Fab heavy chain residue Glu(50), and Arg(45) and Arg(68) of HEL. This interaction has been predicted to play a dominant role in the association on the basis of molecular electrostatics calculations. The association of aspartic acid and glutamine mutants at position 50(H) of the cloned HyHEL-5 Fab with HEL and bobwhite quail lysozyme (BQL), an avian variant bearing an Arg(68) \rightarrow Lys substitution in the epitope, was characterized by isothermal titration calorimetry and sedimentation equilibrium. Affinities for HEL were reduced by 400-fold (E50(H)D) and 40,000-fold (E50(H)Q) ($\Delta\Delta G$ degrees estimated at 4.0 and 6.4 kcal mol⁻¹, respectively). The same mutations reduce affinity for BQL by only 7- and 55-fold, respectively, indicating a reduced specificity for HEL. The loss of affinity upon mutation is in each case primarily due to an unfavorable change in the enthalpy of the interaction; the entropic contribution is virtually unchanged. An enthalpy-entropy compensation exists for each interaction; ΔH degrees decreases, while ΔS degrees increases with temperature. The ΔC_p for each mutant interaction is less negative than the wild-type. Mutant-cycle analysis suggests the mutations present in the HyHEL-5 Fab mutants are linked to those present in the BQL with coupling energies between 3 and 4 kcal mol⁻¹.

Wright A. J., Higginbottom A., Philippe D., Upadhyay A., Bagby S., Read R. C., Monk P. N. and Partridge L. J. (2007) Characterisation of receptor binding by the chemotaxis inhibitory protein of *Staphylococcus aureus* and the effects of the host immune response. *Mol Immunol.* **44**, 2507-2517.

Abstract: The chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS) is reported to bind to the receptors for C5a and formylated peptides and has been proposed as a promising lead for the development of new anti-inflammatory compounds. Here we have examined the receptor specificity and mode of action of recombinant CHIPS(28-149) and also the immune response to CHIPS(28-149) in patients with *S. aureus* infections and in uninfected controls. Recombinant CHIPS(28-149) bound with high affinity to the human C5a receptor (C5aR), but had low affinity for the second C5a receptor, C5L2, and the formyl peptide receptor, FPR. Although ligand binding to C5aR was potently inhibited, CHIPS(28-149) had much weaker effects on ligand binding to C5L2 and FPR. Similarly, CHIPS(28-149) potently inhibited the ligand-induced activation of C5aR but was less potent at inhibition via FPR. NMR studies showed that CHIPS(28-

149) bound directly to the N-terminus of C5aR but not C5L2, and CHIPS(28-149) residues involved in the interaction were identified by chemical shift analysis. All human sera examined contained high titres of IgG and IgA reactivity against CHIPS(28-149), and no correlation was observed between infection status at the time of serum collection and antibody titre. Individual serum samples promoted or inhibited the binding of CHIPS(28-149) to C5aR, or had no effect. IgG depletion of serum samples abrogated the effects on CHIPS binding, demonstrating that these were antibody mediated. Sera from infected individuals were more likely to inhibit CHIPS(28-149) binding than sera from healthy controls. However, high antibody titres correlated well with both inhibition and enhancement of CHIPS(28-149) binding to C5aR; this suggests that the inhibitory effect relates to epitope specificity rather than greater antibody binding. We conclude that CHIPS is likely to be too immunogenic to be used as an anti-inflammatory treatment but that some antibodies against CHIPS may be useful in the treatment of *S. aureus* infections.

Xavier K. A., Shick K. A., Smith-Gil S. J., and Willson R. C. (1997) Involvement of water molecules in the association of monoclonal antibody HyHEL-5 with bobwhite quail lysozyme. *Biophys J* **73**, 2116-2125.

Abstract: Fluorescence polarization spectroscopy and isothermal titration calorimetry were used to study the influence of osmolytes on the association of the anti-hen egg lysozyme (HEL) monoclonal antibody HyHEL-5 with bobwhite quail lysozyme (BWQL). BWQL is an avian species variant with an Arg \rightarrow Lys mutation in the HyHEL-5 epitope, as well as three other mutations outside the HyHEL-5 structural epitope. This mutation decreases the equilibrium association constant of HyHEL-5 for BWQL by over 1000-fold as compared to HEL. The three-dimensional structure of this complex has been obtained recently. Fluorescein-labeled BWQL, obtained by labeling at pH 7.5 and purified by hydrophobic interaction chromatography, bound HyHEL-5 with an equilibrium association constant close to that determined for unlabeled BWQL by isothermal titration calorimetry. Fluorescence titration, stopped-flow kinetics, and isothermal titration calorimetry experiments using various concentrations of the osmolytes glycerol, ethylene glycol, and betaine to perturb binding gave a lower limit of the uptake of approximately 6-12 water molecules upon formation of the HyHEL-5/BWQL complex.

Xiang S. H., Kwong P. D., Gupta R., Rizzuto C. D., Casper D. J., Wyatt R., Wang L., Hendrickson W. A., Doyle M. L., and Sodroski J. (2002) Mutagenic stabilization and/or disruption of a CD4-bound state reveals distinct conformations of the human immunodeficiency virus type 1 gp120 envelope glycoprotein. *J Virol* **76**, 9888-9899.

Abstract: The human immunodeficiency virus type 1 (HIV-1) gp120 exterior envelope glycoprotein is conformationally flexible. Upon binding to the host cell receptor CD4, gp120 assumes a conformation that is recognized by the second receptor, CCR5 and/or CXCR4, and by the CD4-induced (CD4i) antibodies. Guided by the X-ray crystal structure of a gp120-CD4-CD4i antibody complex, we introduced changes into gp120 that were designed to stabilize or disrupt this conformation. One mutant, 375 S/W, in which the tryptophan indole group is predicted to occupy the Phe 43 cavity in the gp120 interior, apparently favors a gp120 conformation closer to that of the CD4-bound state. The 375 S/W mutant was recognized as well as or better than wild-type gp120 by CD4 and CD4i antibodies, and the large decrease in entropy observed when wild-type gp120 bound CD4 was reduced for the 375 S/W mutant. The recognition of the 375 S/W mutant by CD4BS antibodies, which are directed against the CD4-binding region of gp120, was markedly reduced compared with that of the wild-type gp120. Compared with the wild-type virus, viruses with the 375 S/W envelope glycoproteins were resistant to neutralization by IgG1b12, a CD4BS antibody, were slightly more sensitive to soluble CD4 neutralization and were neutralized more efficiently by the 2G12 antibody. Another mutant, 423 I/P, in which the gp120 bridging sheet was disrupted, did not bind CD4, CCR5, or CD4i antibodies, even though recognition by CD4BS antibodies was efficient. These results indicate that CD4BS antibodies recognize conformations of gp120 different from that recognized by CD4 and CD4i antibodies.

Yokota A., Tsumoto K., Shiroishi M., Kondo H., and Kumagai I. (2003) The role of hydrogen bonding via interfacial water molecules in antigen-antibody complexation. The HyHEL-10-HEL interaction. *J Biol Chem* **278**, 5410-5418.

Abstract: To study the role of hydrogen bonding via interfacial water molecules in protein-protein interactions, we examined the interaction between hen egg white lysozyme (HEL) and its HyHEL-10 variable domain fragment (Fv) antibody. We constructed three antibody mutants (I-Y50F, I-S91A, and I-S93A) and investigated the interactions between the mutant Fvs and HEL. Isothermal titration calorimetry

indicated that the mutations significantly decreased the negative enthalpy change (8-25 kJ mol⁻¹), despite some offset by a favorable entropy change. X-ray crystallography demonstrated that the complexes had nearly identical structures, including the positions of the interfacial water molecules. Taken together, the isothermal titration calorimetric and x-ray crystallographic results indicate that hydrogen bonding via interfacial water enthalpically contributes to the Fv-HEL interaction despite the partial offset because of entropy loss, suggesting that hydrogen bonding stiffens the antigen-antibody complex.

Ysern X., Fields B. A., Bhat T. N., Goldbaum F. A., Dall'Acqua W., Schwarz F. P., Poljak R. J., and Mariuzza R. A. (1994) Solvent rearrangement in an antigen-antibody interface introduced by site-directed mutagenesis of the antibody combining site. *J Mol Biol* **238**, 496-500.

Abstract: The three-dimensional structure of a site-directed mutant of the bacterially expressed Fv fragment from monoclonal antibody D1.3, complexed to the specific antigen lysozyme has been determined to a nominal resolution of 1.8 Å using X-ray diffraction data. The replacement of VL Trp92 by Asp allows two water molecules to occupy space taken by Trp92 in the wild-type complex, in agreement with a previous observation that water molecules play an important role in stabilizing this antigen-antibody complex. The equilibrium constant for the binding of the mutant Fv to the antigen decreases by three orders of magnitude (from $2.3 \times 10^8 \text{ M}^{-1}$ to $2.6 \times 10^5 \text{ M}^{-1}$). Titration calorimetry shows that this results from a smaller negative binding enthalpy ($\Delta \Delta H = -16 \text{ kJ mol}^{-1}$ at 24 degrees C), whereas the value of the binding entropy is not affected. Since in the complex between the mutated Fv and antigen the buried area has decreased relative to that of the wild-type Fv by about 150 Å², the contribution of the buried unit area to the decrease in free energy (ΔG_{zero}) is approximately 117 J mol⁻¹ (28 cal mol⁻¹) per Å². The loss of interatomic contacts in replacing Trp by Asp permits an approximate calculation for the contribution of van der Waals interactions made by Trp92 in this complex, which gives an average of 2.1 kJ mol⁻¹ (0.5 kcal mol⁻¹) for contacts between carbon atoms.

Zeder-Lutz G., Zuber E., Witz J., and Van Regenmortel M. H. (1997) Thermodynamic analysis of antigen-antibody binding using biosensor measurements at different temperatures. *Anal Biochem* **246**, 123-132.

Abstract: The thermodynamic parameters of the interaction between hen egg white lysozyme and Fab D1.3 were determined by measuring the temperature dependence of the ratio of its kinetic association and dissociation rate constants. Biosensor technology (BIAcore 2000) was used to measure the rate constants at temperatures ranging from 5 to 40 degrees C. The value of ΔG degrees at 25 degrees C (-49 kJ M⁻¹) calculated by this method was very close to that obtained previously from fluorescence quenching measurements (-48.5 kJ M⁻¹). However, the value of ΔH degrees measured at 25 degrees C by biosensor technology (-35 kJ M⁻¹) was smaller than that determined previously by microcalorimetry (-90 kJ M⁻¹). Another difference was the limited variation of $\ln K$ and ΔG with temperature observed with BIAcore compared to the steady decrease of $\ln K$ with temperature found by calorimetry. Our data showed that the binding reaction was driven only by enthalpy below 23 degrees C, by enthalpy and entropy between 23 and 35 degrees C, and only by entropy above 35 degrees C. This suggests, inter alia, that the contribution from the enthalpy of hydration due to the water molecules present at the interface in the lysozyme-antibody complex is progressively eliminated as the temperature increases. Whereas calorimetric data pertain to all the components present in the sample, including solvent molecules, BIAcore measurements monitor only the physical association and dissociation of the two macromolecular species. The difference between the two sets of data may also reflect the complexity of the binding mechanism between lysozyme and Fab D1.3.

Zhang R., Bowyer A., Eissenthal R. and Hubble J. (2007) A smart membrane based on an antigen-responsive hydrogel. *Biotechnol. Bioeng.* **97**, 976-984.

Abstract: Hydrogel membranes have been fabricated that incorporate antibody/antigen moieties. The permeability of large solutes through these membranes is dependent on the presence of soluble antigen that can compete with the internal interactions between antibody and antigen leading to an increase in gel mesh size. Specifically, the membrane's structure is based on a dextran backbone grafted with a fluorescein isothiocyanate (FITC) antigen and a sheep anti-FITC IgG antibody. The backbone is covalently cross-linked by conjugated divinyl sulfone (DVS) groups. The gel structure is additionally stabilized by affinity crosslinks formed by biospecific interactions between the bound IgG and FITC. FTIR spectra of the gel are consistent with formation of covalent bonds between cysteine groups in the IgG and DVS groups in the dextran. Results obtained using isothermal titration calorimetry (ITC) confirmed the competitive interaction

binding between IgG-FITC-dextran and free sodium fluorescein at pH 5.0. Scanning electron microscopy (SEM) of samples prepared using cryofixation and cryofracturing techniques showed that observed changes in permeability correlate with free fluorescein-dependent structural changes in the gel. Three-dimensional images obtained from confocal laser scanning microscopy show that these changes occur throughout the gel and indicate that SEM results are not artifacts of sample preparation. The permeability of these gels, as shown by blue-dextran (12 kDa) diffusion, increases in response to the presence of free fluorescein of the external medium, which causes competitive displacement of the affinity cross-links. Sequential addition and removal of sodium fluorescein showed that these permeability changes are reversible.