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ORIGINAL PAPER

Hydrophobic interactions are the driving force for the binding of peptide mimotopes and *Staphylococcal* protein A to recombinant human IgG1

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Abstract We studied the interaction of several nonapeptide 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.

Keywords Isothermal titration calorimetry · Antigen binding · Antibodies · Peptide mimotopes · *Staphylococcal* protein A

Abbreviations

ITC	isothermal titration calorimetry
Ig	immunoglobulin
SpA	*Staphylococcal* protein A

Introduction

Immunoglobulin (Ig) proteins (called antibodies) are produced by the mammalian immune system in order to assume one of two roles: immunoglobulins may act as (a) plasma membrane bound antigen receptors on the surface of a B-cell or (b) as antibodies free in cellular fluids functioning to intercept and eliminate antigenic determinants. The structure of an antibody is composed of three fragments, two identical antigen-binding fragments (Fab) and one Fc fragment. Fc contains the binding site for some effector ligands (e.g., Fc receptors, C1q) that activate the body clearance system. *Staphylococcal* protein A (SpA) is a component of the cell surface of *Staphylococcus aureus*. SpA is a 42 kDa protein and composed of five homologous ~58 residue immunoglobulin-binding domains followed by a C-terminal region required for cell wall attachment (Starovasnik et al. 1999). The biological role of SpA is still not fully clear, however, it is believed that it

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plays a role in enhancing the pathogenicity by binding to the Ig and frustrating the immune system (Starovasnik et al. 1999; Jefferis 2002). Antibodies can have two separate binding sites, with which SpA interacts: the ''classical'' binding site on the Fc region of human IgG1, IgG2, and IgG4 and the ''alternate'' binding site found on the Fab part of human IgG, IgM, IgA, and IgE that contain heavy chains of the V_H3 subfamily (Starovasnik et al. 1999). Immobilized protein A matrices are used in affinity chromatography as a rapid one-step purification of antibodies due to specifically binding of protein A to the Fc region of immunoglobulin molecules. This purification procedure is nowadays routinely used in the biotechnological production of therapeutic antibodies for the removal of non-IgG contaminants and allows to achieve purities close to homogeneity.

The antibody binds via its Fab domains specifically to a particular molecular structure of the antigen called antigenic determinant or epitope. Most studies of antigen–antibody interactions reported so far were carried out using whole immunoglobulins (specifically IgG), Fab regions, and single chain Fv regions (Houk et al. 2003). The binding affinity reflects the gain in Gibbs free energy during the binding process when ionic and hydrogen bonds are formed and hydrophobic and van der Waals interactions are maximized. K_a (binding constant) values of a mature antibody for natural antigens (predominantly immunogenic organic molecules, proteins, carbohydrates, and nucleic acid fragments) fall in a wide range of values between 10^4 and 10^{14} M^{-1}, so that $\Delta G°$ can reach values as high as 80 kJ/mol (Houk et al. 2003).

For most antigen–antibody interactions, the binding is associated with a favorable (exothermic) enthalpy change brought by the formation of non-covalent bonds but an unfavorable (negative) entropy change associated with the loss of translational and internal degrees of freedom. By increasing the temperature, $\Delta H°$ increases and is compensated by an increase in $T\Delta S°$ resulting in a small change in $\Delta G°$. This behavior was related to the role of solvent water molecules in the binding process. The binding is also characterized by a high negative ΔC_p-value, which indicates the removal of hydrophobic surface areas from contact with water (Hahn et al. 2001; Welfle et al. 2003). Thermodynamic parameters are used in the modeling and improvement of antibody–antigen interactions.

Large efforts are undertaken nowadays to find and develop new antigenic synthetic mimotopes such as peptides (Hale 1995) or oligosaccharides (Vyas et al. 2003), which mimic the original antigens in their mode of interaction with antibodies. Such analogues would be potentially useful for a simple and reproducible test of antibody activity. In addition, the mimotopes are also beneficial for the selective purification of certain types of antibodies (Hale 1995) (development of new affinity chromatography procedures) and they are more suitable for co-crystallization studies than the original antigen (James et al. 1999). Establishing an ITC quality control procedure using one of these mimotopes can also be considered, by which the affinity and the stoichiometry of the binding of the mimotopes to antibodies are correlated to the bioactivities of the antibodies of interest.

The main aim of our study was (a) to characterize the recognition of five peptide mimotopes (AP-pep, QP-pep, QS-pep, WP-pep, and WS-pep, see Table 1) by the Fab part of recombinant human IgG1 and (b) to investigate the binding of *Staphylococcal* protein A (SpA) to the IgG1 Fc part. The chemical structure of the peptide mimotopes was modified in order to inspect the effect of specific amino acids with regard to the antibody–antigen interaction. Using ITC (isothermal titration calorimetry), the thermodynamic properties were quantified and the effect of temperature and ionic strength on the binding process was studied. Furthermore, the different nature of the Fc (SpA) and Fab (peptide mimotopes) binding sites of the antibody are compared. In addition, this study provides an example of using simple thermodynamic parameters to predict the structure-based changes upon complex formation and the mechanism of the interaction in complex systems.

Materials and methods

Materials

Recombinant human IgG1

Samples were provided by Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany. The concentration of the IgG1 sample solutions were determined by UV-measurement at 279 nm using an extinction coefficient of 1.32 for a 1 mg/ml solution. Purity was determined by size exclusion chromatography. The monomer content of all IgG1 samples was >99%.

The peptide mimotopes

Table 1 were synthesized by Thermo Electron GmbH, Ulm, Germany. The expected molecular weights of all the peptides were confirmed by mass spectrometry.

Table 1 Sequence and abbreviation of the peptide mimotopes

Peptide	Sequence
AP-pep	NH_2-**A**TSS**P**SADA-COOH
QP-pep	NH_2-**Q**TSS**P**SADA-COOH
QS-pep	NH_2-**Q**TSS**S**SADA-COOH
WP-pep	NH_2-**W**TSS**P**SADA-COOH
WS-pep	NH_2-**W**TSS**S**SADA-COOH

The amino acids that were varied are marked by bold letters

Purity was determined by HPLC analysis and was found to be >95% for all peptides. The peptide solutions were freshly prepared by weighing the lyophilized samples, dissolution in the buffer and dilution to the desired concentration.

Recombinant Staphylococcal protein A

SpA (molecular weight 34.3 kDa) was purchased from Amersham Pharmacia. SpA was used as received without any further treatment. The concentration of the SpA sample solutions were determined by UV-measurement at 280 nm using an extinction coefficient of 0.19 for a 1 mg/ml solution.

Buffer

IgG1 and SpA samples were prepared as solutions in a sodium-phosphate buffer (pH 7.2, 137 mM NaCl). The same buffer was used for the preparation of the peptide solutions as well as for any further dilution of IgG1 and SpA samples.

Methods

Concentration determination of peptide solutions using UV spectroscopy

The concentration of the tryptophan-containing peptide solutions, i.e., WP-pep and WS-pep, was determined by measuring their absorbance at 280 nm. The Lambert-Beer law was then applied to calculate the concentration directly using a tryptophan absorption coefficient value of 5,559 l mol^{-1} cm^{-1} (Lottspeich and Zorbas 1998) (Eq. 1):

$$\text{peptide concentration}[M] = \frac{\text{absorbance}}{5,559} \quad (1)$$

where the cell path length is 1 cm.

The method developed by Waddell (1956) was used to determine the concentration of the other peptides containing no aromatic amino acids, i.e., AP-pep, QP-pep and QS-pep. This method is based on measuring the UV-absorption of peptides at shorter wavelengths, namely 215 and 225 nm. Equation 2 was then applied to calculate the peptide concentration, where A_{215} and A_{225} are the respective absorbance values:

$$\text{peptide concentration } (\mu\text{g/ml}) = 144\,(A_{215} - A_{225}) \quad (2)$$

Isothermal titration calorimetry

The calorimetric titration experiments were carried out using a VP-ITC from MicroCal™, Inc. (Northampton, MA).

Titration experiments with peptides

The titration experiments were carried out at 5, 15, 25, 35 and 45°C. To study the effect of salt concentration, different ITC experiments were performed at 15°C using varying NaCl concentrations, namely 137, 300 and 500 mM.

Prior to each experiment, the sample cell and the syringe were rinsed with freshly distilled water and then shortly with buffer. The reference cell was filled with degassed water. The 1.4 ml reaction cell was loaded with the antibody (typically 5 μM) solution, whereas the injection syringe (nominal volume 250 μl) was filled with a peptide solution (typical concentration 212–379 μM).

The instrument was equilibrated at a temperature 5°C below the experimental temperature with an initial delay of 60 s. The reference power and the filter were set to 10 μcal/s and 2 s, respectively.

A typical titration experiment consisted of injecting 2 μl as a first injection of the peptide solution into the reaction cell, followed by 39 injections of 7.5 μl with an injection speed of 0.5 μl/s. The time interval between two consecutive injections was 200 s in order to allow the heat signal to return to the baseline. During the experiments, the sample solution was continuously stirred (290 rpm) by the rotating paddle attached to the end of the syringe needle.

The titration curves were analyzed using the ORIGIN® software provided with the calorimeter. A binding model with identical and independent binding sites was used to fit the data. Data of the first injection were discarded due to inaccurate volume and concentration because of a possible dilution of the peptide solution in the syringe needle during thermal equilibration. Each experiment was repeated 1–5 times under the same conditions in order to increase the accuracy of the results and to ensure their reproducibility. The thermodynamic parameters and errors were then calculated and averaged.

Titration experiments with SpA

The procedure and steps in performing the titration experiments of SpA were the same as for the peptides, unless otherwise specified. The calorimeter reaction cell was filled with 5 μM IgG1 sample solution and titrated by 15 μl volume injections of 20 μM SpA solution using spacing of 200–250 s. The titration was carried out at 5, 15, 25, 35, 45, 55, and 60°C.

Results and discussion

Interaction of IgG1 with the peptide mimotopes

Calorimetric titration curves, binding constants, and binding enthalpies

The interaction of the IgG1 Fab part was studied using five different peptide mimotopes (see Table 1). Variations in the amino acids were performed at position one and/or five of the peptide (referred from the N-terminal to the C-terminal). Figure 1 shows the binding isotherms of the five different mimotopes. WP-pep, the tryptophan-containing peptide, had the highest negative binding enthalpy followed by QP-pep and AP-pep, respectively. The binding of the two peptides to IgG1 where proline–5 was substituted with serine, i.e., QS-pep and WS-pep, was negligible. These two peptides were used as negative controls, indicating that proline-5 was essential for binding. Titrations into buffer were also performed in order to detect the dilution enthalpies of the peptides (data not shown). All dilution enthalpies under different conditions were in the range of 0.8–3.0 kJ/mol.

The saturation limit in the titration curves of all peptides was reached at high peptide to antibody molar ratio, which suggested an interaction with low affinity. For a detailed comparison of the binding isotherms of the three binding peptides (QP-pep, AP-pep and WP-pep) the experimental titration curves are additionally shown in Fig. 2.

The calorimetric titrations were performed over a range of temperature of 5–45°C in 10°C steps to study the influence of temperature on the binding process. Despite the limited temperature range over which the titrations were carried out, we also tried to calculate the temperature dependence of the binding enthalpy, from which the heat capacity changes upon complex formation, ΔC_p, can be calculated. The temperature dependence of WP-pep binding isotherms is displayed in Fig. 3. One can clearly see that the steepness of the titration curves decreased upon increasing the experimental temperature.

For the quantitative determination of the binding enthalpy $\Delta H°$ and the binding constant K_a the titration curves were fitted using a model of identical and independent binding sites as mentioned above. The values of the binding constant K_a at different temperatures as well as the other thermodynamic parameters are summarized in Table 2 for the three peptides QP-pep, AP-pep and WP-pep, which showed significant binding.

The stoichiometry of binding of IgG1 can be determined from the fits of the titration curves. In the case of the WP-peptide the experimental titration curves had sufficient signal to noise ratio to determine the stoichiometry N in the temperature range of 5–35°C, while in all other cases the saturation ratio was fixed to two to allow better fitting of the binding isotherms, because of the lower heats of binding. As shown in Table 2, the experimentally determined number of binding sites per one IgG1 molecule varied between 1.7 and 2.0. However, the theoretical number of binding sites per one IgG molecule should be two. This difference between experimental and theoretical values can have a variety of reasons besides statistical error (Tellinghuisen 2003, 2005), namely aggregation

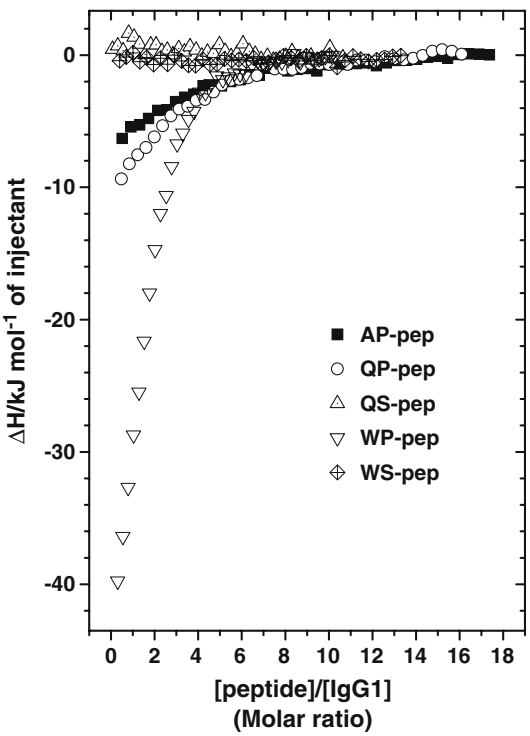

Fig. 1 Calorimetric binding isotherms of the five peptide mimotopes to IgG1 at 15°C

of IgG1 (possibly peptide-induced), denaturation as well as the low binding affinity of some IgG1 molecules due to changes in the binding region, or inexact fitting procedure of the scattered data points. On the other hand other groups have also reported N values in the range 1.72–2.03 for similar systems (Welfle et al. 2003). Larger values of $N > 2$ were not observed. The true reason for the reduced value of N remains therefore unclear at present. In our case, high performance size exclusion chromatography has revealed that the formation of IgG1 aggregation for the used antibody is negligible (data not shown).

As the titration curves in the figures indicate (see also Table 2), all binding reactions were exothermic. WP-pep had the highest binding constants ($K_a \approx 4.6 \times 10^5$ M^{-1} at 15°C). This value was about one order of magnitude higher than that found for QP-pep and AP-pep at the same temperature. QP-pep showed the next strongest binding ($K_a \approx 5.6 \times 10^4$ M^{-1} at 15°C) which was only slightly higher (1.8×) than that of AP-pep ($K_a \approx 3.1 \times 10^4$ M^{-1} at 15°C).

As exhibited in Fig. 4, with increasing temperature the binding affinities of the three peptides decreased. The interaction of AP-pep with IgG1 was the least affected by temperature, as K_a dropped only to 31% of its value by increasing the temperature from 5 to 35°C, followed by much larger changes for QP-pep and WP-pep where K_a dropped to 13 and 9% of its value, respectively.

The effect of temperature on the thermodynamic parameters $\Delta H°$, $-T\Delta S°$ and $\Delta G°$ is displayed in Fig. 4. $\Delta G°$ and $\Delta S°$ for the binding were calculated using the standard equations $\Delta G° = -RT\ln K_a$ and $\Delta S° = (\Delta H° - \Delta G°)/T$, respectively. The temperature dependence of $\Delta H°$ and $T\Delta S°$ of QP-pep was linear over the whole temperature range (5–35°C). Whereas, in case of AP-pep and WP-pep deviations from linear behavior occurred at higher temperature. This was particularly evident for the measurement at 45°C with the peptide WP-pep where a notable decrease in the binding enthalpy was seen. It is possible that this temperature was already too high and that the antibody became slightly damaged.

The results revealed that binding of QP-pep and AP-pep at 5°C were enthalpically as well as entropically-driven, i.e., with favorable contributions of both negative enthalpy and positive entropy. The binding of WP-pep at 5°C, however, was solely driven by enthalpy with a negligible contribution by the entropy change. At temperatures higher than 5°C, the reactions for all peptides were enthalpy-driven, with an unfavorable contribution by a negative entropy change. Both QP-pep and AP-pep had similar $\Delta H°$ and $T\Delta S°$ values, while WP-pep had a higher $\Delta H°$ accompanied by

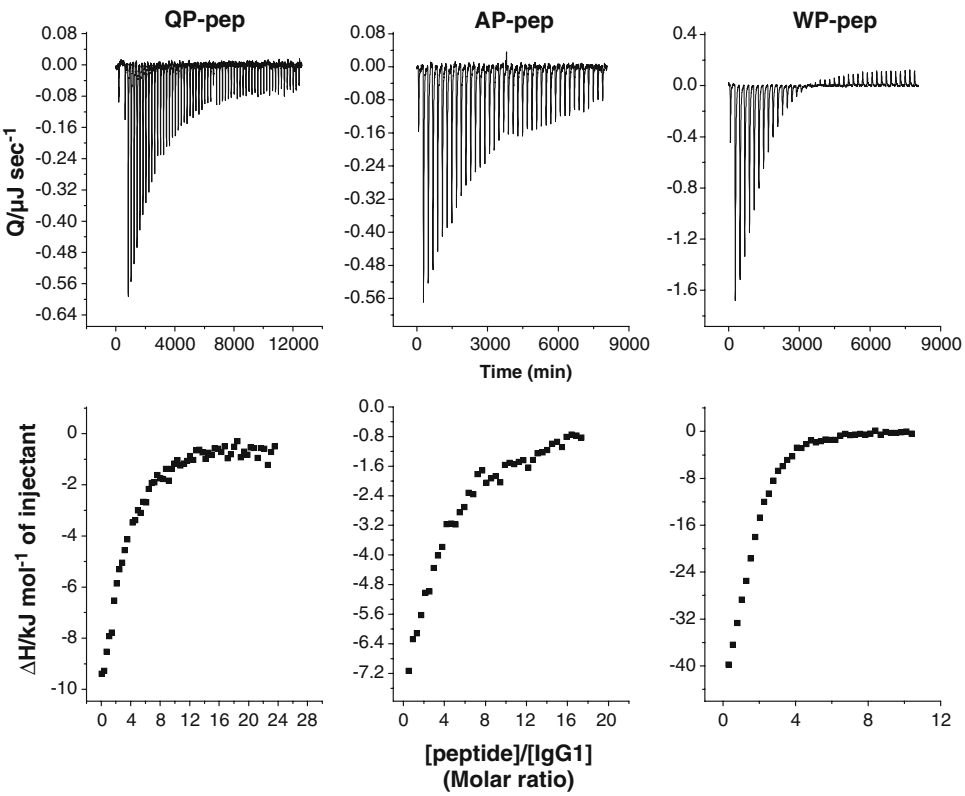

Fig. 2 Experimental power signals and binding isotherms of the three strongly binding peptide mimotopes for titrations performed at 15°C

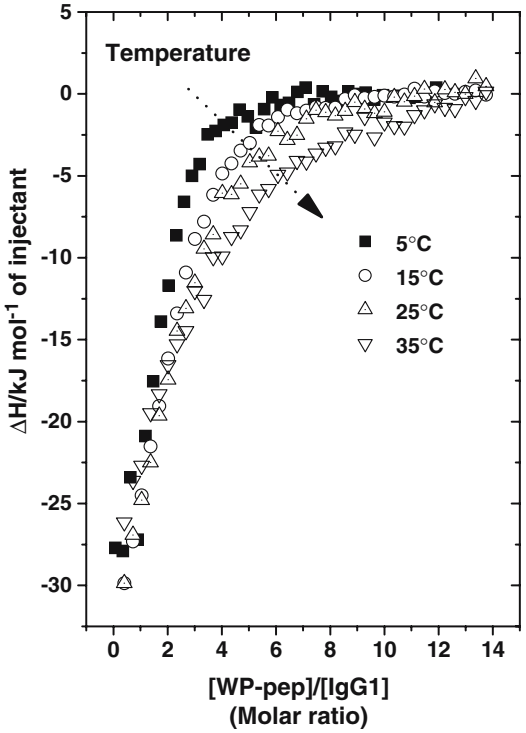

Fig. 3 The binding isotherms of WP-pep to IgG1 determined at different temperatures

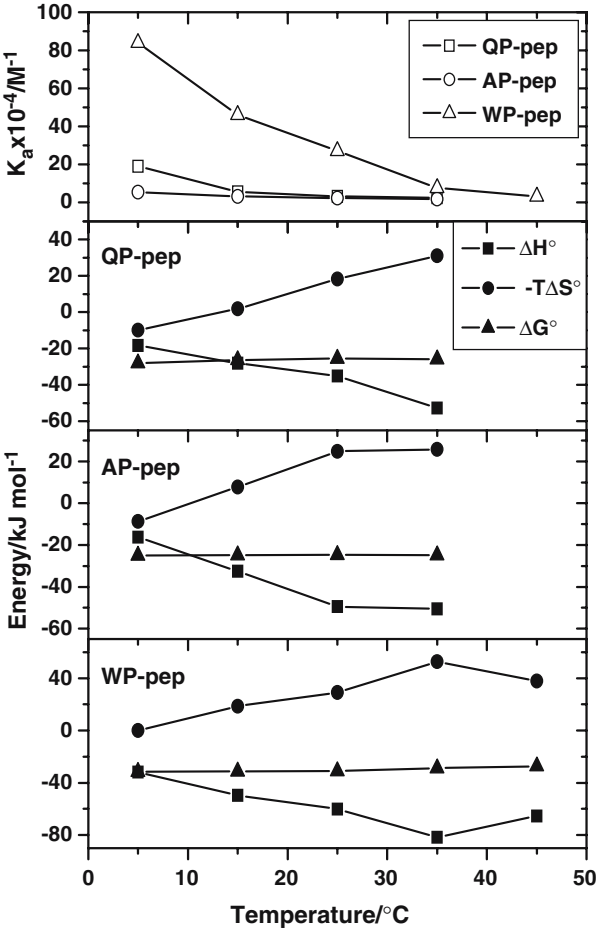

Fig. 4 Temperature dependence of K_a, $\Delta H°$, $-T\Delta S°$ and $\Delta G°$ for the binding of the three strongly binding peptide mimotopes to IgG1

higher $T\Delta S°$ values. $\Delta G°$ remained practically invariant as a function of temperature. The changes in $\Delta G°$ were much smaller than those of $\Delta H°$ and $T\Delta S°$. This was because of the well-known enthalpy–entropy compensation effect.

Thermodynamic characterization of the interaction

Van der Waals interactions and hydrogen bonding are considered as the major potential sources of negative $\Delta H°$ values. The net formation of hydrogen bonds and the decrease in the number of conformations and degrees of freedom are associated on the other hand with a negative $\Delta S°$ (Tame et al. 1998; Dominguez-Pérez et al. 2004). The desolvation of hydrophobic surfaces

Table 2 Thermodynamic parameters of binding of peptide mimotopes to IgG1 at different temperatures

Peptide	T (°C)	N	K_a (M^{-1})	$\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1})	$\Delta G°$ (kJ mol^{-1})
QP-pep	5	2[a]	$1.9 \times 10^5 \pm 1.0 \times 10^4$	–18.3 ± 0.3	9.8 ± 0.2	–28.1 ± 0.1
QP-pep	15	2[a]	$5.6 \times 10^4 \pm 2.5 \times 10^3$	–28.1 ± 0.6	–1.9 ± 0.5	–26.2 ± 0.1
QP-pep	25	2[a]	$3.1 \times 10^4 \pm 1.4 \times 10^3$	–35.1 ± 1.1	–18.2 ± 1.0	–25.5 ± 0.1
QP-pep	35	2[a]	$2.4 \times 10^4 \pm 1.0 \times 10^3$	–52.7 ± 1.5	–31.0 ± 1.3	–25.8 ± 0.1
AP-pep	5	2[a]	$5.3 \times 10^4 \pm 2.6 \times 10^3$	–16.3 ± 0.4	8.8 ± 0.3	–25.1 ± 0.1
AP-pep	15	2[a]	$3.1 \times 10^4 \pm 1.9 \times 10^3$	–32.5 ± 1.1	–7.7 ± 0.9	–24.8 ± 0.1
AP-pep	25	2[a]	$2.2 \times 10^4 \pm 9.1 \times 10^2$	–49.5 ± 1.3	–24.9 ± 1.2	–24.7 ± 0.1
AP-pep	35	2[a]	$1.7 \times 10^4 \pm 1.2 \times 10^3$	–50.6 ± 2.0	–25.7 ± 1.8	–24.9 ± 0.2
WP-pep	5	1.9	$8.4 \times 10^5 \pm 5.2 \times 10^4$	–31.7 ± 0.5	–0.1 ± 0.3	–31.6 ± 0.1
WP-pep	15	1.7	$4.6 \times 10^5 \pm 2.8 \times 10^4$	–49.7 ± 1.1	–18.5 ± 1.0	–31.3 ± 0.1
WP-pep	25	1.7	$2.7 \times 10^5 \pm 1.5 \times 10^4$	–60.1 ± 1.9	–29.1 ± 1.8	–31.0 ± 0.1
WP-pep	35	2.0	$7.6 \times 10^4 \pm 4.4 \times 10^3$	–81.6 ± 3.4	–52.8 ± 3.3	–28.8 ± 0.1
WP-pep	45	2[a]	$3.1 \times 10^4 \pm 2.1 \times 10^3$	–65.3 ± 2.4	–37.9 ± 2.2	–27.3 ± 0.2

[a] N values were fixed to 2 before fitting the data to allow accurate determination of the other parameters

of the protein and the ligand, which involves destruction and rearrangement of the hydrogen bond network in the solvation shell and the following release of water molecules into the bulk solvent where weaker hydrogen bonds are reformed is expected to be the source of unfavorable (positive) $\Delta H°$ and favorable (positive) $\Delta S°$ components (Tame et al. 1998; Dominguez-Pérez et al. 2004). However, the enthalpy change for the desolvation of hydrophobic surfaces is strongly temperature-dependent. At room temperature a large gain in entropy is expected with a negligible change in enthalpy (Blokzijl and Engberts 1993). Only for the peptides AP-pep and QP-pep positive entropy changes were found upon binding, but at lower temperature. At room temperature the entropy changes were either almost zero or relatively small. The increase in solvent entropy expected from the hydrophobic effect was obviously compensated by the large loss of conformational entropy as well as by the loss of rotational-translational degrees of freedom in the complex.

The increase in van der Waals interactions and the formation of hydrogen bonds, which play the major role in the antigen-antibody specificity (Ladbury and Chowdhry 1996; Dominguez-Pérez et al. 2004), are anticipated to be the cause for the negative $\Delta H°$ values at room temperature where the enthalpic contributions from hydrophobic interactions are usually negligible. The strong temperature dependence of $\Delta H°$ indicated that in addition hydrophobic effects are operative (see below).

The correlation between $\Delta H°$ and $T\Delta S°$ can be used to predict some characteristics of the binding mechanism. The more linear the correlation between $\Delta H°$ and $T\Delta S°$ is, the more is the binding driven by hydrophobic effects (Blokzijl and Engberts 1993; and literature cited therein). Plotting $-\Delta H°$ versus $-T\Delta S°$ (Fig. 5) resulted in a fairly linear correlation for the three peptides, and this was expected for the described complexes with ΔC_p values significantly higher than $\Delta S°$ (see below) (Welfle et al. 2003). Since the correlation coefficients are very high for the three mimotopes, one can predict that also hydrophobic interactions played a major role in their binding process.

AP-pep had the best correlation coefficient, followed by WP-pep and QP-pep, respectively, (see Table 3). Consequently, hydrophobic interactions for AP-pep were anticipated to be larger than the other two, followed by WP-pep and QP-pep, respectively. This concurs with the order of hydrophobicity of the mimotopes, where AP-pep is the most hydrophobic among the three, followed by WP-pep and QP-pep, respectively (Kyte and Doolite 1982; Cornette et al. 1987).

In principle, the binding enthalpy can also be determined using the van't Hoff equation $d\ln K_a/d(1/T) = -\Delta H^{vH}/R$ (R is the universal gas constant). A comparison between ΔH^{vH} and the experimentally determined $\Delta H°$ (ΔH^{cal}) could give a hint about the binding mechanism. Similar values of ΔH^{vH} and ΔH^{cal} propose a two-state reaction, whereas smaller ΔH^{vH} values points to a more complicated mechanism. The calorimetrically measured binding enthalpies ΔH^{cal}, however, are highly temperature-dependent. Therefore, the van' Hoff plots are difficult to analyze because they are not linear. For a precise determination of ΔH^{vH} as a function of temperature a high precision for the K_a values, a larger temperature range, and many points are needed. This prerequisite is usually not fulfilled so that this type of analysis yields only approximate results. This is also the case in our experiments. Therefore, we have refrained from performing this type of analysis.

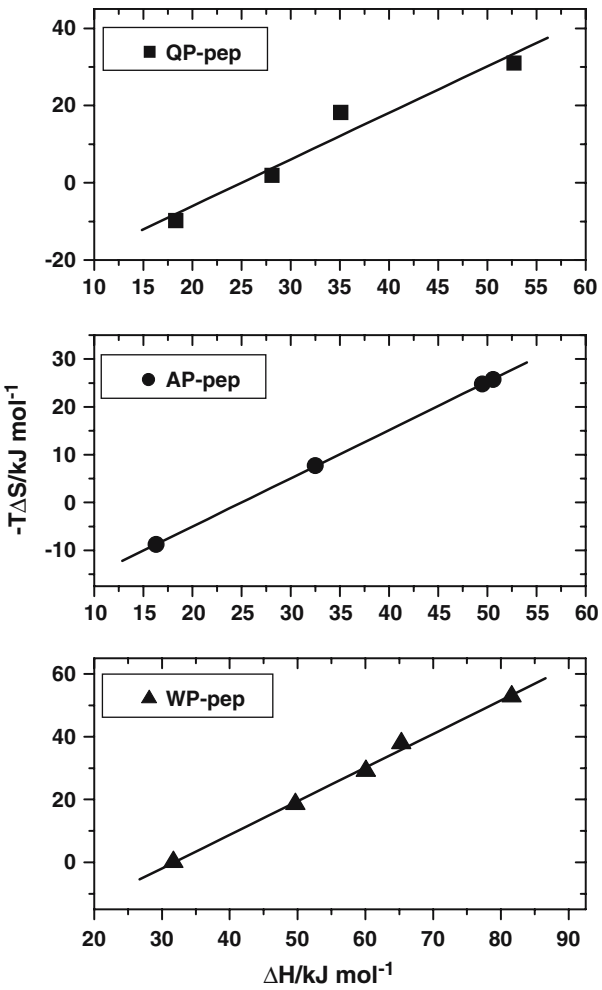

Fig. 5 Correlation between $-\Delta H°$ and $-T\Delta S°$ for the three strongly binding peptides

Table 3 Slopes and correlation coefficients for linear plots of $-\Delta H°$ versus $-T\Delta S°$ for the three strongly binding peptides

Peptide	Slope	Correlation coefficient (R^2)
QP-pep	1.2	0.9494
AP-pep	1.0	0.9999
WP-pep	1.1	0.9958

The binding enthalpies were strongly temperature-dependent, becoming more negative with increasing temperature. The change in specific heat capacity (ΔC_p) of the binding process was calculated from the slope of $\Delta H°$ versus temperature curves (as illustrated in Fig. 6) assuming a temperature independent ΔC_p. ΔC_p values as well as the correlation coefficients of fitting the data points are listed in Table 4. All ΔC_ps were negative, i.e., the complexes had lower heat capacities than the components. The ΔC_p values for the binding of QP-pep and AP-pep were very similar. This indicated a basic similarity in the binding process and thermodynamic properties. In case of WP-pep binding, ΔC_p was about 30% higher than for the other two peptides.

The large negative ΔC_p can be correlated with the burial of hydrophobic surface areas of the Fab binding site of the antibody from exposure to aqueous solvent upon complex formation (Varadarajan et al. 1992; Tame et al. 1998; Gribenko et al. 2002; Dominguez-Pérez et al. 2004). This showed that also hydrophobic interactions of IgG1 with the peptides drove the binding reaction. WP-pep, where the first amino acid is the aromatic amino acid tryptophan, had the highest ΔC_p. Since tryptophan is bulkier than alanine and glutamine together with being highly hydrophobic [the hydrophobicity order is: alanine ≥ tryptophan > glutamine (Kyte and Doolite 1982; Cornette et al. 1987)], this explained its relative high ΔC_p, because larger amounts of water were expected to be released upon complex formation with the WP-peptide. The absolute value of ΔC_p had also a contribution from the change of the exposed polar surface area, which was, however, smaller (see below).

The influence of salt concentration

An increase of the ionic strength induces a charge screening effect. Therefore, studying the effect of ionic strength on the thermodynamics of complex formation is a common method to validate the electrostatic character of interactions (Gribenko et al. 2002). A very strong dependence of binding parameters on salt concentration has been established for biomolecular associations driven by electrostatic interactions. It was

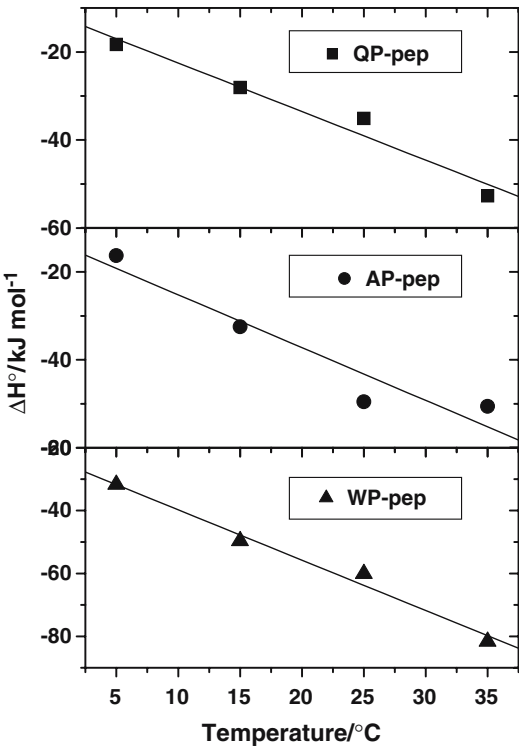

Fig. 6 Plot of $\Delta H°$ versus temperature for the calculation of ΔC_p for the three strongly binding peptides

Table 4 Calculated ΔC_p values and correlation coefficients assuming a linear relation between $\Delta H°$ and temperature

Peptide	ΔC_p (J mol^{-1} K^{-1})	Correlation coefficient (R^2)
QP-pep	–1,102	0.9614
AP-pep	–1,199	0.9097
WP-pep	–1,602	0.984

found that upon increasing the NaCl concentration by 100 mM, K_a was decreased by almost two orders of magnitude (Lundbäck et al. 1996). The change in salt concentration leads mainly to changes in $-T\Delta S°$, whereas $\Delta H°$ appeared to be relatively independent on the salt concentration. The origin of salt dependence is therefore predominantly of entropic origin as most studies have shown (Lundbäck et al. 1996; and literature cited therein).

The effect of salt concentration on the binding affinity of the three peptide mimotopes was investigated by carrying out some ITC experiments using different NaCl concentrations (i.e., 137, 300 and 500 mM) at 15°C. As shown in Fig. 7, the binding of the WP-pep, which was accompanied by the largest burial of IgG1 Fab surface area, was the least affected by changes in NaCl concentration. K_a of WP-pep was decreased only by ca. 25% upon increasing the

NaCl concentration from 137 to 500 mM. The binding constant of QP-pep and AP-pep showed a peculiar salt concentration dependence. At 300 mM NaCl the binding constant for both peptides first increased before decreasing again at the highest NaCl concentration of 500 mM. This is a quite unusual effect, which remains to be explained. However, for pure electrostatic binding one would expect a strong decrease of the binding constant in any case. From the experimental data for the changes in solvation of surfaces and from the salt dependence of the binding constant, one can again see that the interaction of the peptides with IgG1 Fab part is mostly of hydrophobic origin.

Interaction of IgG1 with SpA

The binding isotherm of the interaction of the IgG1 Fc part with SpA at 15°C is exhibited in Fig. 8. The SpA binding isotherm was characterized by a steeper step-like change of released heat per injection than that of the mimotopes, which lead to 2–4 orders of magnitude higher K_a values (~3.1×10^8 M^{-1} at 15°C). The enthalpy change $\Delta H°$ associated with the binding of SpA to IgG1 was much more negative than that of the mimotopes (~ –225.5 kJ mol^{-1} at 15°C) and compensated with a high unfavorable $\Delta S°$ value (~ –619.6 J mol^{-1} K^{-1} at 15°C). Recombinant SpA (34.3 kDa) has a much higher molecular weight than the mimotopes (~1 kDa). Therefore, it is reasonable that the binding of SpA is connected with the formation of a higher number of hydrogen bonds and increased hydrophobic interactions and, accordingly, to be associated with a much higher favorable enthalpy change and an unfavorable entropy change than the mimotopes.

The titration experiments were repeated at the same conditions using different temperatures (5 to 60°C), to gain knowledge about the temperature dependence of the thermodynamic parameters of binding of IgG1 to SpA. The binding isotherms of SpA at different temperatures are displayed in Fig. 9. Upon increasing the temperature the steepness of the titration curves decreased, while, the heat associated with binding process and the reaction stoichiometry increased. A model of independent binding sites was used to fit the curves and the results are summarized in Table 5. Each IgG1 molecule has two binding sites for SpA in the Fc region, which are located in the joint region between the CH_2 and CH_3 domains of each heavy chain (Radaev et al. 2001; and literature cited therein). However, the experimentally determined stoichiometry factor N for the interaction of IgG1 with SpA was 0.6 (see Table 5). This could probably be related to the experimental

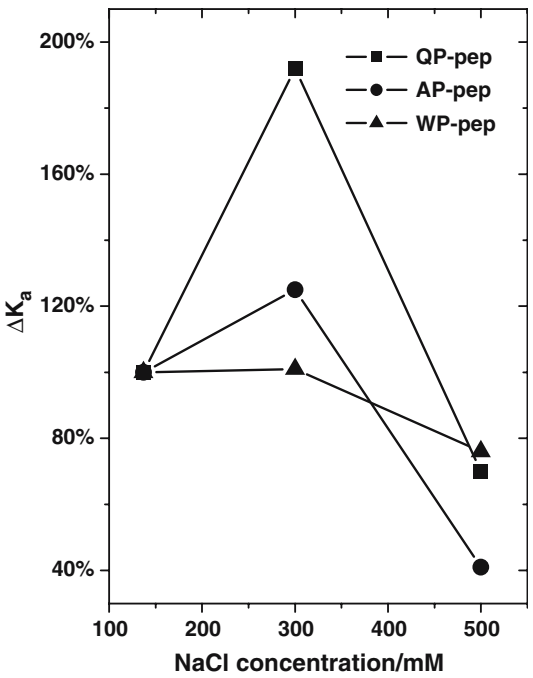

Fig. 7 Relative changes in binding constant as a function of NaCl concentration for the three strongly binding peptides

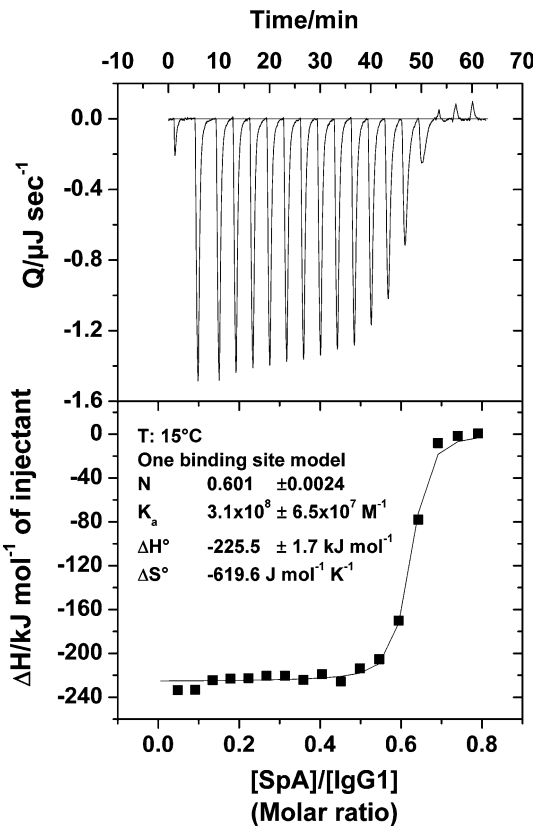

Fig. 8 Binding isotherm for a titration of a solution of 20 μM SpA into 5 μM IgG1 at 15°C

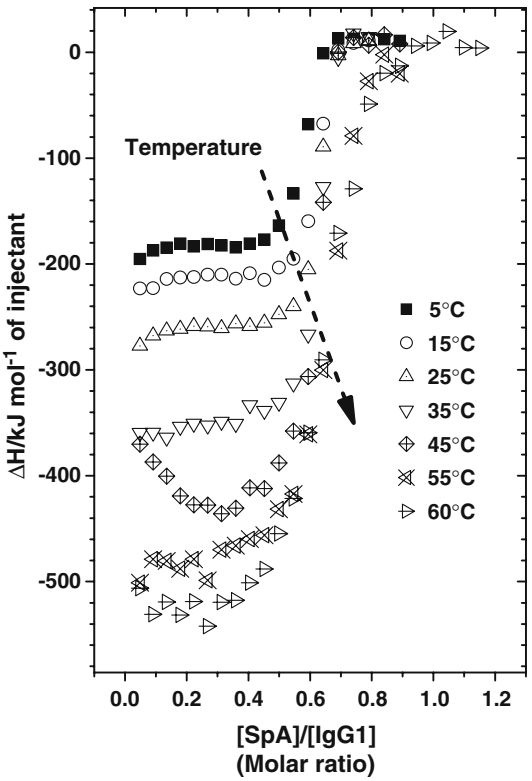

Fig. 9 Binding isotherms for binding of SpA to IgG1 determined at different temperatures

design of the ITC experiments, where SpA was titrated into an excess of IgG1. A comparable stoichiometry was reported by Langone et al. (1985) where a SpA:IgG complex of ratio of 2:4 was observed when IgG was present in excess, whereas with an excess of SpA, the stoichiometry of the complex became 1:1. It was also found that the preformed 2:4 and 1:1 complexes can be interconverted by adding excess SpA and IgG, respectively (Langone et al. 1985).

The binding affinity of SpA showed an unusual temperature dependence. As illustrated in Fig. 10 (see also Table 5), K_a of SpA increased by increasing the temperature from 5 to 25°C, after which it started decreasing over the temperature range 25–60°C. The maximum K_a was observed at 25°C (3.5×10^8 M^{-1}).

In Fig. 10 one can see clearly the strong linear temperature dependence of $\Delta H°$ and $T\Delta S°$, since both highly increased upon increasing the temperature, nevertheless, $\Delta G°$ increased only slightly. The binding of SpA to IgG1 Fc part was highly enthalpy driven over the entire experimental temperature range (5–60°C), as expected for such interactions between two high molecular weight proteins. $\Delta H°$ values were compensated and almost canceled by the associated unfavorable $T\Delta S°$, therefore $\Delta G°$ values remained in a narrow energy range (44.3–50.9 kJ mol^{-1}).

The correlation between $-\Delta H°$ and $-T\Delta S°$ was linear (see Fig. 11) with a slope and R^2 values very close to one, and this evidenced a hydrophobic-driven binding of IgG1 Fc part to SpA.

The binding of IgG1 to SpA was associated with ΔC_p value of –6,420 J mol^{-1} K^{-1} (Fig. 12), which was more than 400% higher than that of the mimotopes. This high ΔC_p value signified a burial of a large hydrophobic surface area upon binding of SpA to the antibody Fc region.

In comparison with the peptide mimotopes, SpA is larger and has much higher binding affinity. Consequently, SpA binding is anticipated to involve more hydrophobic interactions and to be associated with burial of larger hydrophobic surface areas than the mimotopes. This justifies the significant higher ΔC_p of SpA than those of the mimotopes.

Estimation of structural changes upon complex formation

Folding of proteins and also ligand binding are usually accompanied by large reductions in water-accessible nonpolar surface area due to the removal of nonpolar amino acid side chains from water (Livingstone et al. 1991). However, also polar surface areas can be shielded from water contacts during this process. Several methods have been proposed in order to characterize the structural changes of proteins, specifically the change in both water-accessible polar (ΔA_{pol}) and nonpolar (ΔA_{np}) surface areas (Murphy and Freire

Table 5 Thermodynamic parameters of binding of SpA to IgG1 determined at different temperatures

T (°C)	N	K_a (M^{-1})	$\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1})	$\Delta G°$ (kJ mol^{-1})
5	0.56	$2.1 \times 10^8 \pm 4.1 \times 10^7$	–198.5 ± 1.7	–154.2 ± 1.3	–44.3 ± 0.4
15	0.60	$3.1 \times 10^8 \pm 6.5 \times 10^7$	–225.5 ± 1.7	–178.7 ± 1.3	–46.9 ± 0.5
25	0.60	$3.5 \times 10^8 \pm 7.5 \times 10^7$	–272.0 ± 2.0	–223.2 ± 1.5	–48.8 ± 0.5
35	0.60	$2.8 \times 10^8 \pm 6.5 \times 10^7$	–365.9 ± 3.3	–316.1 ± 2.8	–49.8 ± 0.5
45	0.60	$2.3 \times 10^8 \pm 7.0 \times 10^7$	–437.5 ± 7.3	–386.6 ± 6.6	–50.9 ± 0.7
55	0.64	$6.7 \times 10^7 \pm 1.2 \times 10^7$	–483.2 ± 5.1	–434.0 ± 4.6	–49.2 ± 0.5
60	0.64	$3.8 \times 10^7 \pm 4.8 \times 10^6$	–541.4 ± 5.1	–493.0 ± 4.8	–48.3 ± 0.3

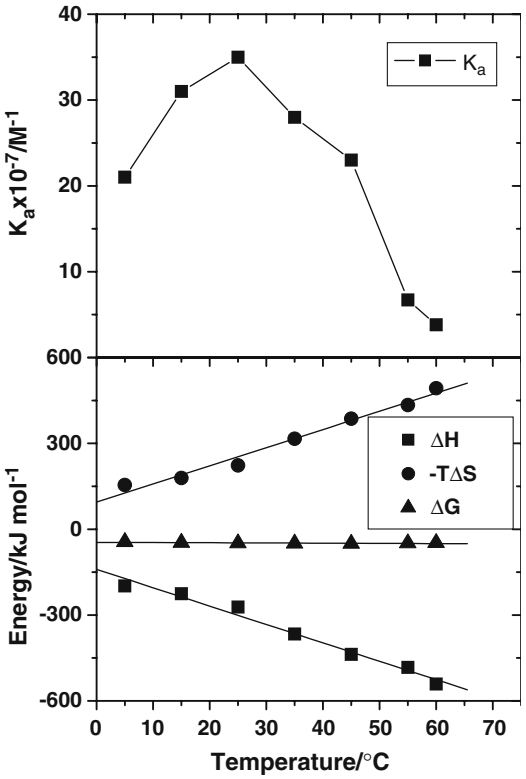

Fig. 10 Temperature dependence of K_a, $\Delta H°$, $-T\Delta S°$ and $\Delta G°$ for binding of SpA to IgG1

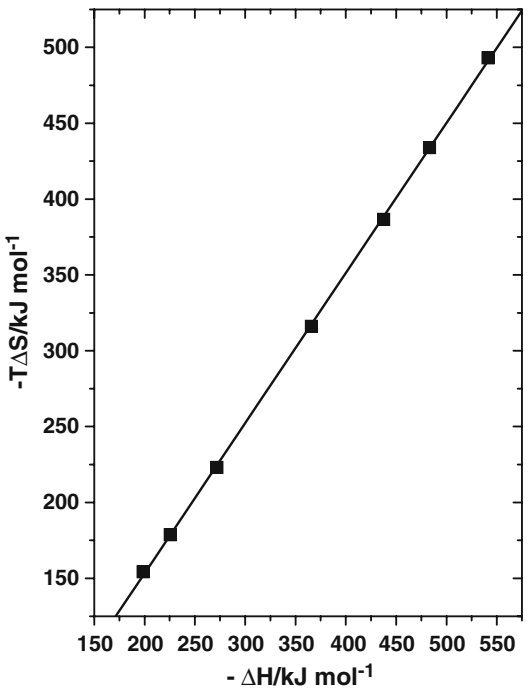

Fig. 11 The correlation between $-\Delta H°$ and $-T\Delta S°$ for binding of SpA to IgG1

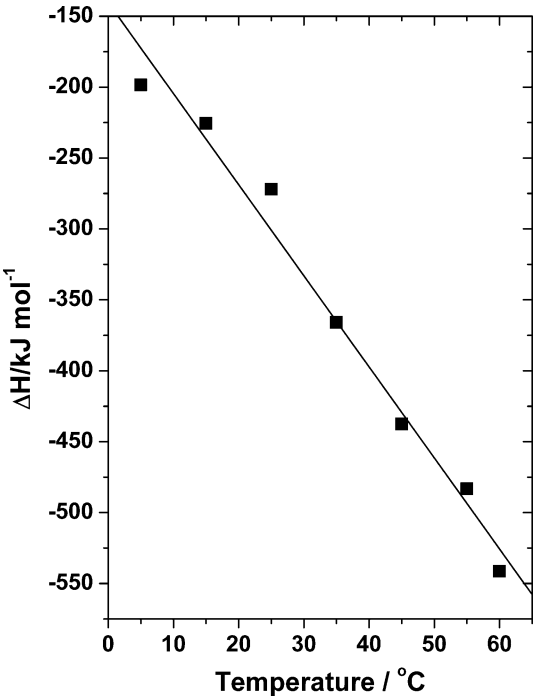

Fig. 12 Plot of $\Delta H°$ versus temperature for the calculation of ΔC_p for SpA

1992; Spolar and Record 1994). These methods are based on the values of both thermodynamic parameters $\Delta H°$ and ΔC_p, after it could be shown that they are directly correlated with the change in solvent accessible surface area (Livingstone et al. 1991; Murphy 1999). The same methods can be used the other way around to calculate binding and folding energetics using structural details (Murphy 1999; Welfle et al. 2003).

Here, we applied the method developed by Murphy and Freire (1992) to estimate both ΔA_{np} and ΔA_{pol} values associated with the binding of SpA and the three peptide mimotopes using Eqs. 3 and 4.

$$\Delta C_p = 1.88\Delta A_{np} - 1.09\Delta A_{pol} \quad (3)$$

The coefficients are in units of J K^{-1} mol^{-1} $Å^{-2}$.

$$\Delta H°(60°C) = -35.3\Delta A_{np} + 131\Delta A_{pol} \quad (4)$$

Here the coefficients are in units of J mol^{-1} $Å^{-2}$. $\Delta H°(60°C)$ is the enthalpy change for denaturation at 60°C, which is the median denaturation temperature of the model protein (Murphy 1999). Δ The binding enthalpy at 60°C $\Delta H°(60°C)$ for SpA was measured experimentally, while for the three mimotopes $\Delta H°(60°C)$ was calculated by extrapolating their $\Delta H°$ values determined experimentally to 60°C assuming a linear temperature dependence of $\Delta H°$. The results of $\Delta H°(60°C)$, ΔA_{np} and ΔA_{pol} are presented in Table 6.

For SpA and the three peptides, the change in water-accessible nonpolar surface area ranges from 53 to 55% of the whole change in surface area. This is consistent with other literature values (Mandiyan et al. 1996; Dominguez-Pérez et al. 2004). As expected, the order of ΔA_{total}, ΔA_{np} and ΔA_{pol} agrees with the order of ΔC_p values, i.e., the higher the ΔC_p value the higher is the change in total water-accessible surface area. The estimated changes in exposed surface area for the binding of the three peptides are relatively high. For instance, for the WP-peptide the total solvent accessible surface area in the extended conformation is ca. 1,230 Å^2, whereas the total area change upon binding is almost –3,000 Å^2. This is a very large discrepancy, because for the peptide alone the change in solvent accessible surface cannot be larger than the estimated 1,230 Å^2. These differences could in principle be due to inaccurate $\Delta H°$(60°C) values, which were calculated assuming a linear temperature dependence of $\Delta H°$. However, $\Delta H°$(60°C) influences mainly the change in polar accessible surface area, whereas ΔC_p has a large influence on ΔA_{np}. For instance, a 50% reduction of the $\Delta H°$(60°C) value reduces ΔA_{pol} also by almost 50%, whereas ΔA_{np} is reduced by only 18%. Another possible explanation is that the used semiempirical equation does not describe the studied system appropriately, as reported for other similar systems (Welfle et al. 2003).

In general $\Delta S°$ is composed of three terms:

$$\Delta S° = \Delta S°_{solv} + \Delta S°_{conf} + \Delta S°_{mix} \quad (5)$$

where $\Delta S°_{solv}$ is the contribution to $\Delta S°$ arising from restructuring of solvent (release of water molecules upon binding), $\Delta S°_{conf}$ is the entropy change arising from changes in conformational degrees of freedom of the backbone and side chain groups, and $\Delta S°_{mix}$ is the entropy contribution arising from changes in translational, rotational, and vibrational degrees of freedom upon binding (Murphy 1999).

$\Delta S°_{solv}$ can be calculated using Eq. 6:

$$\Delta S°_{solv} = \Delta C_p \ln \frac{T}{T_s^*} \quad (6)$$

where T is the absolute temperature and T_S^* is the temperature of 385 K at which the polar and nonpolar contributions to the entropy changes are considered to be zero (Murphy et al. 1994; Murphy 1999; Hahn et al. 2001).

One of the methods to estimate $\Delta S°_{mix}$ of biomolecular associations in water is using the cratic entropy contribution as shown in Eq. 7:

$$\Delta S°_{mix} = R \ln \frac{1}{55.5} \quad (7)$$

where R is the universal gas constant and 55.5 is the molarity of water (Amzel 1997; Murphy 1999).

$\Delta S°_{conf}$ was calculated by subtracting $\Delta S°_{mix}$ and $\Delta S°_{solv}$ from $\Delta S°$.

The $\Delta S°$ values determined experimentally at 25°C of SpA and the three peptides were resolved into their three components using the equations discussed above. The results are displayed in Table 7.

In summary, $\Delta S°$ is the sum of unfavorable conformational and mixing contributions, which are not fully compensated by a favorable contribution of the solvation entropy, and this agrees with similar calculations cited in the literatures (Hahn et al. 2001).

Conclusions

At present, there is still inadequate information on the various types of biomolecular associations, including antigen–antibody binding and other antibody interactions. This impedes the advancement in many areas which are strongly dependent on data obtained from intensive characterization of biomolecular associations. Because of the limited number of studies that have discussed the thermodynamics of antibody–antigen interactions we have attempted to

Table 6 $\Delta H°$(60°C), ΔA_{np}, ΔA_{pol} and ΔA_{total} for SpA and the three strongly binding peptides

Ligand	$\Delta H°$(60°C)	ΔA_{np}		ΔA_{pol}		ΔA_{total}
	(kJ mol^{-1})	(Å^2)	(%)	(Å^2)	(%)	(Å^2)
QP-pep	–77.6[a]	–1,102	55	–889	45	–1,991
AP-pep	–85.2[a]	–1,203	55	–974	45	–2,177
WP-pep	–119.9[a]	–1,639	55	–1,357	45	–2,995
SpA	–541.4	–6,887	53	–5,989	47	–12,876

[a] $\Delta H°$(60°C) values were calculated by extrapolating the $\Delta H°$ values determined experimentally for the three peptides to 60°C assuming a linear temperature dependence of $\Delta H°$

Table 7 Values of $\Delta S°$ and its different components calculated at 25°C for SpA and the three binding peptides

Ligand	$\Delta S°$ (J mol^{-1} K^{-1})	$\Delta S°_{mix}$ (J mol^{-1} K^{-1})	$\Delta S°_{solv}$ (J mol^{-1} K^{-1})	$\Delta S°_{conf}$ (J mol^{-1} K^{-1})
QP-pep	–61	–33	282	–310
AP-pep	–83	–33	307	–357
WP-pep	–98	–33	410	–475
SpA	–749	–33	1,644	–2,360

advance the knowledge in this field by investigating the interaction of IgG1 Fab and IgG1 Fc subunits with some synthetic peptide mimotopes and with *Staphylococcal* protein A (SpA), respectively. For this purpose we used ITC, the method of choice to directly quantify the complete set of thermodynamic parameters of the binding equilibrium, which then provides insight into the stability, specificity and stoichiometry of the biomolecular interactions. ITC experiments can be carried out in a variety of ways (see Henzl et al. 2002; Markova and Hallén 2004) and the only limiting requirement of this technique is a sufficiently large enthalpy change for the binding of the ligand to the macromolecule.

We determined the thermodynamic profiles for the interactions of the peptide mimotopes and the *Staphylococcal* protein A under different conditions. The thermodynamic data gave insight into the influence of the amino acid sequence of the peptide mimotope with regard to the antibody-peptide interaction. Moreover, the results were used for monitoring the structural changes accompanied the binding process. Our results correspond to those published before on other systems (Bhat et al. 1994; Murphy 1999; Hahn et al. 2001; Vyas et al. 2003; Welfle et al. 2003). The binding of SpA and the peptide mimotopes was mainly driven by enthalpic effects which over-compensated the unfavorable negative change in entropy upon binding. This overcompensation effect increased with temperature. Hydrophobic effects were found to play a major role in driving the binding processes. The unfavorable change in entropy was mainly due to loss of conformational freedom whereas the solvation changes lead to favorable entropy changes.

This study showed that ITC is a suitable method for studying biomolecular interactions even in complicated systems. The complete analysis of the thermodynamic functions at different temperatures leads to bettter insight with regard to contributions arising from conformational as well as solvation changes occurring upon binding.

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