Transition state thermodynamic analysis using Biacore T100

Providing information crucial for predicting molecular recognition and structure-based drug design

- Kinetic and thermodynamic data from one system, revealing how and why molecular interactions occur at specific rates

- Transition state thermodynamics provide detailed information about the molecular mechanisms that drive interactions

- Software wizards evaluate kinetic rate constants and thermodynamic parameters from a single experiment

- No waiting for equilibrium: thermodynamic parameters derived from association and dissociation rate constants

- Data unaffected by solvent conditions
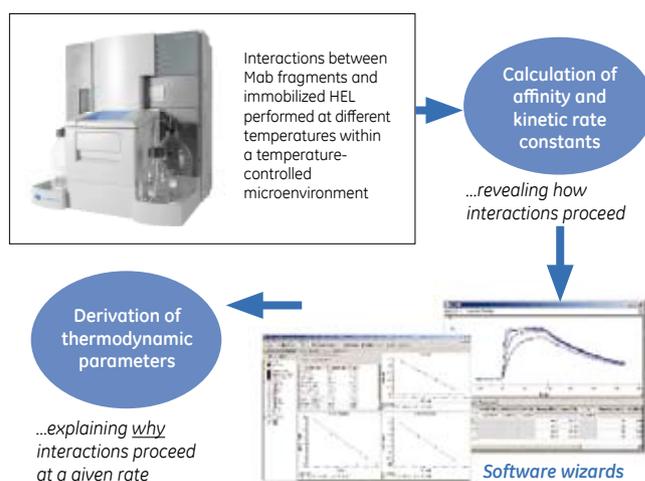
- Minimal sample consumption

Figure 1. Thermodynamic assays on Biacore T100; (1) Samples are injected over a target protein immobilized on a sensor surface. Each interaction is analyzed at several temperatures; (2) Affinity and kinetic rate constants for each interaction are automatically calculated; (3) Software wizards integrate affinity and kinetic rate constants into thermodynamic equations to calculate $\Delta G°$, $\Delta H°$ and $\Delta S°$ at equilibrium, as well as during complex association and dissociation ($\Delta G°^{\ddagger}$, $\Delta H°^{\ddagger}$ and $\Delta S°^{\ddagger}$).

Why study thermodynamics?

Biomolecular interactions may be defined at several levels. The simplest quantitative descriptor, affinity, is defined by the affinity constant, K_D, a measure of the strength of binding at equilibrium. This term may be further resolved into kinetic descriptors of rate of association (defined by the association rate constant (k_a) and dissociation (k_d).

Kinetic information, however, does not reveal the molecular mechanisms underlying the rates at which a complex forms and dissociates. Although structural aspects of complementarity may be revealed by technologies such as X-ray crystallography or NMR, the character and quantitative contributions of non-covalent forces within the binding site can only be rationalized by studying interaction thermodynamics. Fully understanding molecular recognition by being able to predict binding energetics from the three-dimensional structure of protein complexes through thermodynamic analysis may well provide the basis for structure-based molecular design of drugs and engineered antibodies.

Thermodynamic profiling of interactions between drug candidates and targeted receptors has already proven its value as an indicator of drug function. There are several examples in the literature showing that agonists and antagonists can be differentiated according to their thermodynamic profiles (enthalpy or entropy driven), a phenomenon known as thermodynamic discrimination (for a review, see reference 1).

WHAT DO THERMODYNAMICS REVEAL ABOUT AN INTERACTION?

Kinetic characterization of an interaction gives information about the rate of complex recognition and stability, but to understand *why* the interaction proceeds at these rates, it is necessary to define the thermodynamics of the system. The magnitude of K_D is determined by the free energy difference, $\Delta G°$, between the associated and unassociated states of the interacting molecules, according to the equation:

$$\Delta G° = RT\ln K_D \quad (1)$$

where R and T are the universal gas constant and absolute temperature, respectively. From this equation, it follows that high-affinity interactions (small K_D) require large negative changes in free energy. $\Delta G°$, therefore, is a measure of likelihood of complex formation, but it reveals little about the forces driving the interaction. For this, it is necessary to look separately at the two terms, enthalpy change ($\Delta H°$) and entropy change ($\Delta S°$), which comprise $\Delta G°$ according to another fundamental equation:

$$\Delta G° = \Delta H° - T\Delta S° \quad (2)$$

$\Delta H°$ is the total heat evolved or absorbed due to the formation and disruption of non-covalent bonds such as salt bridges, hydrogen bonds or van der Waals interactions. $\Delta S°$, on the other hand, may be regarded as a measure of the change in the degree of freedom with which the interacting molecules, including the surrounding solvent, and their parts (e.g. amino acid side chains) can move, rotate or vibrate.

$\Delta S°$ also reflects solvent reorganization when solvent is displaced from the interaction interface or, occasionally, captured between two interacting surfaces. Solvent reorganization is believed to dominate $\Delta S°$. Displacement of water from a hydrophobic surface, for example, increases entropy and so interactions characterized by large positive $\Delta S°$ are often interpreted as involving a contact between hydrophobic surfaces.

Kinetics and thermodynamics on one system

The ability to simultaneously deliver kinetic as well as thermodynamic data specifically associated with complex formation and dissociation is a unique feature of Biacore™ T100. In the worked example shown here, the kinetics and transition state thermodynamics of interactions between hen egg lysozyme (HEL) and variable fragments of native and mutant monoclonal anti-HEL antibody Fab fragments are characterized. In addition, data from Biacore T100 are compared with an analysis of the same interaction using ITC, the most commonly used technology for equilibrium thermodynamics, showing that both systems deliver similar equilibrium data in terms of absolute values and precision. By running kinetics at different temperatures, Biacore T100 is then used to evaluate the enthalpic and entropic terms of the transition state of the interaction.

Fitting affinity and kinetic rate constants to thermodynamic equations

Equilibrium thermodynamics

From equations 1 and 2 it follows that

$$\ln K_D = \Delta H°/RT - \Delta S°/R \quad (3)$$

By measuring K_D at several temperatures and plotting $\ln K_D$ against reverse temperature, $\Delta H°$ and $\Delta S°$ may be calculated from the slope and Y-axis intercept of the resulting straight line, known as a van't Hoff plot (Figure 2).
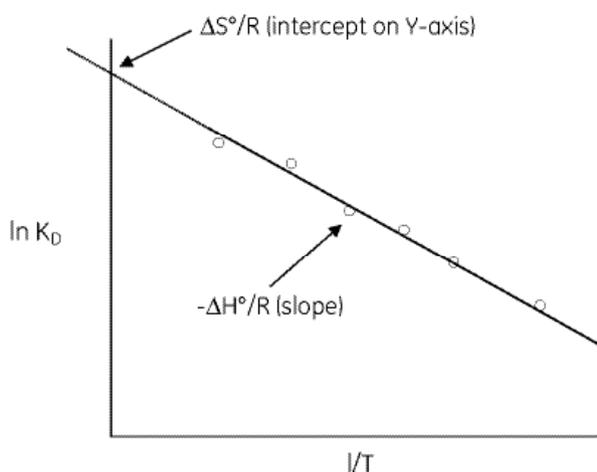

Figure 2. A sample van't Hoff plot, showing the relationship between K_D and temperature.

Transition state thermodynamics

K_D is given by the ratio of the dissociation and association rate constants:

$$K_D = k_d/k_a \quad (4)$$

Two different binders with the same steady-state affinity (and, consequently, same $\Delta G°$), can have a very different kinetic profile. An energy barrier known as activation free energy or $\Delta G°^{\ddagger}$ must be overcome in the transition between unassociated reactants and a complex; it is the height of this energy barrier that determines the magnitudes of k_a and k_d (Figure 3) according to the Eyring equation:

$$k = (k_B T/\hbar)\, exp\, (-\Delta G°^{\ddagger}/RT) \quad (5)$$

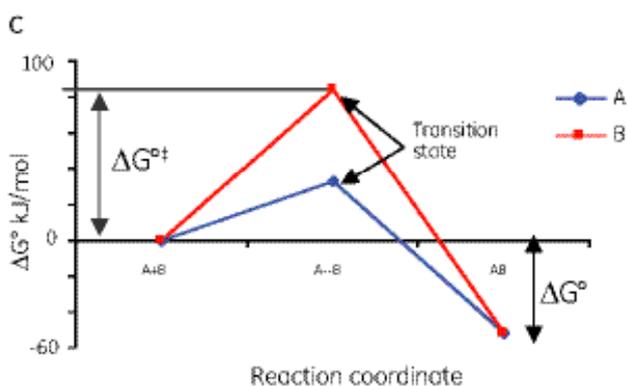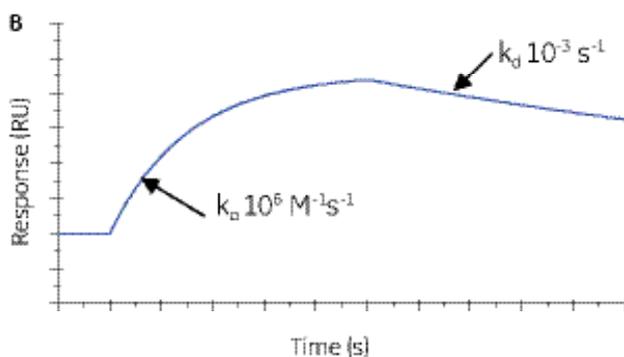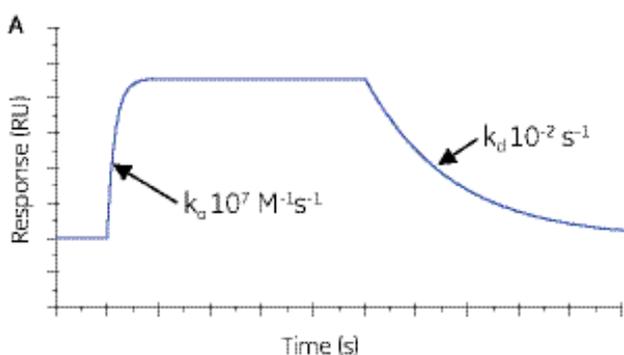where k_B is the Boltzmann constant and $\hbar$ is Planck's constant.

Figure 3. Two binding and dissociation profiles are illustrated in sensorgrams A and B. Although both interactions are characterized by K_D of 10^{-9} M, the association and dissociation rates are very different. The free energy profiles of the interactions are depicted in C and show how a higher energy barrier must be overcome under the conditions that lead to slower association and dissociation rates.

Deriving transition state thermodynamics from a sensorgram

Again, $\Delta G°^{\ddagger}$ is comprised of an enthalpy and entropy term. Equation 5 may be rewritten as

$$k = (k_B T/\hbar)\, exp\, (\Delta S°^{\ddagger}/R - \Delta H°^{\ddagger}/RT) \quad (6)$$

As with equilibrium thermodynamic parameters, activation enthalpy and entropy can help quantify the contribution of particular structural features to the association and dissociation rates.

Equation 6 may be rewritten as

$$ln\, k/T = \Delta S°^{\ddagger}/R - \Delta H°^{\ddagger}/RT + ln\, kB/\hbar \quad (7)$$

By measuring k at several temperatures and plotting $ln\, k/T$ vs $1/T$, $\Delta H°^{\ddagger}$ and $\Delta S°^{\ddagger}$ may be calculated from the slope and Y-axis intercept of the resulting straight line, known as an Eyring plot (Figure 4).
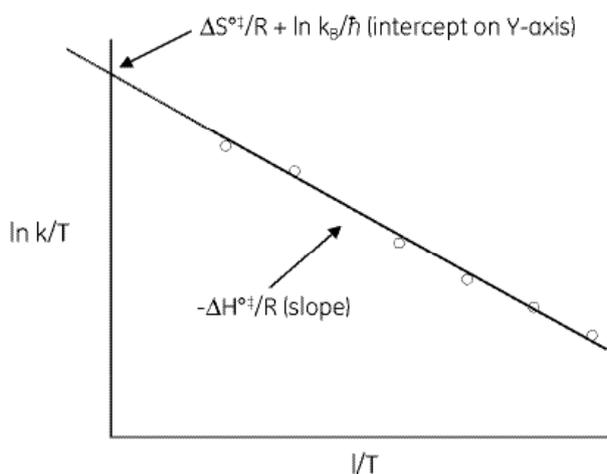

Figure 4. A sample Eyring plot. By fitting rate constants, k_a or k_d, to the Eyring equation (equation 7), values for transition state thermodynamic parameters ($\Delta H°^{\ddagger}$ and $\Delta S°^{\ddagger}$) can be derived from the slope and the extrapolated intercept of the line on the Y-axis, respectively, of a straight line fitted to the data.

Results

Hen egg lysozyme (HEL) was immobilized on the sensor surface of Sensor Chip CM5 using amine coupling. Samples for interaction analysis (variable domains (Fv) of an anti-HEL monoclonal antibody and two mutants, mut 1 and mut 2) were prepared by serial dilution in HBS-EP buffer and injected over the surface. Interactions were monitored across a range of temperatures from 13°C to 45°C, allowing the interactions to be thermodynamically characterized. Figure 5 illustrates the effect of temperature on interaction kinetics for wildtype and mut 1. The kinetic and equilibrium constants, automatically calculated using Biacore T100 evaluation wizards, are presented in the Appendix.
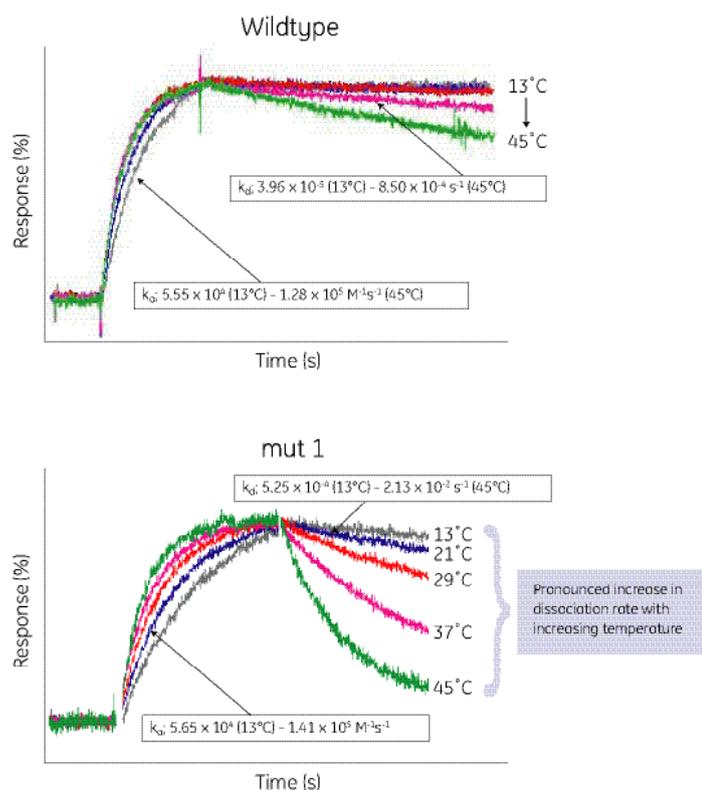
Two patterns are immediately apparent from a visual inspection of these sensorgrams. Firstly, increasing temperature caused an increase in both the association and dissociation rates. Secondly, the effect of increased temperature, especially on mut 1, was greater on the dissociation rate than on association. Both mutants studied had reduced affinity for HEL (see Appendix). In both cases, the reduction was due almost entirely to an increase in the dissociation rate while the association rate was only marginally affected.

The affinity and kinetic data derived from the sensorgrams were then fitted to equations 3 or 7 via evaluation wizards in Biacore T100 software to automatically obtain values for the equilibrium and transition state thermodynamic parameters for the interactions (Figure 6).

Figure 5. Sensorgram overlays for wildtype and mut 1 injected over HEL at temperatures between 13°C and 45°C. Sensorgrams were normalized to 100% at the end of the injections.
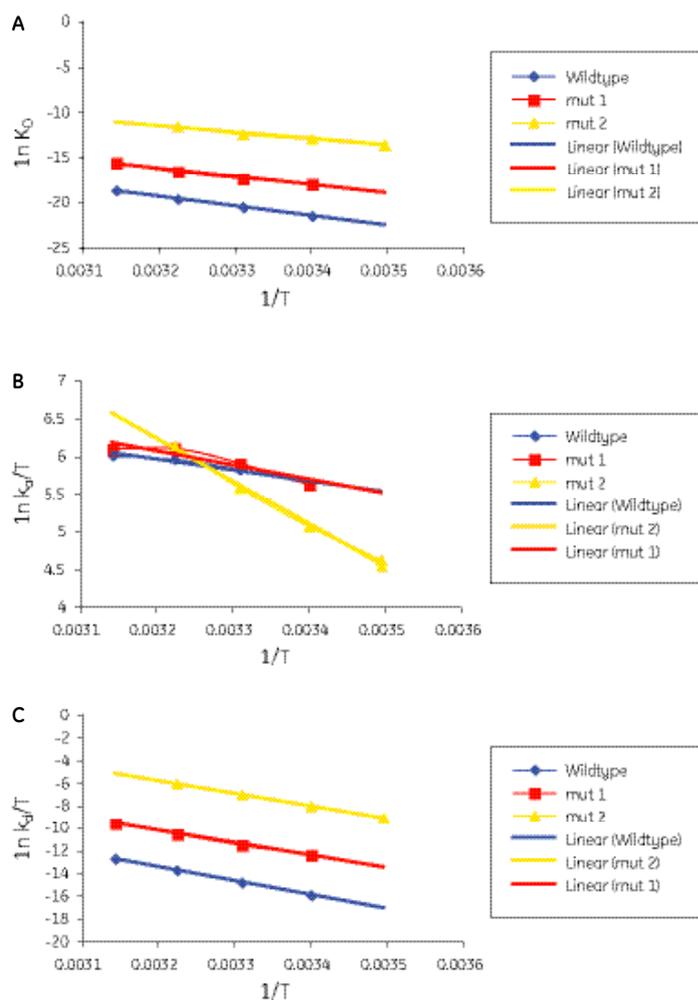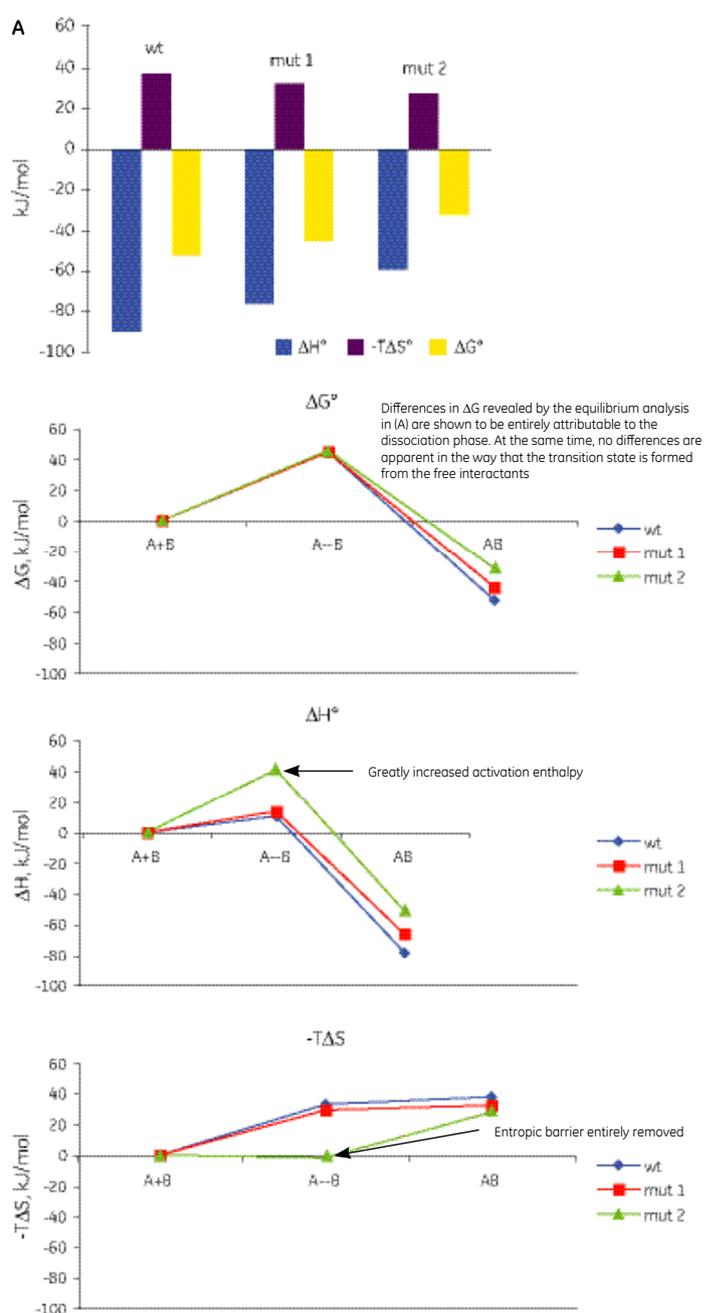

Figure 6. van't Hoff plot (A) and Eyring plots (B and C) of the experimental data. The Eyring plots are derived from Eyring equations comprising association rate constants, k_a (B) and dissociation rate constants, k_d (C). See Figures 2 and 4 for an explanation of how thermodynamic parameters are evaluated from these plots.

Table 1. Thermodynamic parameters of the interactions studied using Biacore T100. Data are derived from the van't Hoff plots and Eyring plots shown in Figure 6. Data from ITC experiments are shown in parentheses.

Interaction phase	Thermodynamic parameter	Wildtype	mut 1	mut 2
Equilibrium	$\Delta H°$ (kJ mol^{-1})	-90 (-92)	-76 (-75)	-59 (-60)
	$\Delta S°$ (J (K*mol)$^{-1}$)	-126 (-136)	-107 (-93)	-92 (-79)
	$\Delta G°$ (kJ mol^{-1}) at 25°C	-52 (-50)	-44 (-47)	-32 (-36)
	$T\Delta S°$	-37 (-41)	-32 (-28)	-27 (-24)
Association	$\Delta H°^{\ddagger}$	12	16	47
	$\Delta S°^{\ddagger}$	-110	-97	6
	$\Delta G°^{\ddagger}$	45	45	46
	$T\Delta S°$	-33	-29	2
Dissociation	$\Delta H°^{\ddagger}$	102	92	93
	$\Delta S°^{\ddagger}$	16	12	52
	$\Delta G°^{\ddagger}$	97	89	78
	$T\Delta S°$	5	4	15

The values of the thermodynamic parameters estimated from linear fits of the data on the van't Hoff and Eyring plots shown in Figure 6 are presented above in Table 1. Equilibrium data obtained using ITC are also shown for comparison. The two techniques gave similar results, confirming that label-free interaction analysis is a reliable alternative to ITC for equilibrium thermodynamic studies. The data further demonstrated that the interaction in this case study was enthalpically driven and entropically unfavorable for both wild type and mutant antibodies. The mutations increased $\Delta G°$ (and, therefore, decreased affinity) by increasing $\Delta H°$. The entropic term was not significantly affected by the mutations.

The data generated from Biacore T100 experiments run over several temperatures are also shown in graphic form in Figure 7.

Figure 7. Thermodynamic characterization of the interaction between wildtype or mutant Mabs and HEL at equilibrium (A) and in transition (B-D). A+B, A-B and AB represent initial state, transition state and complex, respectively. The results demonstrate an additional value of kinetic over equilibrium data and of thermodynamic analysis over a range of temperatures compared to analysis at a single temperature. The data shown in panel B cannot be obtained by equilibrium analysis, but only by running kinetics at a single temperature. One further step, running kinetics at different temperatures, allows $\Delta G°^{\ddagger}$ to be split into enthalpic and entropic terms (C, D). In this example, the activation pattern of mut 2 is shown to be very different from that of both wildtype and mut 1, implying that association may occur via a different interaction mechanism.

By resolving the interactions into association and dissociation phases, it was possible to thermodynamically characterize the transition state. The mutations affected only the dissociation rates, implying that the differences in the binding energies are due to different free energy barriers for dissociation, whereas the activation free energies of association for the wild type and mutant antibodies are similar. However, if the activation free energy of association is resolved into enthalpy and entropy, a significant difference in the activation pattern of mut 2, compared to both wildtype and mut 1, is revealed. Replacement of Tyr 50 with phenylalanine (mut 1) does not affect the enthalpy and entropy of activation, while the substitution of alanine residues for Tyr 33 and Tyr 53 (mut 2) entirely removes the entropic barrier, a change compensated by an increased activation enthalpy. This suggests that the association of mut 2 with HEL may follow a very different molecular mechanism from that governing both wild type and mut 1.

The difference is apparent only if activation free energy of association is resolved into its enthalpic and entropic components, illustrating an additional advantage of thermodynamic analysis over kinetic analysis at a single temperature.

Summary

- Thermodynamic analysis with Biacore T100 showed how mutations in variable fragments of a monoclonal antibody affected the kinetics of their interactions with hen egg lysozyme.

- Running the analyses over a series of temperatures enabled thermodynamic characterization, showing that the interaction was enthalpically driven and entropically unfavorable for both wildtype and mutant antibodies.

- Interactions were resolved into association and dissociation phases, making it possible to thermodynamically characterize the transition state. The mutations affected only the dissociation rates, implying that the differences in the binding energies were due to different free energy barriers for dissociation, whereas the activation free energies of association for the wildtype and mutant antibodies were similar.

- Resolving the activation free energy of association into enthalpy and entropy revealed differences in the activation pattern of specific mutations. The difference was apparent only when activation free energy of association was resolved into its enthalpic and entropic components, illustrating an additional advantage of thermodynamic analysis over kinetic analysis at a single temperature.

- This level of information could only be provided by integrating kinetic rate constants for interactions calculated over a range of temperatures into thermodynamic equations. The thermostated flow cell compartment in Biacore T100 enabled the rapid acquisition of these data while the software wizards automatically evaluated the entropic and enthalpic terms comprising the activation free energy of association.

- Comparison with ITC suggested that Biacore T100 is a reliable alternative to ITC for equilibrium thermodynamic studies. In addition, Biacore T100 delivered transition state thermodynamics, information impossible to obtain with ITC.

Methods

Instrument

All experiments were run on Biacore T100, featuring an integrated degasser allowing problem-free kinetic measurements at temperatures up to 45°C, as well as a temperature-controlled flow cell and sample compartment.

Model system

Interactions between HEL and variable region fragments of a monoclonal anti-HEL antibody (HyHEL-10 Fv and two mutants, single mutant LY50F (mut 1 - replacement of tyrosine residue 50 with phenylalanine) and double mutant HY33AY53A (mut 2 - substitution of alanine residues 33 and 53 with tyrosines) were characterized for kinetic and thermodynamic parameters.

Interaction analysis

HEL was immobilized on Sensor Chip CM5 by amine coupling. Samples were prepared by serial dilution in buffer and injected over the prepared sensor surface. The sensor surface was regenerated between runs by a 1-minute pulse of glycine solution, pH 1.5.

Thermodynamics assay

HBS-EP was used as the running buffer throughout. Data were taken from a three to five independent experiments at temperatures of 13°C, 21°C, 29°C, 37°C and 45°C. Dissociation of the wildtype antibody at 13°C was too slow for reliable kinetic data to be obtained and this temperature point was therefore excluded from the analysis. Data from the interaction involving mut 2 at 45°C was also excluded, as dissociation was too rapid to accurately determine.

Data evaluation

The kinetic data were evaluated using Biacore T100 evaluation wizards. At each temperature and for each analyte, the last (if more than one) blank reference-subtracted sensorgram was subtracted from reference-subtracted sensorgrams for non-zero analyte concentrations. The resulting curves were fitted to the Langmuir 1:1 interaction model with local R_{max}. The affinity constant, K_D, as well as association and dissociation rate constants, k_a and k_d, were obtained and fitted to the linear forms of the van't Hoff and Eyring equations to obtain estimates for $\Delta H°$ and $\Delta S°$ as well as $\Delta H°^{‡}$ and $\Delta S°^{‡}$, respectively.

References

1. Borea, P.A., Varani, K., Gessi, S., Merighi, S., Dal Piaz, A., Gilli, P. and Gilli, G. Receptor binding thermodynamics at the neuronal nicotinic receptor. Current Topics in Medicinal Chemistry 4: 361-8 (2004)

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Appendix

Affinity and kinetic rate constants for interactions between variable fragments of monoclonal anti-HEL antibody (wildtype, mut 1 and mut 2) and HEL.

Analyte (Fv fragment from Mab)	Temperature (°C)	k_a (M^{-1}s^{-1})	k_d (s^{-1})	K_D (M^{-1})
Wildtype	21	8.22×10^4	3.38×10^{-5}	4.12×10^{-10}
	29	1.02×10^5	1.04×10^{-4}	1.05×10^{-9}
	37	1.19×10^5	3.07×10^{-4}	2.61×10^{-9}
	45	1.28×10^5	8.50×10^{-4}	6.64×10^{-9}
mut 1	21	8.15×10^4	1.15×10^{-3}	1.44×10^{-8}
	29	1.11×10^5	2.84×10^{-3}	2.58×10^{-8}
	37	1.38×10^5	7.79×10^{-3}	5.69×10^{-8}
	45	1.41×10^5	2.13×10^{-2}	1.52×10^{-7}
mut 2	13	2.66×10^4	3.00×10^{-2}	1.19×10^{-6}
	13	2.87×10^4	2.90×10^{-2}	1.07×10^{-6}
	21	4.60×10^4	8.51×10^{-2}	2.06×10^{-6}
	29	7.93×10^4	2.45×10^{-1}	3.48×10^{-6}
	37	1.43×10^5	6.65×10^{-1}	8.34×10^{-6}

k_a = association rate constant, k_d = dissociation rate constant, K_D = affinity constant. Values are means of three to five replicates.

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