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Thermodynamic characterization of affinity maturation: the D1.3 antibody and a higher-affinity mutant

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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 fivefold 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. © **1998 John Wiley & Sons, Ltd.**

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Introduction

Significant therapeutic uses for antibodies have been explored, but many require higher affinity than is typically produced by the immune system (Foote and Eisen, 1995). For example, anticancer agents have been complexed to antibodies against tumor specific antigens; however, very high affinity ($K_d < 1 \times 10^{-9}$ M) is needed to obtain sufficient tumor retention for efficacy (Schier *et al.*, 1996a,b). Much previous research has focused on generating large random libraries of mutant antibodies, then searching for the rare one with improved affinity (e.g. Lowman *et al.*, 1991; Hawkins *et al.*, 1992, 1993; Barbas et al., 1994; Schier *et al.*, 1996a,b). What has not been done is to exhaustively trace the thermodynamic development of such an affinity-matured antibody. Purely rational design of antibodies is likely a very distant prospect. However, improved understanding of the thermodynamic pathways followed during affinity maturation may help identify and bias selection methods towards those paths with the best affinity potential. Joyce (1997) points out the danger of a germline antibody not sampling a broad enough range of conformations in the affinity maturation process. Similarly, selection processes favoring particular thermodynamic mechanisms for affinity maturation may implicitly incorporate affinity limitations. Characterization of the thermodynamic pathway followed by an affinity-matured antibody may help future guidance of selection conditions towards the most productive pathways.

For this study we used D1.3, a monoclonal antibody against hen egg-white lysozyme (HEL). Using multiple rounds of affinity or kinetic selection with phage display, Hawkins *et al.* (1993) identified mutants of the single-chain variable region fragment (scFv) of D1.3 with improved binding affinity for HEL. By combining these mutations, Hawkins and co-workers created the mutant M3 with six amino acid changes and a fivefold higher affinity for HEL.

Experimental

Materials

HEL, phosphatidyl choline, and bovine ubiquitin were from Sigma (St Louis, MO, USA). Acrylamide (40%) was from Bio-Rad (Hercules, CA, USA). scFvs (D1.3 and M3) were expressed in *E. coli* (HB101) and purified on an HEL–sepharose affinity column prepared from CNBr-linked sepharose. Concentrations of protein solutions were determined by absorbance at 205 nm (Scopes, 1974) and/or 280 nm.

Fluorescence Quench Titrations

There is a large quench in fluorescence when HEL and D1.3

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(or M3) bind. By titrating known concentrations of the antigen with the antibody, the equilibrium dissociation constant was measured. Fluorescence was measured with an SPF-500C spectrofluorometer (Spectronic Instruments, Inc., Rochester, NY, USA) operated in ratio mode using an internal fluorescence standard. The cuvette block was temperature-controlled at 25 °C by a circulating water bath.

To reduce protein adsorption, cuvettes were soaked overnight in a 400 mg l^{-1} solution of phosphatidyl choline, then rinsed in water and air-dried. Titrations were performed in HBS (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA). Ubiquitin, a protein with very low fluorescence, was added (at 30 mg l^{-1}) to the HBS as a carrier protein to further reduce adsorption to cuvette walls (Foote and Winter, 1992).

For a titration a cuvette would contain 20–30 nM antibody in 2.5 ml. Aliquots (10 µl) of a 2 µM solution of HEL were added to the cuvette and allowed to reach equilibrium for 8 min before each fluorescence measurement. An identical blank titration of HEL into HBS was performed. The excitation was at 280 nm, with a 1 nm slit to reduce photobleaching. Emission was measured at 346 nm (the peak for D1.3), with a 20 nm slit to maximize signal. The fluorescence was measured for 10 s and averaged, and the excitation shutter was closed to prevent photobleaching between measurements.

The fluorescence quench F_q was defined as the fluorescence of the titration mixture, less the fluorescence of the antibody alone, less the fluorescence of the corresponding HEL concentration from the blank titration. The dissociation equilibrium constant is defined by assuming the binding model

$$K_d = \frac{[Ab][H]}{[AbH]}$$

Using this, the fluorescence quench is described by

$$F_q = \frac{1}{2}\Delta f\,[([Ab]_0 + [H]_t + K_d) - \sqrt{([Ab]_0 + [H]_t + K_d)^2 - 4[Ab]_0[H]_t}]$$

where Δf is the molar fluorescence change upon complexation with lysozyme; [Ab], [H] and [AbH] are the concentrations of antibody, HEL and complex respectively, $[Ab]_0$ is the initial antibody concentration and $[H]_t$ is the total HEL concentration (free and in complex). A non-linear least squares fit was performed to estimate the parameters K_d and Δf, with the HEL concentration as the independent variable and the fluorescence quench as the dependent variable.

Isothermal Titration Calorimetry

Titrations were performed using the MCS microcalorimeter (Wiseman *et al.* 1989) from MicroCal Inc. (Northampton, MA, USA). The scFv and HEL were dialyzed overnight in the same vessel. The buffer used was 10 mM sodium phosphate (pH 7.1) with 150 mM NaCl. The proteins were centrifuged for 10 min at 16 000 *g* to remove aggregates, then degassed. For a typical experiment, 0.1 mM HEL solution was titrated into a 5 µM scFv solution. Following a 2 µl preinjection, 15 injections of 10 µl each were made. Including a correction for the heat of dilution, the enthalpy of binding was calculated by ORIGIN (MicroCal Inc.).

Acrylamide Quenching of Fluorescence

A cuvette was coated with phosphatidyl choline, then filled with HBS containing ubiquitin (30 mg l^{-1}). Protein was added to the cuvette from concentrated stocks to yield the following concentrations: 100 nM scM3, 200 nM D1.3, 200 nM HEL, 100 nM scM3 + 100 nM HEL or 200 nM scD1.3 + 200 nM HEL. The cuvettes with HEL and antibody together were allowed sufficient time to reach equilibrium.

Fluorescence was measured at an excitation of 280 nm and an emission of 346 nm in a cell thermostatted to 25 °C. Successive aliquots (10, 15 or 20 µl) of 40% acrylamide were added to the cuvettes and the fluorescence was measured. Fluorescence data were corrected for the dilution of the protein due to the increase in volume (about 5%) over the course of the titration. Fluorescence intensities were also corrected for the absorbance of incident light due to the acrylamide (McClure and Edelman, 1967). Finally, for the complexes a correction was made for the fraction of protein not in complex at equilibrium, by subtracting the fluorescence contribution of the free protein as measured in the titrations with one protein only.

Results and Discussion

By fluorescence quench titrations we determined the binding equilibrium constants (K_d) at 25 °C to be 22 (± 10) × 10^{-9} M for D1.3 and 4.2 (± 1.7) × 10^{-9} M for M3. Thus, in terms of the free energy, $\Delta\Delta G$ of binding ($\Delta G_{mutant} - \Delta G_{wild\text{-}type}$) is −1.0 kcal mol^{-1}. Isothermal titration calorimetry was used to measure the change in enthalpy upon binding. At 25 °C, ΔH of binding for D1.3 and M3 is essentially the same, within accuracy limits of the measurements (−21.9 and −22.2 ± 0.6 kcal mol^{-1} respectively). Therefore the increased affinity can be largely attributed to entropic effects (Table 1).

Murphy *et al.* (1995) have developed a theoretical deconvolution of the entropy into the component contributions from the solvation (ΔS_{solv}), configurational (ΔS_{conf})

Table 1. Thermodynamics of binding show that affinity improvement of mutant M3 is predominantly entropic

	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔC_p (kcal mol^{-1} K^{-1})	$-T\Delta S$ (kcal mol^{-1})
D1.3	−10.4 (±0.3)	−21.9	−0.35 (±0.01)	+11.5
M3	−11.4 (±0.2)	−22.2 (±0.6)	−0.38 (±0.03)	+10.8 (±0.6)
ΔΔ	−1.0 (±0.4)	−0.3	−0.03 (±0.03)	−0.7

Data at 25 °C. All negative values in table represent favorable contributions to binding; ΔΔ represents mutant minus wild-type (M3 - D1.3). Numbers in parentheses are standard deviations (or standard error in the slope for ΔC_p).

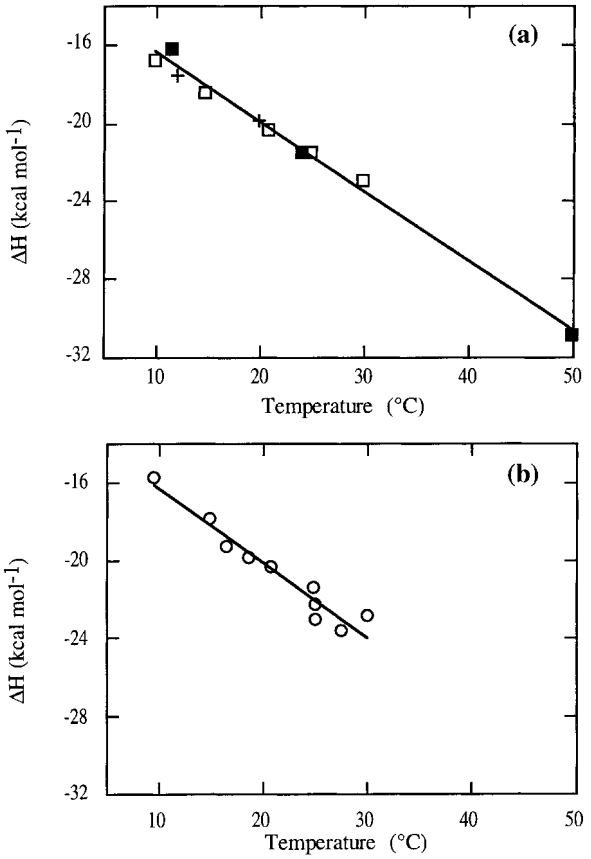

Figure 1. Enthalpy change upon binding antibody to HEL. The slope of each plot is the heat capacity change upon binding. (a) Wild-type D1.3. Open squares are for the Fv and crosses are for the scFv, demonstrating the same behavior. Full squares are data from Schwarz *et al.* (1995) for Fv D1.3 to show agreement. (b) Mutant scFv M3.

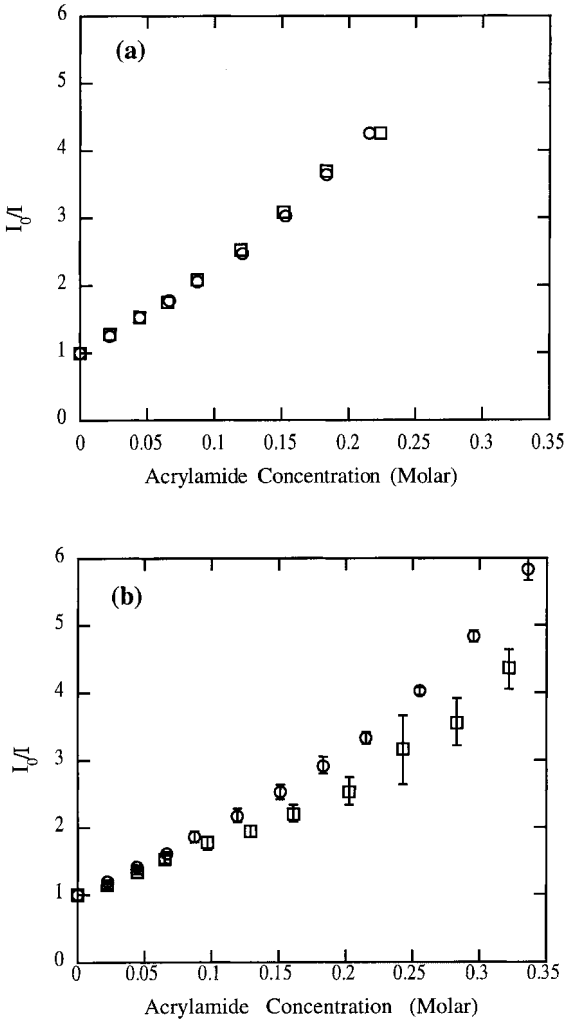

Figure 2. Stern–Volmer plots comparing the effect of adding acrylamide as a fluorescence quencher to wild-type or mutant antibody solutions. I_0/I is the ratio of the fluorescence intensity in the absence of acrylamide to the fluorescence intensity in the presence of acrylamide. (a) For scFv antibodies only. Squares represent D1.3 and circles represent M3. Degree of quenching is indistinguishable. (b) For the complexes of scFv antibodies with HEL. Squares represent D1.3–HEL and circles represent M3–HEL. The M3–HEL complex is significantly more quenched than the D1.3–HEL complex.

and cratic (ΔS_{crat}) entropy terms, with cratic entropy approximately constant (Novotny *et al.* 1989). ΔS_{solv} accounts for the change in entropy upon binding resulting from solvent effects, including solvent binding or release. The solvation contribution to the entropy change at temperature T (K) is

$$\Delta S_{solv} = \Delta C_p \ln\left(\frac{T}{385}\right)$$

Thus, to evaluate the entropic improvement of M3, additional calorimetry was done to obtain the heat capacity change of binding for each antibody (Fig. 1). Within limits of experimental uncertainty, ΔC_p was the same for the wild-type as the mutant (-0.35 ± 0.01 and -0.38 ± 0.03 kcal mol^{-1} respectively). The uncertainty in the calculated ΔS_{solv} (due to the uncertainty in ΔC_p) is larger than the entire binding free energy gain of the mutant, making it impossible to use this method to distinguish between solvation and configurational entropy gains.

To attempt to separate the entropy contributions experimentally, Stern–Volmer plots were constructed (Fig. 2). Acrylamide was used to quench the fluorescence of the scFv antibodies alone or while in complex with HEL. The upward curvature of the plots (which could indicate static quenching or result from the large number of fluorophores in these antibodies) makes it impractical to fit Stern–Volmer constants (Eftink and Ghiron, 1981). However, it is clear that scFv D1.3 and M3 alone are quenched indistinguishably, while the M3–HEL complex is more quenched than the D1.3–HEL complex at the same acrylamide concentration. This indicates that the fluorophores in the M3–HEL complex are more accessible to a bulk phase quencher than the fluorophores in the D1.3–HEL complex. This provides preliminary support for the M3–HEL complex being less rigid, which would imply that at least some of the entropic gain is configurational.

To further probe the solvent entropy changes of the mutant, osmotic stress experiments are being conducted. X-ray crystallography has identified 48 bound water molecules in the D1.3–HEL interface (Bhat *et al.* 1994). Goldbaum *et*

al. (1996) have also shown that D1.3 takes up water molecules upon binding. We will compare the solvent uptake upon binding of D1.3 to M3 to investigate the possible changes in solvation entropy. We are also isolating various combinations of the six amino acid changes of M3 in order to determine which are significant to the affinity improvement.

Further affinity maturation attempts via yeast surface display (Boder and Wittrup, 1997) will indicate whether M3 can be mutated to higher affinity or has approached the limits for its particular thermodynamic pathway. Finding a higher-affinity mutant may allow more concrete conclusions about the various entropic contributions, as the changes would be larger. Affinity maturation starting from the parent D1.3 will also be pursued under varying selection conditions to attempt to force alternative thermodynamic affinity maturation pathways.

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