Supplemental Material

Human TNF-α amino acid sequence:

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  1 VRSSSRTPSD KPVAHVVANP QAEGQLQWLN RRANALLANG VELRDNQLVV PSEGLYLIYS
 61 QVLFKGQGCP STHVLLTHTI SRIAVSYQTK VNLLSAIKSP CQRETPEGAE AKPWYEPIYL
121 GGVFQLEKGD RLSAEINRPD YLDFAESGQV YFGIIAL
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Extinction coefficient used for determination of concentration: $\varepsilon_{TNF(278nm)} = 60500\ M^{-1}cm^{-1}$

Fab of adalimumab amino acid sequence:

Light chain:

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  1 DIQMTQSPSS LSASVGDRVT ITCRASQGIR NYLAWYQQKP GKAPKLLIYA ASTLQSGVPS
 61 RFSGSGSGTD FTLTISSLQP EDVATYYCQR YNRAPYTFGQ GTKVEIKRTV AAPSVFIFPP
121 SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT
181 LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC
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Heavy chain:

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  1 EVQLVESGGG LVQPGRSLRL SCAASGFTFD DYAMHWVRQA PGKGLEWVSA ITWNSGHIDY
 61 ADSVEGRFTI SRDNAKNSLY LQMNSLRAED TAVYYCAKVS YLSTASSLDY WGQGTLVTVS
121 SASTKGPSVF PLAPSSKSTS GGTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS
181 SGLYSLSSVV TVPSSSLGTQ TYICNVNHKP SNTKVDKKVE PKSC
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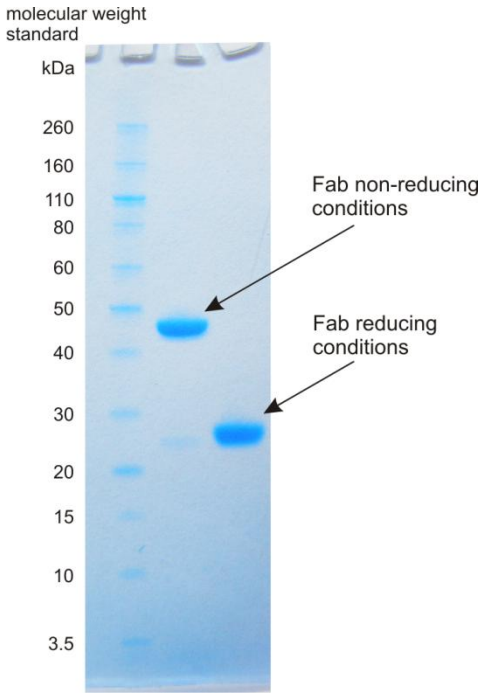
Extinction coefficient used for determination of concentration: $\varepsilon_{Fab(278nm)} = 66400\ M^{-1}cm^{-1}$

SDS PAGE electrophoresis:

SDS PAGE electrophoresis was employed to confirm suitability of the monomolecular denaturation model used for the interpretation of urea induced unfolding curves. The data suggests that Fab is composed of two polypeptide chains of similar length (20-30 kDa) connected with a disulphide bond. This is also in accordance with the amino acid sequence data presented above.



SI-Figure 1. SDS PAGE electrophoresis of Fab under reducing and non-reducing conditions.

Thermodynamic Analysis of Experimental Data

Global Model Analysis of Urea-induced Unfolding Curves Monitored by CD-Urea denaturation of Fab, TNF-α and the Fab-TNF-α complex can be successfully described in terms of a two-state or three-state model. The models can be defined as follows,

$$\mathrm{F} \xleftrightarrow{K_{\mathrm{F}(T,u)}} \mathrm{F^D}$$

$$\mathrm{T_3} \xleftrightarrow{K_{\mathrm{T1}(T,u)}} \mathrm{I_T} \xleftrightarrow{K_{\mathrm{T2}(T,u)}} 3\mathrm{T^D}$$

$$\mathrm{T_3F_3} \xleftrightarrow{K_{\mathrm{TF1}(T,u)}} \mathrm{I_{TF}} \xleftrightarrow{K_{\mathrm{TF2}(T,u)}} 3\mathrm{T^D} + 3\mathrm{F^D}$$

Models 1-3

where F, T_3 and T_3F_3 represent Fab monomer, TNF-α trimer and TNF-α-Fab_3 heterohexamer in their native (N) states, respectively, whereas superscript D denotes the proteins in their denatured (D) states. The transitions to the intermediate states I_T and I_{TF} accompanying the denaturation of T_3 and T_3F_3 complex is assumed to be monomolecular. The apparent equilibrium constants in Models 1-3 are functions of temperature (T) and urea concentration (u) and can be defined as: $K_{\mathrm{F}(T,u)} = [\mathrm{F^D}]/[\mathrm{F}]$, $K_{\mathrm{T1}(T,u)} = [\mathrm{I_T}]/[\mathrm{T_3}]$, $K_{\mathrm{T2}(T,u)} = [\mathrm{T^D}]^3/[\mathrm{I_T}]$, $K_{\mathrm{TF1}(T,u)} = [\mathrm{I_{TF}}]/[\mathrm{T_3F_3}]$ and $K_{\mathrm{TF2}(T,u)} = [\mathrm{T^D}]^3[\mathrm{F^D}]^3/[\mathrm{I_{TF}}]$. The quantities in the square brackets represent the corresponding equilibrium molar concentrations that are dependent on T and u. According to the models the measured molar ellipticity ($[\Theta]_{(T,u)}$) at a given wavelength, T and u can be expressed in terms of the corresponding contributions $[\Theta]_{\mathrm{N}(T,u)}$, $[\Theta]_{\mathrm{I}(T,u)}$ and $[\Theta]_{\mathrm{D}(T,u)}$ that characterize states N, I, and D as follows,

$$[\Theta]_{(T,u)} = [\Theta]_{\mathrm{N}(T,u)}\alpha_{\mathrm{N}(T,u)} + [\Theta]_{\mathrm{I}(T,u)}\alpha_{\mathrm{I}(T,u)} + [\Theta]_{\mathrm{D}(T,u)}\alpha_{\mathrm{D}(T,u)} \qquad \text{(SI-Eq. 1)}$$

where $\alpha_{\mathrm{N}(T,u)}$, $\alpha_{\mathrm{I}(T,u)}$ and $\alpha_{\mathrm{D}(T,u)}$ represent fractions of proteins in states N, I and D respectively, at given T and u. In the case of F denaturation , the fractions are defined as $\alpha_{\mathrm{N}(T,u)} = \alpha_{\mathrm{F}(T,u)} = [\mathrm{F}]/[\mathrm{F}]_t$ and $\alpha_{\mathrm{D}(T,u)} = \alpha_{\mathrm{F^D}(T,u)} = [\mathrm{F^D}]/[\mathrm{F}]_t$, in the case of T_3 denaturation as $\alpha_{\mathrm{N}(T,u)} = \alpha_{\mathrm{T_3}(T,u)} = [\mathrm{T_3}]/[\mathrm{T_3}]_t$ and $\alpha_{\mathrm{D}(T,u)} = \alpha_{\mathrm{T^D}(T,u)} = 1/3[\mathrm{T^D}]/[\mathrm{T_3}]_t$ and in the case of T_3F_3 denaturation $\alpha_{\mathrm{N}(T,u)} = \alpha_{\mathrm{T_3F_3}(T,u)} = [\mathrm{T_3F_3}]/[\mathrm{T_3}]_t$, whereas $\alpha_{\mathrm{D}(T,u)}$ and $[\Theta]_{\mathrm{D}(T,u)}$ are defined as $\alpha_{\mathrm{D}(T,u)} = \alpha_{\mathrm{T^D}(T,u)} = \alpha_{\mathrm{F^D}(T,u)}$ and $[\Theta]_{\mathrm{D}(T,u)} = 3[\Theta]_{\mathrm{T^D}(T,u)} + 3[\Theta]_{\mathrm{F^D}(T,u)}$, respectively ($[F]_t$, $[T_3]_t$ and $[T_3F_3]$ represent total Fab, TNF-α trimer and Fab-TNF-α complex concentrations, respectively). While $[\Theta]_{(T,u)}$ $[\Theta]_{\mathrm{N}(T,u)}$, $[\Theta]_{\mathrm{D}(T,u)}$ can be obtained from the experiment ($[\Theta]_{(T,u)}$ is the measured signal, while $[\Theta]_{\mathrm{N}(T,u)}$ and $[\Theta]_{\mathrm{D}(T,u)}$ can be estimated at any measured T as linear functions of u from pre- and post-transitional base lines), $\alpha_{\mathrm{I}(T,u)}$ and $\alpha_{\mathrm{D}(T,u)}$ can be calculated from the model, as shown below. For two state denaturation of F the second term in SI-Eq. 1 is equal to zero. In the case of T_3 or T_3F_3 denaturation $[\Theta]_{\mathrm{I}(T,u)}$ was included in the global modeling as a linear function of T independent of u. $\alpha_{\mathrm{I}(T,u)}$ and $\alpha_{\mathrm{D}(T,u)}$ can be connected to the thermodynamics of unfolding through proposed models (Models 1-3) and the general characteristics of urea denaturation that the standard Gibbs free energy of unfolding ($\Delta G^o_{i(T,u)}$) for any transition i (i = F, T1, T2, TF1 or TF2) and T appears to be a linear function of u

$$\Delta G^o_{i(T,u)} = \Delta G^o_{i(T)} - m_i \cdot u \qquad \text{(SI-Eq. 2)}$$

where m_i is an empirical parameter correlated strongly to the amount of protein surface area-exposed to the solvent upon denaturation (1) and assumed to be temperature-independent. $\Delta G^o_{i(T)}$ is the standard Gibbs free energy of unfolding in the absence of urea (u = 0) that may be expressed in terms of corresponding standard Gibbs free energy ($\Delta G^o_{i(T_0)}$) and standard enthalpy of unfolding ($\Delta H^o_{i(T_0)}$) at a reference temperature T_0 = 37 ºC and standard heat capacity of unfolding ($\Delta C^o_{P,i}$), assumed to be temperature-independent, through the Gibbs-Helmholtz relation (integrated form).

$$\Delta G^o_{i(T)} = \Delta G^o_{i(T_0)} T/T_0 + \Delta H^o_{i(T_0)}\left[1 - T/T_0\right] + \Delta C^o_{P,i}\left[T - T_0 - T\ln\left(T/T_0\right)\right] \qquad \text{(SI-Eq. 3)}$$

It follows from SI-Eq. 2 and SI-Eq. 3 that the model (adjustable) parameters $\Delta G^o_{i(T_0)}$, $\Delta H^o_{i(T_0)}$, $\Delta C^o_{P,i}$, and m_i define $\Delta G^o_{i(T,u)}$ and thus the corresponding $K_{i(T,u)}$ [$K_{i(T,u)} = \exp\left(-\Delta G^o_{i(T,u)}/RT\right)$]. Consequently, they specify the populations of species in solution ($K_{i(T,u)} = f(\alpha_{j(T,u)}; j = N, I, D)$, $\sum_j \alpha_{j(T,u)} = 1 \Rightarrow \alpha_{j(T,u)}$) and the model function (SI-Eq. 1) at any u and T. The best global fit values of $\Delta G^o_{i(T_0)}$, $\Delta H^o_{i(T_0)}$ and $\Delta C^o_{P,i}$ (Table 1) obtained using the nonlinear Levenberg-Marquardt regression procedure (2) were used to estimate $\Delta G^o_{i(T)}$ (from SI-Eq. 3), $\Delta H^o_{i(T)}$ from the Kirchhoff's law (integrated form),

$$\Delta H^o_{i(T)} = \Delta H^o_{i(T_o)} + \Delta C^o_{P,i}\left(T - T_0\right) \qquad \text{(SI-Eq. 4)}$$

and the corresponding entropy contribution, $T\Delta S^o_{i(T)}$ from the general relation

$$\Delta G^o_{i(T)} = \Delta H^o_{i(T)} - T\Delta S^o_{i(T)} \qquad \text{(SI-Eq. 5)}$$

Global Model Analysis of ITC Binding Curves-Model function describing binding of Fab to three equivalent independent binding sites on TNF-α was fitted to the sets of ITC curves measured at various temperatures as described below. The model function at a given T can be defined as follows (3,4)

$$\Delta H_T = \Delta H^o_{i(T)}\left(\partial\bar{\nu}/\partial r\right)_{P,T,n_1} \qquad \text{(SI-Eq. 6)}$$

where $\Delta H^o_{i(T)}$ is the standard enthalpy of Fab binding to the binding site of TNF-α, $\bar{\nu}$ is an average number of Fab bound on TNF-α ($\bar{\nu} = \sum_{i=1}^{n} i\left[T_3F_i\right]/\left[T_3\right]_t$), and r is a molar ratio between total concentration of Fab and TNF-α in the measuring cell. The derivative in SI-Eq. 6 can be expressed as $\left(\partial\bar{\nu}/\partial r\right)_{P,T,n_1}$ = (1/2)(1+[3-r-c]/[r^2-2r(3-c)+(1+c)^2]$^{1/2}$), where c = 1/($K_{(T)}$[T_3]$_t$), $K_{(T)}$ is an apparent constant of Fab binding to any of the three binding sits on TNF-α and [T_3]$_t$ is the total concentration of TNF-α in the measuring cell. The corresponding standard Gibbs free energy $\Delta G^o_{i(T^o)}$ and standard enthalpy $\Delta H^o_{i(T^o)}$ at T_0 = 37 ºC and standard heat capacity of binding $\Delta C^o_{P,i}$ (assumed to be temperature independent) define $\Delta G^o_{i(T)}$ and $\Delta H^o_{i(T)}$ at any T by SI-Eqs. 3 and 4. Thus the values of adjustable parameters $\Delta G^o_{i(T^o)}$, $\Delta H^o_{i(T^o)}$ and $\Delta C^o_{P,i}$ completely define the temperature dependence of the binding constant [$K_{i(T)}$ = exp(-$\Delta G^o_{i(T^o)}$/RT)], the model function (SI-Eq. 6) at any T and

consequently the corresponding thermodynamic profiles (Fig. 6 in the main text). The best fit values of the adjustable parameters (Table 1 in the main text) were obtained using the nonlinear Levenberg-Marquardt regression procedure (2). It should be mentioned that a significant curvature of the ITC curve at Fab/TNF-α molar ratio $r \approx 3$ (Fig 2) from which a safe binding constant can be estimated is observed only at the highest measured temperature. Therefore, one may be a bit skeptical about the accuracy of the obtained binding constant. However, since the global model gives good description of all ITC isotherms, the one with the curvature and the others with noticeable breaks at $r \approx 3$, we believe that the corresponding estimate of the binding constant is reasonable.

Structural interpretation of TNF-α-Fab association- Numerous recent studies of protein unfolding and protein-protein binding processes have shown that the corresponding enthalpy ($\Delta H^o_{i(T)}$) and heat capacity ($\Delta C^o_{P,i}$) changes can be parameterized in terms of changes in solvent accessible polar (ΔA_P) and nonpolar (ΔA_N) surface areas accompanying these processes (1,5-9). Such a parameterization is based on the estimation of the nonpolar (A_N) and polar (A_P) solvent-accessible areas of proteins in the initial (unbound) and final (bound) states. A_N and A_P of TNF-α-Fab complex were calculated from structural model obtained by molecular modeling (described in the main text) by applying the program NACCESS version 2.1 using the probe size of 1.4 Å (10). A_N and A_P of unbound TNF-α and Fab were obtained from the structural model of the complex by deleting the coordinates of either of the binding partners and by applying the same program as for the complex. $\Delta A_{N,\,BIND}$ and $\Delta A_{P,\,BIND}$ were estimated as a difference between A_N (A_P) of the TNF-α-Fab complex and the sum A_N (A_P) of the unbound TNF-α and Fab. The heat capacity ($\Delta C^o_{P,BIND}$) and enthalpy changes ($\Delta H^o_{BIND(T)} = \Delta H^o_{BIND(T_H)} + \Delta C^o_{P,BIND}(T - T_H)$) accompanying binding can be expressed as the sum of nonpolar (subscript N) and polar (subscript P) contributions (5-9).

$$\Delta C^o_{P,BIND} = \Delta C^o_{P,BIND,N} + \Delta C^o_{P,BIND,P} = a\Delta A_{N,BIND} + b\Delta A_{P,BIND} \qquad \text{(SI-Eq. 7)}$$

$$\Delta H^o_{BIND(T)} = \Delta H^o_{BIND(T),N} + \Delta H^o_{BIND(T),P} = \\ = \left[c + a(T - T_H)\right]\Delta A_{N,BIND} + \left[d + b(T - T_H)\right]\Delta A_{P,BIND} \qquad \text{(SI-Eq. 8)}$$

Parameters a = 0.45 calmol^{-1}K^{-1}Å^{-2}, b = -0.26 calmol^{-1}K^{-1}Å^{-2}, c = -8.44 calmol^{-1}Å^{-2}, and d = 31.4 calmol^{-1}Å^{-2} are obtained from Murphy and Freire (5) and Xie and Freire (6), whereas $\Delta H^o_{i(T_H)}$ is parameterized as $\Delta H^o_{i(T_H)} = c\Delta A_N + d\Delta A_P$ and represents the enthalpy of unfolding observed with most global proteins at their median transition temperature of T_H = 60 ºC. The entropy change ($\Delta S^o_{BIND,T}$) accompanying binding processes can be expressed as follows,

$$\Delta S^o_{BIND,T} = \Delta C^o_{P,BIND} \ln(T / T_S) + \Delta S^o_{R+T} \qquad \text{(SI-Eq. 9)}$$

where first term in SI-Eq. 9 is an estimate of change in solvation entropy upon binding and $T_S \approx 112$ ºC is the reference temperature at which solvation entropy is assumed to be zero (7). The second term ΔS^o_{R+T} = -50 cal mol^{-1}K^{-1} is an estimate of the translational and rotational entropy loss accompanying a rigid-body association (8). Empirical relations between structural and thermodynamic parameters in combination with experimentally obtained quantities enable us to parse the thermodynamics of binding into a contribution of rigid-body association (ΔF^o_{BIND}) and contribution of conformational change (ΔF^o_{CONF}; see Eq. 1 in the main text).

$$\Delta F^o = \Delta F^o_{BIND} + \Delta F^o_{CONF}, \qquad \text{(SI-Eq. 10)}$$

where $F = G, H, S, C_P$. ΔF^o was obtained from the ITC experiments. ΔF^o_{BIND} was calculated as mentioned above while ΔF^o_{CONF} was estimated as $\Delta F^o_{CONF} = \Delta F^o - \Delta F^o_{BIND}$. The calculated $\Delta C^o_{P,CONF}$ and $\Delta H^o_{CONF(T)}$ values enable us to estimate changes in solvent accessible polar and nonpolar surface areas accompanying conformation change ($\Delta A_{P,CONF}$, $\Delta A_{N,CONF}$) by employing SI-Eqs. 7 and 8.

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