Supplemental Material

**Human TNF-α amino acid sequence:**

1 VRSSSRTPSD KPVAHVVANP QAEGQLQWLNRANALLANG VELRDNQLVV PSEGLYLIYS
61 QVLFKSQGCPS THVLLITHTISRIAVSYQTK VNLLSAIKSP CQRETPEGAE AKPWEPIYL
121 GGVFQLEKGD RLSEAINRPDYLDFAESGQV YF GI AL

Extinction coefficient used for determination of concentration: $\epsilon_{\text{TNF}}(278\text{nm}) = 60500$ M$^{-1}$cm$^{-1}$

**Fab of adalimumab amino acid sequence:**

**Light chain:**

1 DIQMTQSPSS LSASVGDRVT ITCRASQGIR NYLAWYQQKP GKaPKLLIYA ASTLQSGVFS
61 RFSGSGSGTD FTLTISLQLP EDVATYYCQR YNRAFYTFGQ GTKVEIKRTVALPSVFIFFP
121 SDEQLKSGTA SVVCLNNFYY PREAKVQWKV DNLQSGNSQ ESVTEQDSKD STYSLSSTLT
181 LSKADYKHKV YVACEVTHQG LSSPVTKSFN RGEC

**Heavy chain:**

1 EVQLVESGGG LVQPGRSLRL SCAASGFTFD DYAMHWVRQA PGKLEWVSA ITWNSGHIDY
61 ADSVEGRFTI SRDNKNSLY LQMNSLRAED TAVYYCAKVS YLSTASSLDY WGGQTLVTVS
121 SASTKGPVSF PLAPSSTSKSTGGTAALCLVL KDYPFEPVTV SWNSGALTSG VHTFPAVLQS
181 SGLYSLSSVV TVFSSSLGTQ TYICNVNHKP SNTKVDKKEV PKSC

Extinction coefficient used for determination of concentration: $\epsilon_{\text{Fab}}(278\text{nm}) = 66400$ 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,

\[
F \leftrightarrow \frac{K_{T_u(a)}}{K_{T_u(a)}} F^D
\]

\[
T_3 \leftrightarrow \frac{K_{T^{T_3}(a)}}{K_{T^{T_3}(a)}} I_T \leftrightarrow \frac{K_{T^{T_3}(a)}}{K_{T^{T_3}(a)}} 3T^D
\]

\[
T_3F_3 \leftrightarrow \frac{K_{T^{T_3(T_3)}(a)}}{K_{T^{T_3(T_3)}(a)}} I_{T_3F_3} \leftrightarrow \frac{K_{T^{T_3(T_3)}(a)}}{K_{T^{T_3(T_3)}(a)}} 3T^D + 3F^D
\]

**Models 1-3**

where \( F, T_3 \) and \( T_3F_3 \) represent Fab monomer, TNF-α trimer and TNF-α-Fab3 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 \( I_{T_3F_3} \) 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: \( K_{T^{T_3}(T_u)} = \frac{[F_3]}{[F]} \),

\[
K_{T^{T_3(T_3)}(T_u)} = \frac{[I_T]}{[T_3]}, \quad K_{T^{T_3(T_3)}(T_u)} = \frac{[I_{T_3F_3}]}{[T_3F_3]} \quad \text{and} \quad K_{T^{T_3(T_3)}(T_u)} = \frac{[3T^D + 3F^D]}{[I_{T_3F_3}]}.
\]

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 \((\theta)_{T_u,a}\) at a given wavelength, \( T \) and \( u \) can be expressed in terms of the corresponding contributions \( [\theta]_{N(T_u)}, [\theta]_{I(T_u)} \) and \( [\theta]_{D(T_u)} \) that characterize states N, I, and D as follows,

\[
[\theta]_{T_u,a} = [\theta]_{N(T_u)} \alpha_{N(T_u)} + [\theta]_{I(T_u)} \alpha_{I(T_u)} + [\theta]_{D(T_u)} \alpha_{D(T_u)} \quad \text{(SI-Eq. 1)}
\]

where \( \alpha_{N(T_u)}, \alpha_{I(T_u)} \) and \( \alpha_{D(T_u)} \) represent fractions of proteins in states N, I and D respectively, at a given \( T \) and \( u \). In the case of F denaturation, the fractions are defined as \( \alpha_{N(T_u)} = \frac{[F]}{[F]} \),

\[
\alpha_{I(T_u)} = \frac{[F^D]}{[F]}, \quad \text{in the case of } T_3 \text{ denaturation as } \alpha_{N(T_u)} = \alpha_{T_3(T_u)} = \frac{[T_3]}{[I_T]} \quad \text{and} \quad \alpha_{D(T_u)} = \frac{[T_3]}{[T_3]},
\]

\[
\alpha_{I(T_u)} = \frac{[T_3]}{[T_3]}, \quad \text{whereas } \alpha_{D(T_u)} \quad \text{and} \quad [\theta]_{D(T_u)} \quad \text{are defined as}
\]

\[
\alpha_{D(T_u)} = \frac{[T_3]}{[T_3]}, \quad \text{and} \quad [\theta]_{D(T_u)} = \frac{[\theta]_{F^D(T_u)}}{[F^D]} + \frac{3[\theta]_{F^D(T_u)}}{[F^D]}, \quad \text{respectively (}[F], \quad [T_3], \quad \text{and } [T_3F_3] \text{ represent total Fab, TNF-α trimer and Fab-TNF-α complex concentrations, respectively). While } [\theta]_{N(T_u)}, [\theta]_{I(T_u)}, \quad \text{and } [\theta]_{D(T_u)} \text{ can be obtained from the experiment (}[\theta]_{I(T_u)} \text{ is the measured signal, while } [\theta]_{N(T_u)} \text{ and } [\theta]_{D(T_u)} \text{ can be estimated at any measured } T \text{ as linear functions of } u \text{ from pre- and post-transitional base lines), } \alpha_{I(T_u)} \quad \text{and} \quad \alpha_{D(T_u)} \text{ can be calculated from the model, as shown below. For two state denaturation of F the second term in SI-Eq. 1 is equal to zero. In the case of } T_3 \text{ or } T_3F_3 \text{ denaturation } [\theta]_{I(T_u)} \text{ was included in the global modeling as a linear function of } T \text{ independent of } u.
\]

\[\alpha_{I(T_u)} \text{ and } \alpha_{D(T_u)} \text{ can be connected to the thermodynamics of unfolding through proposed models (Models 1-3) and the general characteristics of urea denaturation that the standard Gibbs free energy of unfolding } (\Delta G_{T_u(a)}) \text{ for any transition } i (i = F, T_1, T_2, TF1 \text{ or TF2) and } T \text{ appears to be a linear function of } u.
\]

\[
\Delta G_{T_u(a)} = \Delta G_{T_u} - m_i \cdot u \quad \text{(SI-Eq. 2)}
\]
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. $\Delta G_{i(T)}^o$ is the standard Gibbs free energy of unfolding in the absence of urea ($\mu = 0$) that may be expressed in terms of corresponding standard Gibbs free energy ($\Delta G_{i(T_0)}^o$) and standard enthalpy of unfolding ($\Delta H_{i(T_0)}^o$) at a reference temperature $T_0 = 37 \, ^\circ C$ and standard heat capacity of unfolding ($\Delta C_{i,T}^o$), assumed to be temperature-independent, through the Gibbs-Helmholtz relation (integrated form).

\[
\Delta G_{i(T)}^o = \Delta G_{i(T_0)}^o \frac{T}{T_0} + \Delta H_{i(T_0)}^o \left[ 1 - \frac{T}{T_0} \right] + \Delta C_{i,T}^o \left[ T - T_0 - T \ln \left( \frac{T}{T_0} \right) \right]
\]  

(SI-Eq. 3)

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

\[
\Delta H_{i(T)}^o = \Delta H_{i(T_0)}^o + \Delta C_{i,T}^o \left( T - T_0 \right)
\]  

(SI-Eq. 4)

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

\[
\Delta G_{i(T)}^o = \Delta H_{i(T)}^o - T \Delta S_{i(T)}^o
\]  

(SI-Eq. 5)

**Global Model Analysis of ITC Binding Curves**-Model function describing binding of Fab to three equivalent independent binding sites on TNF-\(\alpha\) 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)

\[
\Delta H_T = \Delta H_{i(T)}^o \left( \frac{\bar{v}}{\bar{r}} \right)_{P,T,n_i}
\]  

(SI-Eq. 6)

where $\Delta H_{i(T)}^o$ is the standard enthalpy of Fab binding to the binding site of TNF-\(\alpha\), $\bar{v}$ is an average number of Fab bound on TNF-\(\alpha\) ($\bar{v} = \sum_i \left[ T_i F_i \right] / \left[ T_i \right]$), and $r$ is a molar ratio between total concentration of Fab and TNF-\(\alpha\) in the measuring cell. The derivative in SI-Eq. 6 can be expressed as

\[
\left( \frac{\bar{v}}{\bar{r}} \right)_{P,T,n_i} = \frac{1}{2} (1+3-r-c)((r^2-2r(3-c)+(1+c)^2)^{1/2}),
\]

where $c = 1/(K(T_3)T_3)$, $K(T_3)$ is an apparent constant of Fab binding to any of the three binding sites on TNF-\(\alpha\) and $[T_3]$ is the total concentration of TNF-\(\alpha\) in the measuring cell. The corresponding standard Gibbs free energy $\Delta G_{i(T)}^o$ and standard enthalpy $\Delta H_{i(T)}^o$ at $T_0 = 37 \, ^\circ C$ and standard heat capacity of binding $\Delta C_{i,T}^o$ (assumed to be temperature independent) define $\Delta G_{i(T)}^o$ and $\Delta H_{i(T)}^o$ at any $T$ by SI-Eqs. 3 and 4. Thus the values of adjustable parameters $\Delta G_{i(T)}^o$, $\Delta H_{i(T)}^o$ and $\Delta C_{i,T}^o$ completely define the temperature dependence of the binding constant $[K_{i(T)} = \exp(-\Delta G_{i(T)}^o/RT)]$, 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 (\( \Delta H^\alpha_{\text{rel}} \)) and heat capacity (\( \Delta C^\alpha_{p,i} \)) changes can be parameterized in terms of changes in solvent accessible polar (\( \Delta A_p \)) and nonpolar (\( \Delta A_N \)) 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. \( \Delta A^N_{\text{BIND}} \) and \( \Delta A^P_{\text{BIND}} \) 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 (\( \Delta C^\alpha_{p,BIND} \)) and enthalpy changes (\( \Delta H^\alpha_{\text{BIND}} = \Delta H^\alpha_{\text{BIND}(T)} \) + \( \Delta C^\alpha_{p,BIND} (T - T_H) \)) accompanying binding can be expressed as the sum of nonpolar (subscript N) and polar (subscript P) contributions (5-9).

\[
\Delta C^\alpha_{p,BIND} = \Delta C^\alpha_{p,BIND,N} + \Delta C^\alpha_{p,BIND,P} = a \Delta A^N_{\text{BIND}} + b \Delta A^P_{\text{BIND}} 
\]  
(SI-Eq. 7)

\[
\Delta H^\alpha_{\text{BIND}(T)} = \Delta H^\alpha_{\text{BIND}(T),N} + \Delta H^\alpha_{\text{BIND}(T),P} = 
\]
\[
= [c + a(T - T_H)] \Delta A^N_{\text{BIND}} + [d + b(T - T_H)] \Delta A^P_{\text{BIND}} 
\]  
(SI-Eq. 8)

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

\[
\Delta S^\alpha_{\text{BIND},T} = \Delta C^\alpha_{p,BIND} \ln(T/T_S) + \Delta S^\alpha_{R,T} 
\]  
(SI-Eq. 9)

where first term in SI-Eq. 9 is an estimate of change in solvation entropy upon binding and \( T_S \approx 112 \text{ ºC} \) is the reference temperature at which solvation entropy is assumed to be zero (7). The second term \( \Delta S^\alpha_{R,T} = -50 \text{ cal mol}^{-1} \text{ K}^{-1} \) 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 (\( \Delta F^\alpha_{\text{BIND}} \)) and contribution of conformational change (\( \Delta F^\alpha_{\text{CONF}} \); see Eq. 1 in the main text).

\[
\Delta F^\alpha = \Delta F^\alpha_{\text{BIND}} + \Delta F^\alpha_{\text{CONF}}, 
\]  
(SI-Eq. 10)
where $F = G, H, S, C_P$. $\Delta F^o$ was obtained from the ITC experiments. $\Delta F^o_{\text{BIND}}$ was calculated as mentioned above while $\Delta F^o_{\text{CONF}}$ was estimated as $\Delta F^o_{\text{CONF}} = \Delta F^o - \Delta F^o_{\text{BIND}}$. The calculated $\Delta C^o_{p,\text{CONF}}$ and $\Delta H^o_{\text{CONF}(T)}$ values enable us to estimate changes in solvent accessible polar and nonpolar surface areas accompanying conformation change ($\Delta A^o_{p,\text{CONF}}, \Delta A^o_{n,\text{CONF}}$) by employing SI-Eqs. 7 and 8.

References: