Supporting Information

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SI Materials and Methods

Surface Plasmon Resonance (SPR) Experiments. Before use, the chip surface was prepared by flowing HBS buffer [10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20 (pH 7.4)] at 100 μL/min and performing three injections of 20 μL of hydrochloric acid solution (N/10), sodium hydroxide solution (N/10), and 0.5% (wt/vol) SDS. Receptor coupling involved first activating the surface by flowing 25 μL of NHS:EDC (75 mg/mL:11.5 mg/mL) at a flow rate of 5 μL/min. Next, 20 μL of 18 mg/mL 2-(2-pyridinyldithio)ethaneamine hydrochloride (PDEA) was injected over all flow cells. Human growth hormone receptor (hGHR) (2–4 μL of 1 μM) was diluted to 100 μL of total volume of 10 mM sodium acetate so that the final receptor concentration was 20–40 nM (pH 4.5); 25–50 μL was then injected over sample flow cells (FC2, 3, and 4; leaving FC1 as a reference cell). The receptor was always coupled so that a maximum resonance units (RU) upon hormone binding reached a maximum value between 20 and 60 RU. After receptor coupling, uncoupled functional groups were blocked with 30 μL of 50 mM reduced glutathione in 50 mM sodium acetate, 1 M NaCl (pH 4.5). Human prolactin receptor (hPRLR) was coupled to the surface with an engineered cysteine (C184A/T207C) by using a procedure outlined in ref. 1, which includes both a glutathione and ethanolamine blocking step to eliminate unreacted SH and NH groups, respectively.

All experiments were carried out at a flow rate of 50 μL/min. Experiments typically included duplicate injections of ≈6 different concentrations of hormone (using 2-fold dilutions starting from 75 to 200 nM) injected over the three sample cells (FC2, 3, and 4 and one reference FC1). Blank (buffer) injections were dispersed between every 3–4 hormone injections to optimize blank subtractions. The chip surface was regenerated with 5-μL injections of 4.5 M $MgCl_2$. Data were subjected to baseline/blank injection normalization by using the program SCRUBBER (BioLogic Software). Data were then fit globally by using the 1:1 Langmuir model using the program CLAMP (2). Transition state (TS) thermodynamics parameters (theory described below) were investigated by determining the temperature dependence of the association and dissociation rates over a range of 15–40 °C. Urea dependence studies (examining association and dissociation binding kinetics) were investigated over a range of 0–0.8 M urea.

TS Thermodynamics. TS theory describes the activation free energy, or TS free energy of association (or dissociation), with the following relationship:

$$\Delta G^{\ddagger}_{on} = -RT\ln\left(\frac{k_{on}h}{k_B T}\right) \qquad [s1]$$

where $\Delta G^{\ddagger}_{on}$ is the TS free energy for association (or dissociation), R is the gas constant, T is the temperature (on Kelvin scale), k_{on} is the association rate, h is the Plank constant (6.625×10^{-34} Js), and k_B is the Boltzmann constant (1.3805×10^{-23}J/K). $\Delta G^{\ddagger}_{on}$ possesses both enthalpic ($\Delta H^{\ddagger}_{on}$) and entropic components ($\Delta S^{\ddagger}_{on}$).

$$\Delta G^{\ddagger}_{on} = \Delta H^{\ddagger}_{on} - T\Delta S^{\ddagger}_{on} \qquad [s2]$$

The temperature dependence of $\Delta H^{\ddagger}_{on}$ and $\Delta S^{\ddagger}_{on}$ are described by the $\Delta C^{\ddagger}_{p,on}$:

$$\begin{aligned}\Delta H^{\ddagger}_{on} &= \Delta H^{\ddagger}_{on,ref} + \Delta C^{\ddagger}_{p}(T - T_{ref})\\ \Delta S^{\ddagger}_{on} &= \Delta S^{\ddagger}_{on,ref} + \Delta C^{\ddagger}_{on}\ln(T/T_{ref})\end{aligned} \qquad [s3]$$

where T_{ref} is the reference temperature (25 °C for the work presented here) and $\Delta H^{\ddagger}_{on,ref}$ and $\Delta S^{\ddagger}_{on,ref}$ are the TS enthalpy and entropy at the reference temperature, respectively. The above equations were combined to the following equation:

$$\ln\left(\frac{k_{on}}{T}\right) = \frac{\Delta S^{\ddagger}_{on} - \Delta C^{\ddagger}_{p,on}}{R} + \ln\left(\frac{h}{k_B}\right) + \frac{\Delta C^{\ddagger}_{p,on} - \Delta H^{\ddagger}_{on}/T_{ref}}{R}\left(\frac{T_{ref}}{T}\right) - \frac{\Delta C^{\ddagger}_{p,on}}{R}\ln\left(\frac{T_{ref}}{T}\right). \qquad [s4]$$

Similarly, the following includes the dissociation parameter:

$$\ln\left(\frac{k_{off}}{T}\right) = \frac{\Delta S - \Delta C^{\ddagger}_{p,off}}{R} + \ln\left(\frac{h}{k_B}\right) + \frac{\Delta C^{\ddagger}_{p,on} - \Delta H^{\ddagger}_{off}/T_{ref}}{R}\left(\frac{T_{ref}}{T}\right) - \frac{\Delta C^{\ddagger}_{p,off}}{R}\ln\left(\frac{T_{ref}}{T}\right). \qquad [s5]$$

These two relationships were used in fitting the temperature-dependent SPR k_{on} and k_{off} values, respectively. A temperature range of 15–40 °C was examined experimentally. The program KaleidaGraph was used in fitting the data, when expressed as ln k/T as a function of $1/T$, to determine the TS thermodynamics parameters.

For both hGHv and AS1, the slow rate of dissociation at lower temperatures prevented obtaining accurately the dissociation thermodynamics (including $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta C^{\ddagger}_{p}$. However, comparison of dissociation thermodynamics (for both WT hGH and AS2) calculated from equilibrium and association thermodynamic values compared well with dissociation thermodynamics determined experimentally, suggesting that dissociation thermodynamics for hGHv and AS1 could be reliably calculated using the existing data. Therefore, dissociation thermodynamics were determined by using the association thermodynamics (determined here) and the equilibrium thermodynamics [determined previously (3)] by using the following relationships.

$$\begin{aligned}\Delta H^{o}_{eq} &= \Delta H^{\ddagger}_{on} - \Delta H^{\ddagger}_{off}\\ \Delta S^{o}_{eq} &= \Delta S^{\ddagger}_{off} - \Delta S^{\ddagger}_{off}\\ \Delta C^{o}_{p,eq} &= \Delta C^{\ddagger}_{p,on} - \Delta C^{\ddagger}_{p,off}.\end{aligned} \qquad [s6]$$

Error Analysis. Errors are presented as ±1 SD. When necessary, such as calculation of $\Delta S^{\ddagger}_{assoc}$ and $\Delta C^{\ddagger}_{p,off}$, error was propagated according to the following (4):

$$(df)^2 = \left[\left(\frac{\partial f}{\partial x}\right)^2_y (dx^2) + \left(\frac{\partial f}{\partial y}\right)^2_x (dy^2) + \ldots\right]. \qquad [s7]$$

Mutational Analysis of Binding Kinetics. Single alanine mutants of both hGH and hGHR interface were explored (5, 6). These include alanine point mutations at hGH residues M14, H18, H21, Q22, F25, D26, Q29, Y42, L45, Q46, P48, S51, E56, P61, S62, N63, R64, E65, Q68, Y164, R167, K168, D171, K172, E174,

T175, F176, R178, I179, R183, and E186 and hGHR residues: R43, E44, R70, W76, S98, S102, I103, W104, I105, P106, E120, K121, D126, E127, D164, I165, Q166, W169, V171, Q216, R217, N218, and S219.

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5. Bernat B, Sun M, Dwyer M, Feldkamp M, Kossiakoff AA (2004) Dissecting the binding energy epitope of a high-affinity variant of human growth hormone: cooperative and additive effects from combining mutations from independently selected phage display mutagenesis libraries. *Biochemistry* 43:6076–6084.
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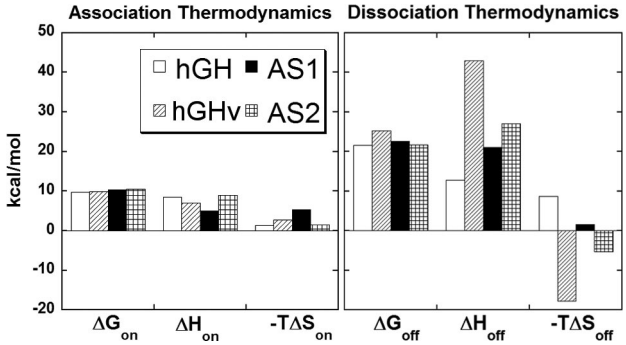

Fig. S1. TS thermodynamics for hormone–receptor binding, which includes wild-type hormone, the phage-optimized hGHv, and two alanine-shaved hormones, AS1 and AS2. Both association (*Left*) and dissociation (*Right*) TS thermodynamics are presented.

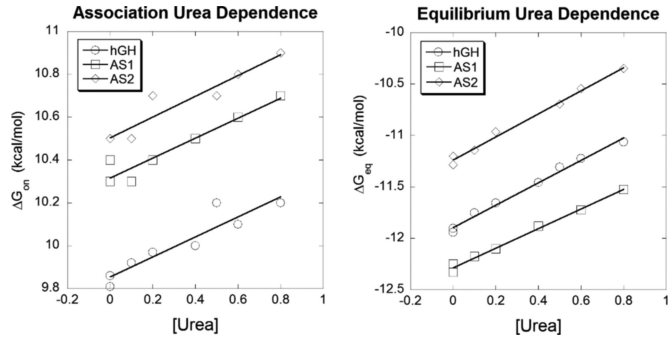

Fig. S2. Urea dependence of the binding association rate (*Left*) and equilibrium constant (*Right*) for hGH and the two alanine-shaved variants.

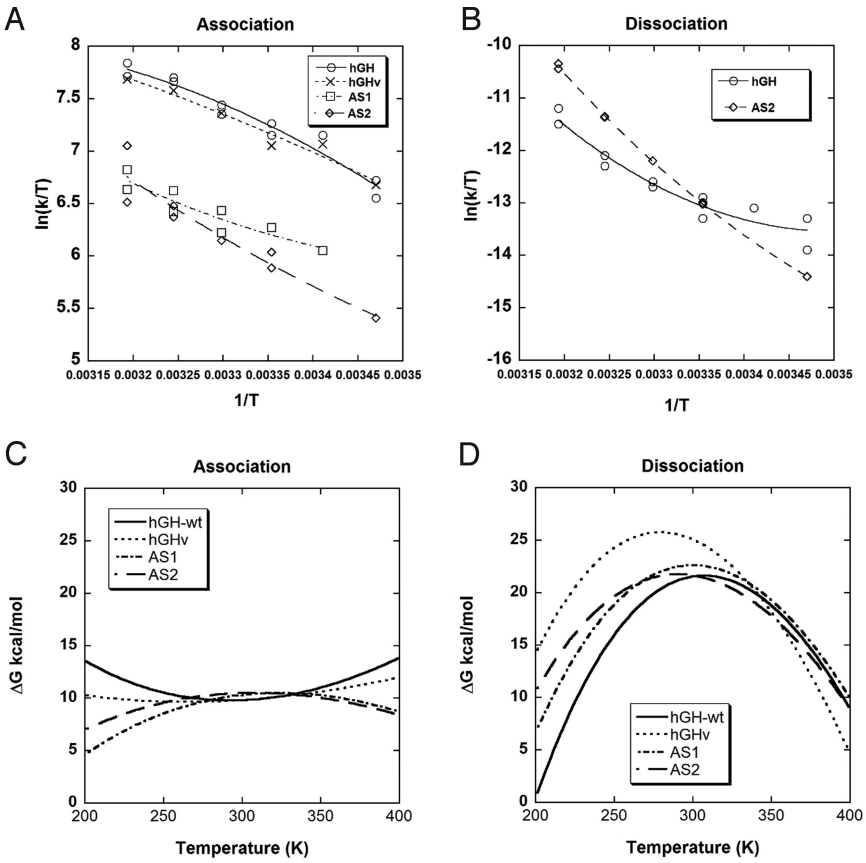

Fig. S3. Temperature dependence of growth hormone–receptor association and dissociation. (*A* and *B*) Raw data plots of ln k/T vs. $1/T$ for association (*A*) and dissociation (*B*). Temperature (T) units are in the Kelvin scale. (*C* and *D*) Extrapolated free energy barriers are displayed for association (*C*) and dissociation (*D*) as a function of temperature for the different hGH variants. The stability curves (*C* and *D*) are obtained from the TS association and dissociation thermodynamic values (i.e., $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta C_p^{\ddagger}$) as described in *SI Materials and Methods*. Data are extrapolated over a wide temperature range ranging from 200 to 400 K.