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Compensatory Energetic Mechanisms Mediating the Assembly of Signaling Complexes Between Interleukin-2 and its α, β, and γ_c Receptors

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Interleukin-2 is a key immuno-regulatory cytokine whose actions are mediated by three different cell surface receptors: the α, β and the "common γ" (γ_c) chains. We have undertaken a complete thermodynamic characterization of the stepwise assembly cycle for multiple possible combinations of the receptor–ligand, and receptor–receptor interactions that are necessary for formation of the high-affinity IL-2/$\alpha\beta\gamma_c$ signaling complex. We find an entropically favorable high affinity interaction between IL-2 and its α receptor, a moderately entropically favorable low affinity interaction between IL-2 and its β receptor, and no interaction between IL-2 and the shared receptor, γ_c. Formation of the stable intermediate trimolecular complexes of IL-2 with α and β receptors, as well as IL-2 with β and γ_c receptors proceeds through enthalpy–entropy compensation mechanisms. Surprisingly, we see a moderate affinity interaction between the unliganded receptor α and β chains, suggesting that a preformed $\alpha\beta$ complex may serve as the initial interaction complex for IL-2. Reconstitution of the IL-2/R$\alpha\beta\gamma_c$ high-affinity quaternary signaling complex shows it to be assembled through cooperative energetics to form a 1:1:1:1 assembly. Collectively, the favorable entropy of the bimolecular interactions appears to be offset by the loss in rigid body entropy of the receptor components in the higher-order complexes, but overcome by the formation of increasingly enthalpically favorable composite interfaces. This enthalpic mechanism utilized by γ_c contrasts with the favorable entropic mechanism utilized by gp130 for degenerate cytokine interaction. In conclusion, we find that several energetically redundant pathways exist for formation of IL-2 receptor signaling complexes, suggesting a more complex equilibrium on the cell surface than has been previously appreciated.

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Introduction

Interleukin-2 (IL-2)[1] is a prototypical member of the four-helix bundle hematopoietic cytokine superfamily[1–3] that includes interleukin-6, growth hormone, erythropoietin, and many others. This superfamily, originally described by Bazan *et al*.[3] is subdivided into short and long-chain members, of which IL-2 resides in the former group. IL-2 was initially identified as a key regulatory molecule in T-cell proliferation[4] and natural killer cell activity[5] and thus was originally denoted as T-cell stimulatory factor. The biological activities of IL-2 are many, and diverse, and a number of these activities have made IL-2 a very compelling protein for clinical use in a variety of immune-related diseases.[6] Currently, IL-2 (known clinically as

Abbreviations used: IL-2, interleukin-2; IL2-Rα, IL-2 α receptor; IL2-Rβ, IL-2 β receptor; IL2-Rγ_c, common IL-2 γ receptor; ITC, isothermal titration calorimetry MALS, multi-angle light scattering; SPR, surface plasmon resonance; CHR, cytokine binding homology region; BCA, bicinchoninic acid.

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Proleukin) is one of the most effective biological agents in the treatment of metastatic renal cell carcinoma and metastatic melanoma.[7] Although the downstream signaling pathways of IL-2 receptors are known to proceed through JAK/STAT pathways,[8] uncertainties about the nature of the molecular interactions between IL-2 and its receptors still exist. Given the immunological relevance of IL-2 we have undertaken a comprehensive effort to study the thermodynamic basis of complex formation with its receptors, to complement previous kinetic studies.[9,10]

The biological activities of IL-2 are mediated by three single-transmembrane, cell surface receptors: the IL-2-specific IL2-Rα and IL2-Rβ chains, and the promiscuous Rγ_c (review[11]). The cDNA of the IL2-Rα subunit (p55, Tac)[12] encodes a single mature polypeptide chain of 251 amino acid residues that folds into two sushi-style domains connected by an extended 30 amino acid linker. IL2-Rα is not involved in signaling but its presence enhances receptor affinity and it may play a role in the regulation of the lymphoid compartment.[13] The binding of IL-2 to IL2-Rα can be blocked with monoclonal antibodies, termed anti-Tac, which have been proven to be clinically effective as immunosuppressive agents.[14] The larger 75 kDa IL2-Rβ (p75),[15] is a member of the hematopoietic growth factor receptor family.[3] The key structural feature of these signaling receptors is a cytokine binding homology region (CHR) comprised of two fibronectin repeats where the C-terminal domain encodes a WSXWS motif and the N-terminal domain shows a distinct disulfide-bonding pattern.

The third receptor component of the IL-2 signaling complex is the "common gamma chain" (γ_c), which is the principal signaling receptor within the complex.[16] IL2-Rγ_c is also utilized as a signaling receptor component by IL-4, IL-7, IL-9, IL-15, and IL-21 in combination with their cytokine-specific receptor α or β chains.[17] γ_c along with the common β_c and gp130 form the three principal shared signaling receptors for cytokines. As such, IL2-Rγ_c is endowed with striking cross-reactivity for diverse cytokines. The use of a common signaling chain by immunoregulatory cytokines ensures functional redundancy and pleiotropy in their biological activities.

There have been no thermodynamic studies of cytokine receptor interactions involving IL-2, or any of the cytokines, which use the shared γ_c (IL-2, IL-15, IL-13, IL-4, IL-7, and IL-9). An important question is the energetic basis for the ability of γ_c to cross-react with such a diverse array of cytokines, as well as assemble multipartite signaling complexes. The majority of data describing ligand–receptor interactions within the IL-2 system has been derived from cell-based assays and a comprehensive series of kinetic analyses from the Ciardelli group using surface plasmon resonance.[10,18] From these studies, the affinity of IL-2 for each of its individual receptor subunits was measured to be modest. The IL2-Rα and IL2-Rβ subunits exhibit dissociation constants (K_d) of approximately 10 nM and 450 nM, respectively.[19,20] The interaction between IL-2 and the γ_c subunit is still controversial and was determined to be very weak ($K_d > 50$ μM).[21] Physiologically, these subunits act in concert to generate binding sites with much greater affinity for the ligand.[9,22–24] On NK-cells IL2-Rβ and IL2-Rγ_c also form a heterodimeric complex in the presence of ligand to create an intermediate-affinity site having a $K_d \approx 1$ nM.[19] When all three subunits are present, such as on the surface of activated T-cells, a heterotrimeric high-affinity site is generated with a $K_d \approx 10$ pM.[22] Different groups have studied the affinity and kinetics of interactions between IL-2 and its receptors using different formats for different combinations of receptors, but there has not yet been any thermodynamic characterization. Such thermodynamic data can give detailed information about the chemical and structural nature of the receptor–ligand interactions.

Recently, experimental evidence indicates that the anti-tumor efficacy of IL-2 is mediated by distinct subsets of T cells, which express structurally unique forms of the IL-2 receptor.[25] Further, it appears that these different forms of the IL-2 receptor may dissociate efficacy from toxicity. Thus, it has become a desirable goal to understand, in detail, the thermodynamic basis of assembly of the various low, intermediate, and high-order complexes of IL-2 with its receptors as a complement to ongoing efforts to crystallize the IL-2 receptor complexes, for which no structural information currently exists.

We have undertaken a complete thermodynamic characterization of the assembly cycle for multiple possible combinations between IL-2 and its three, different receptor subunits, using isothermal titration calorimetry (ITC) and multi-angle light scattering (MALS). We find a transition from highly favorable entropy for formation of the bimolecular complex between IL-2 and IL2-Rα to a more enthalpy-driven process commensurate with the formation of composite interfaces in the higher-order IL2-R$\alpha\beta\gamma_c$ signaling complex. Thus, the thermodynamic basis for the positive cooperativity in formation of the quaternary signaling complex is achieved through compensation of loss in entropy by increasingly enthalpically favorable receptor–receptor, and receptor–ligand intermolecular interactions. We show positive cooperativity for formation of the IL-2 complex with IL2-Rα and IL2-Rβ. We also find low affinity binding of IL2-Rα to IL2-Rβ in the absence of IL-2, supporting the idea of a preformed IL2-Rα/IL2-Rβ complex, which would serve as the initial interaction complex for IL-2 on the surface of activated T-cells. These studies reveal important new features, as well as confirm some previous notions, about the stepwise mechanism of IL2-R assembly on activated T and NK-cells. Further, they define a unique entropy–enthalpy compensation mechanism used by the shared γ_c to cross-react

with multiple cytokines of differing surface structure and chemistry.

Results

Thermodynamics of complex formation between IL-2 and its individual receptor subunits

In order to carry out our proposed studies, we expressed human IL-2 as well as the extracellular domains of Rα, Rβ and Rγ_c using a baculovirus expression system (Figure 1(A)). Each of the proteins is secreted into the culture media using an insect-derived leader peptide. The individual, unliganded proteins behave according to their expected molecular masses as analyzed by gel filtration chromatography. Each of the molecules is extensively *N*-glycosylated, thereby eliminating concern about the behavior of non-glycosylated forms expressed in *Escherichia coli*. To study the assembly energetics of the ternary IL-2 signaling complex we dissected all possible binding combinations between IL-2 and the extracellular domains of its three individual receptor subunits using ITC (Figure 1). Each experiment is presented with a schematic model describing the interactions studied, a representative ITC trace (I), an FPLC chromatogram along with SDS-PAGE gel of the contents from the ITC cell after the titration (II) and the measured and calculated thermodynamic values (III). Thus, all experiments described here show both measured *N* values by ITC as well as gel filtration and SDS-PAGE to verify complex formation and to rule out protein precipitation in the ITC-cell. Additionally, the thermodynamic experiments of the binary ligand–receptor complexes were performed at three different temperatures to enable the calculation of heat capacity, ΔC_p (more temperatures are not possible due to the limited supply of recombinant proteins). ΔC_p is an approximate measure of buried hydrophobic surface area and can also serve as a signature of conformational rearrangements upon binding when ΔC_p is anomalously large.

In the first series of experiments, IL-2 was titrated into IL2-Rα ectodomain at 10 °C, 15 °C and 20 °C with the representative ITC trace at 15 °C (shown in Figure 1(AI)). The interaction of IL-2 with IL-2Rα shows a stoichiometry of 1:1 with a dissociation constant (K_d) of 10 nM, consistent with previous observations.[19] We find that the binary interaction is largely entropy driven ($\Delta S = 18.38$ cal/mol K), presumably as a result of desolvation, but also displays a favorable enthalpy of $\Delta H = -5.23$ kcal/mol, suggesting that hydrophilic interactions also play a significant role in complex formation. The enthalpy change upon binding was shown to decrease linearly with temperature, indicating that the association reaction is characterized, in this temperature range, by a constant heat capacity change, ΔC_p, of -519 cal/mol K (Figure 1(AIII)).

The titration of IL-2 into its receptor β-subunit ectodomain (Figure 1(B)) shows a binding stoichiometry of 1:1, consistent with previous studies.[10] The measured affinity of IL-2 with IL-2Rβ of $K_d \sim 144$ nM, is significantly lower than observed for the IL-2/IL2-Rα interaction but higher than determined previously (450 nM) from a competitive receptor binding assay.[20] The interaction between IL-2 and IL2-Rβ displays both an overall favorable enthalpy ($\Delta H = -7.6$ kcal/mol) and entropy ($\Delta S = 4.79$ cal/mol K). In comparison to the IL-2/IL2-Rα titration, the IL-2/IL2-Rβ interaction shows a greater enthalpic contribution while being less reliant on the entropic term (Figure 1(BIII)). The heat capacity change (ΔC_p) was calculated to be -597 cal/mol K.

In the absence of complete structural information, buried surface can be approximated based on an empirical calculation of $\Delta C_p = 0.45A_{apol} - 0.26A_{pol}$, where A_{apol} represents the change in apolar and A_{pol} represents the change in polar surface buried upon complex formation.[26] Using these parameters, we calculated the total buried surface area between IL-2 and IL2-Rα, and IL-2 and IL2-Rβ, to be ~ 2700 Å^2 and ~ 3100 Å^2, respectively. These calculated values for overall buried surfaces are somewhat higher compared to normal buried surface area for typical hydrophobic, rigid body interactions.[27] This suggests that, in addition to the hydrophobic effect, there are likely to be some structural rearrangements occurring upon binding, either domain movements or conformational adjustments of flexible loops. Structural plasticity is known from the human growth hormone system to be a common feature of cytokine receptor interactions.[28]

The titration of IL-2 into IL2-Rγ_c ectodomain showed no detectable interaction at 15 °C and 20 °C, nor was any complex formation detected by FPLC sizing chromatography. This observation contrasts previous SPR experiments[29] and antibody precipitations of whole cell lysates that showed a modest interaction between the two proteins.[21] The discrepancy may lie in the very weak affinity of IL-2 for IL2-Rγ_c, which is supported by a competitive binding assay[30] where the affinity of IL-2 for IL2-Rγ_c was measured to be $K_d > 50$ μM, which is above the protein concentrations used in our ITC experiments (and therefore below our detection limit). We conclude that there is not a biologically relevant interaction affinity between IL-2 and Rγ_c.

Receptor–receptor binding interactions within the IL-2 signaling complex

To test the possibility that a population of the IL-2 receptors may exist as preformed complexes on the cell surface, in the absence of IL-2, we carried out all possible combinations of ITC titrations between the extracellular domains of

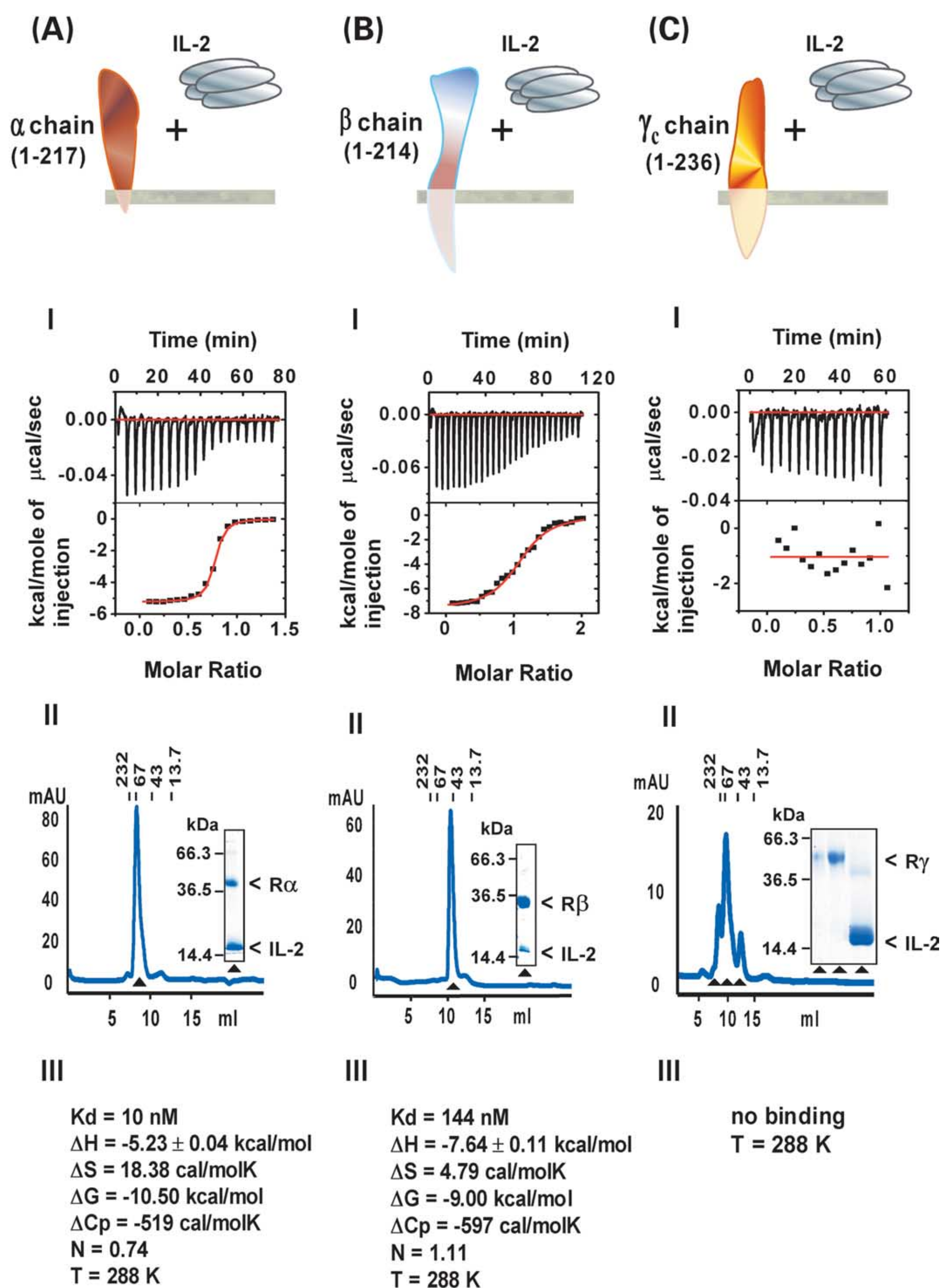


Figure 1. Isothermal titration calorimetry (ITC) of IL-2 with (A) IL2-Rα, (B) IL2-Rβ and (C) IL2-Rγ_c. Each experiment in Figures 1 through 3 is presented in a multi-panel format that includes a schematic model of the experiment, (I) a representative ITC trace, (II) an FPLC chromatogram and SDS-PAGE l of the contents of the ITC cell and (III) a list of the measured and calculated thermodynamic values. In each titration experiment, the concentration of protein in the cell was 2–4 µm with a seven- to tenfold higher concentration in the syringe. Data points from each titration are fit with a best-fit curve with non-linear least-squares fitting using the ORIGIN-software. (A) The titration of IL-2 into IL2-Rα formed a 1:1 complex that is largely entropy driven ($\Delta S = 18.38$ cal/mol K) with a moderate enthalpic ($\Delta H = -5.23(\pm 0.04)$ kcal/mol) contribution. (B) IL-2 also forms a 1:1 complex with IL2-Rβ; however, the affinity (144 nM) is much weaker than for IL-2/IL-2 Rα and the interaction is enthalpy driven ($\Delta H = -7.64(\pm 0.11)$ kcal/mol) and relies less on entropic contributions (4.79 cal/mol K). The heat capacity (ΔC_p) calculated for (A) and (B) was −519 cal/mol K and −597 cal/mol K, respectively. (C) No binding is observed for IL-2 with IL2-Rγ_c.

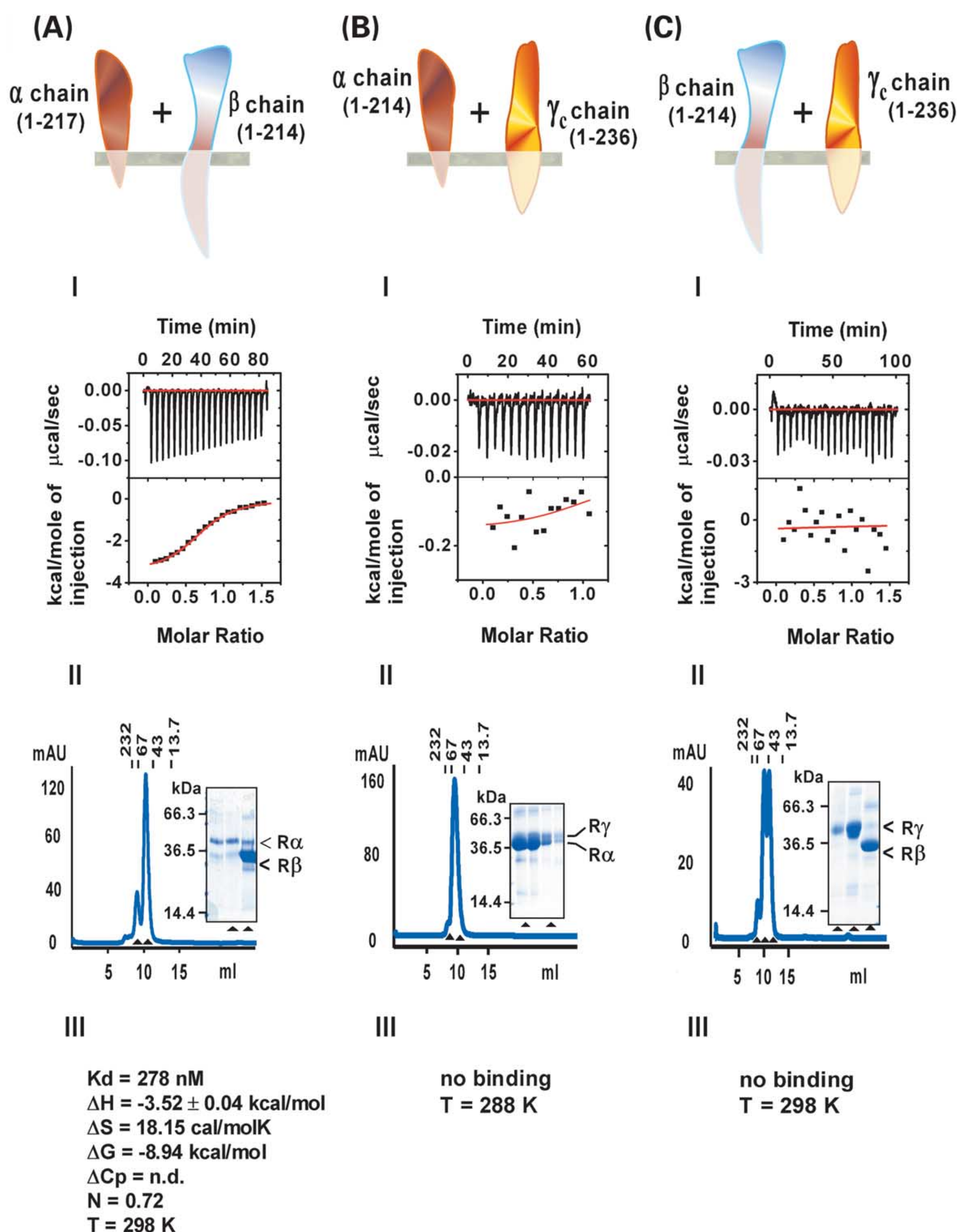


Figure 2. Receptor–receptor interactions. (A) IL2-Rα forms a 1:1 complex with IL2-Rβ of moderate affinity ($K_d \sim 278$ nM) in the absence of IL-2. The interaction is largely entropy driven ($\Delta S = 18.15$ cal/mol K) suggesting burial of hydrophobic surfaces. The FPLC chromatogram and SDS-PAGE indicate that the IL2-Rα/IL2-Rβ complex is of low affinity with only a small complex peak visible on the FPLC chromatogram relative to the excess Rβ. (B) and (C) IL2-Rγ_c does not appear to form a complex with either IL2-Rα or IL2-β. n.d., not determined.

IL2-Rα, IL2-Rβ and IL2-Rγ_c (Figure 2). First we titrated IL2-Rα into IL2-Rβ at 25 °C (Figure 2(A)). A low affinity binding event was observed ($K_d \sim 278$ nM) with favorable enthalpy ($\Delta H = -3.52$ kcal/mol) and entropy ($\Delta S = 18.15$ cal/mol K) resulting in $\Delta G = -8.94$ kcal/mol. We find a 1:1 stoichiometry of binding consistent with previously published reports.[10] Due to the low affinity of this interaction, a large excess of Rβ was necessary to drive the titration to saturation, and this is reflected in the excess unliganded Rβ apparent in the second peak of the gel filtration

profile, with the first peak containing all of the Rα in a Rα/Rβ complex through mass action.

Titrations of IL2-Rγ_c into either IL2-Rα or IL2-Rβ showed no detectable binding (Figure 2(B) and (C)). Our thermodynamic data reflect kinetic studies done by Liparoto *et al.*,[31] who used SPR to characterize the binding activity between IL2-Rγ_c and components of the IL-2 receptor complex. That IL2-Rγ_c is not separately binding to any individual module of the IL-2 receptor complex suggests that IL2-Rγ_c needs a composite binding interface between the ligand IL-2 and/or IL2-Rα and IL2-Rβ. This observation prompted our next series of ITC experiments studying the thermodynamics of the higher-order assembly of IL-2 receptor variants.

Thermodynamic assembly of the higher-order IL-2 receptor complex variants

At least two different biologically relevant IL-2 receptor variants are present in two affinity states on IL-2 responsive cells. High affinity receptor complexes exist as heterotetramers (IL-2 plus -Rα, -Rβ and -Rγ_c) on activated T-cells, while the intermediate affinity receptor complex is comprised of IL-2 plus -Rβ and -Rγ_c on NK-cells and monocytes.[5] To study the thermodynamic basis for these alternate higher-order assemblies, we titrated monomeric components into preformed, purified bimolecular complexes. By using this approach we were able to effectively deconvolute a multi-body problem into a binary interaction.

In the first series of experiments, IL2-Rβ was titrated into a pre-formed IL-2/IL2-Rα complex (Figure 3(A)). Initially, IL-2 and IL2-Rα were mixed stoichiometrically and the complex purified over a FPLC gel filtration column to ensure a binary 1:1 complex was used in the titration. The titration of IL2-Rβ into the pre-formed complex showed a moderate binding affinity of $K_d \sim 63$ nM, which is significantly higher compared to the separate binding of IL2-Rβ to IL-2 ($K_d \sim 144$ nM), or to IL2-Rα ($K_d \sim 278$ nM). Despite the N value of 0.64 (IL-2/Rα to Rβ), we are certain that this three-way complex is 1:1:1, since it runs at ~67 kDa by gel filtration, the IL-2/Rα complex was preformed for the titration, and 1:1:1 is consistent with SPR analysis.[32] Also, the individual IL-2/Rα and IL-2/Rβ are each 1:1. Thus, the N value reflects the collective imprecisions inherent in the protein concentration measurements of three heavily glycosylated proteins in the complex. These data indicate a positive cooperativity for IL2-Rβ binding to the "composite" IL-2/IL2-Rα binary complex, suggesting that IL2-Rβ interacts with both IL-2 and IL2-Rα. Myszka *et al.*,[9] saw a similar effect of cooperativity when they tested the kinetics of IL-2 binding to IL2-Rα and IL2-Rβ, where the effect of IL2-Rβ was to decrease the off-rate of IL-2 binding to the trimeric complex of IL-2, IL2-Rα, and IL2-Rβ.[9] Both the enthalpy and entropy of binding contribute favorably to the interaction of IL2-Rβ with the IL-2/IL2-Rα complex, resulting in a free energy of $\Delta G = -9.5$ kcal/mol; however, the entropic contribution is considerably lower compared to the separate binding of IL2-Rβ to IL2-Rα or to IL-2 (Figures 1(A) and 2(A)). The less favorable entropy is likely the result of the loss of rotational and translational freedom associated with fixation of multiple rigid bodies[33,34] as the IL2-Rβ chain enters the higher-order complex. Another possible explanation is an altered composite interface that is less entropically favorable for binding.

In our initial binary ITC experiments (Figure 1), we did not measure a significant affinity for IL2-Rγ_c with either IL-2 or IL2-Rα. To test whether IL2-Rγ_c requires a composite binding epitope to participate in the higher-order complex, we used the pre-formed complex of IL-2 and IL2-Rα. The ITC titrations at 10 °C and 15 °C (Figure 3(B); data not presented for titration at 10 °C) show no measurable interaction, indicating that IL2-Rα plays no role in binding of IL2-Rγ_c within the higher-order IL-2 signaling complex.

In some NK-cells and monocytes that lack IL2-Rα, IL2-Rγ_c may form a complex with IL2-Rβ in the presence of IL-2 ligand, resulting in a signaling complex called the intermediate-affinity site.[5,31] We designed an ITC titration to examine the thermodynamic parameters for binding of IL2-Rγ_c to a pre-formed complex comprised of IL-2 and IL2-Rβ (Figure 3(C)). As shown in Figure 3(CIII), IL2-Rγ_c binds with high affinity ($K_d = 1.5$ nM) to the IL-2/IL2-Rβ complex. At 25 °C, we find that both, the enthalpy, $\Delta H = -10.6$ kcal/mol, as well as the entropy of binding, $\Delta S = 4.85$ cal/mol K, contribute favorably to the free energy, $\Delta G = -12.04$ kcal/mol. Since we know that IL2-Rγ_c is not binding to unbound IL-2, or IL2-Rβ, the binary complex of IL-2 and IL2-Rβ must provide a composite epitope required to recruit IL2-Rγ_c.

On antigen stimulated CD4$^+$ T-cells, all three IL-2-receptor subunits are expressed and together with IL-2, comprise the high-affinity signaling complex.[35] In our final ITC titration experiment, we assembled the complete quaternary IL-2-signaling complex by titrating the IL2-Rγ_c chain into a pre-formed complex of IL-2/IL2-Rα/IL2-Rβ (Figure 4). We find a strong binding affinity ($K_d = 12$ nM) between IL2-Rγ_c and the three-way complex with the interaction being almost completely enthalpy driven, $\Delta H = -10.25$ kcal/mol with very little entropic contribution $\Delta S = 0.72$ cal/mol K. Since we know that IL2-Rα plays no direct role in binding of IL2-Rγ_c within the high affinity IL-2 signaling complex, we would expect that the titration of IL2-Rγ_c into the two-way complex between IL-2 and IL2-Rβ and into the three-way complex comprised of IL-2, IL2-Rα and IL2-Rβ should give the same numerical values for K_d, ΔH and ΔS. However, surprisingly only the value for the binding enthalpy is the same between these two ITC titrations, clearly indicating

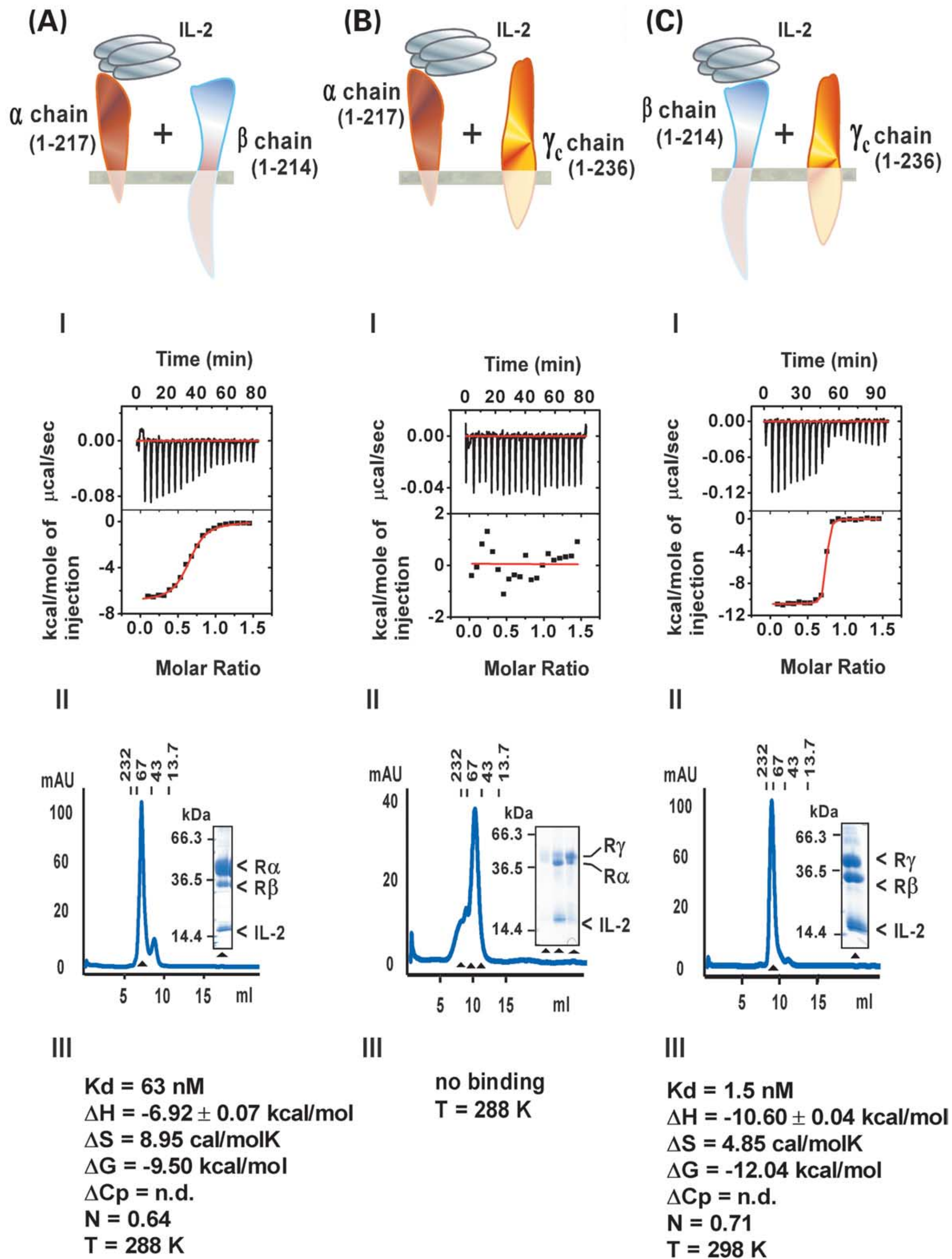
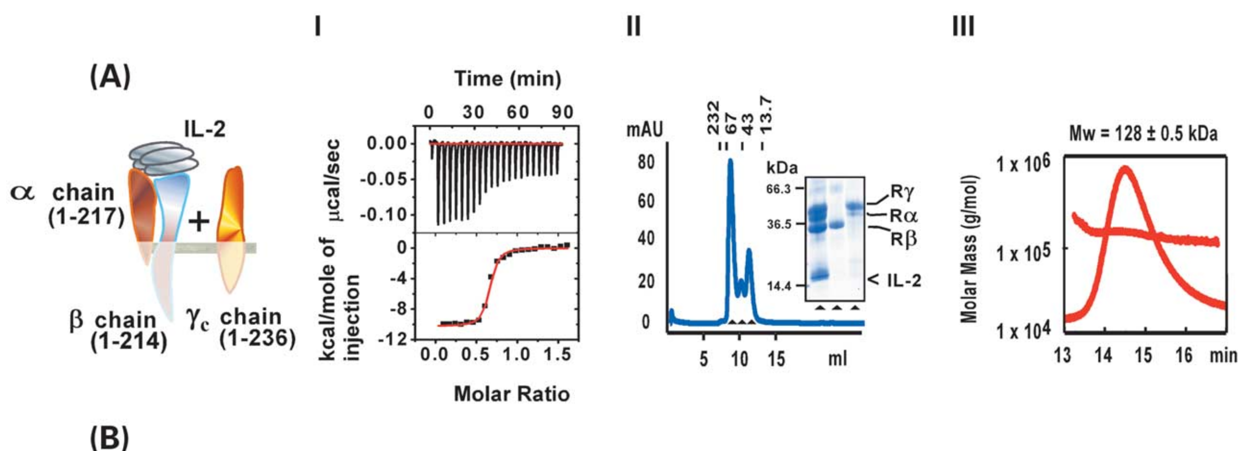


Figure 3. Higher-order assembly of IL-2 receptor complexes. (A) IL2-Rβ binds to the preformed IL-2/IL2-Rα complex with affinity $K_d \sim 63$ nM with both enthalpically ($\Delta H = -6.92(\pm 0.07)$ kcal/mol) and entropically ($\Delta S = 8.95$ cal/mol K) favorable energetics. The affinity is greater than the binding of IL2-Rβ to IL-2 or free IL2-Rα, indicating that IL2-Rβ is cooperatively associating with the IL-2/IL2-Rα complex. (B) IL2-Rγ_c shows no affinity for a preformed IL-2/IL2-Rα complex consistent with IL2-Rα not being involved in binding of IL2-Rγ_c in the final signaling complex. (C) IL2-Rγ_c does, however, form a stable complex with preformed IL-2/IL2-Rβ binary complex with high affinity ($K_d = 1.5$ nM) with enthalpically ($\Delta H = -10.6(\pm 0.04)$ kcal/mol) favorable energetics. Hence, IL-2 and IL2-Rβ must provide a composite binding interface before IL2-Rγ_c can be recruited to build up the intermediate-affinity signaling complex. n.d., not determined.



(B)

Table 1: Thermodynamic Parameters

binding partners	temp (K)	Kd (nM)	stoichiometry (n)	ΔH (kcal/mol)	ΔS [cal/(molK)]	ΔG (kcal/mol)	ΔCp [cal/(molK)]
IL-2/Rα/Rβ + Rγ	288	12	0.64	-10.25 ± 0.13	0.72	-10.40	n.d.

Figure 4. Thermodynamic assembly of the quaternary IL-2 signaling complex. (A) IL2-Rγ_c binds to the preformed IL-2/IL2-Rα/IL2-Rβ complex with high affinity ($K_d = 12$ nM) that is stable on the FPLC sizing column. SDS-PAGE clearly shows the presence of the four components of the ternary complex.MALS determined the molecular mass of the signaling complex to be 128 kDa, consistent with a 1:1:1:1 assembly including one copy each of IL-2, IL2-Rα, IL2-Rβ and IL2-Rγ_c. (B) The binding reaction is largely enthalpy ($\Delta H = -10.25$ ($\pm$0.13) kcal/mol) driven, with little entropic ($\Delta S = 0.72$ cal/mol K) contribution. n.d., not determined.

an energetic cooperativity that is not apparent from the additive measurements of the individual bimolecular and trimolecular interactions. The lack of contribution of ΔS to the binding of IL2-Rγ_c to the three-way complex is perhaps due to the loss of rotational and translational entropy, as observed for binding of IL2-Rβ to the preformed IL-2/IL2-Rα complex (Figure 3(A)). However, the higher-order complexes may assemble structurally altered composite interfaces that are less entropically favorable for binding, but provide superior composite enthalpic contributions as the means of overcoming the unfavorable loss of entropy of the components. Thus, there appears to be significant favorable energetic cooperativity conferred by the increasingly multipartite interfaces. The molecular mass of the final IL-2 signaling complex was measured by MALS; (Figure 4(AIII)) to be 128 kDa (±3% accuracy), consistent with a heterotetrameric assembly including one copy each of IL-2, IL2-Rα, IL2-Rβ and IL2-Rγ_c.

Discussion

IL-2 is one of the most compelling cytokines with regards to promise for human cancer and immunotherapy, but so far clinical results have been less predictable than anticipated. A major challenge to improving IL-2 as a therapeutic is a lack of structural and thermodynamic understanding of the assembly of its receptor complexes. Different compositions of its receptor complexes are associated with different functional, and therefore clinical outcomes. Despite the crystal structure of IL-2 being solved more than 15 years ago,[1] there exists no structural information of IL-2 with any of its receptors. However, in the absence of such direct detailed structural information, we have used ITC to study the stepwise thermodynamic assembly of IL-2 with IL2-Rα, IL2-Rβ and IL2-Rγ_c. We can then use these data, combined with previous data on the kinetics of IL-2-receptor interactions, to rationalize structural models of the IL-2 signaling complex[36] and the extensive repertoire of mutagenesis studies.[37–39]

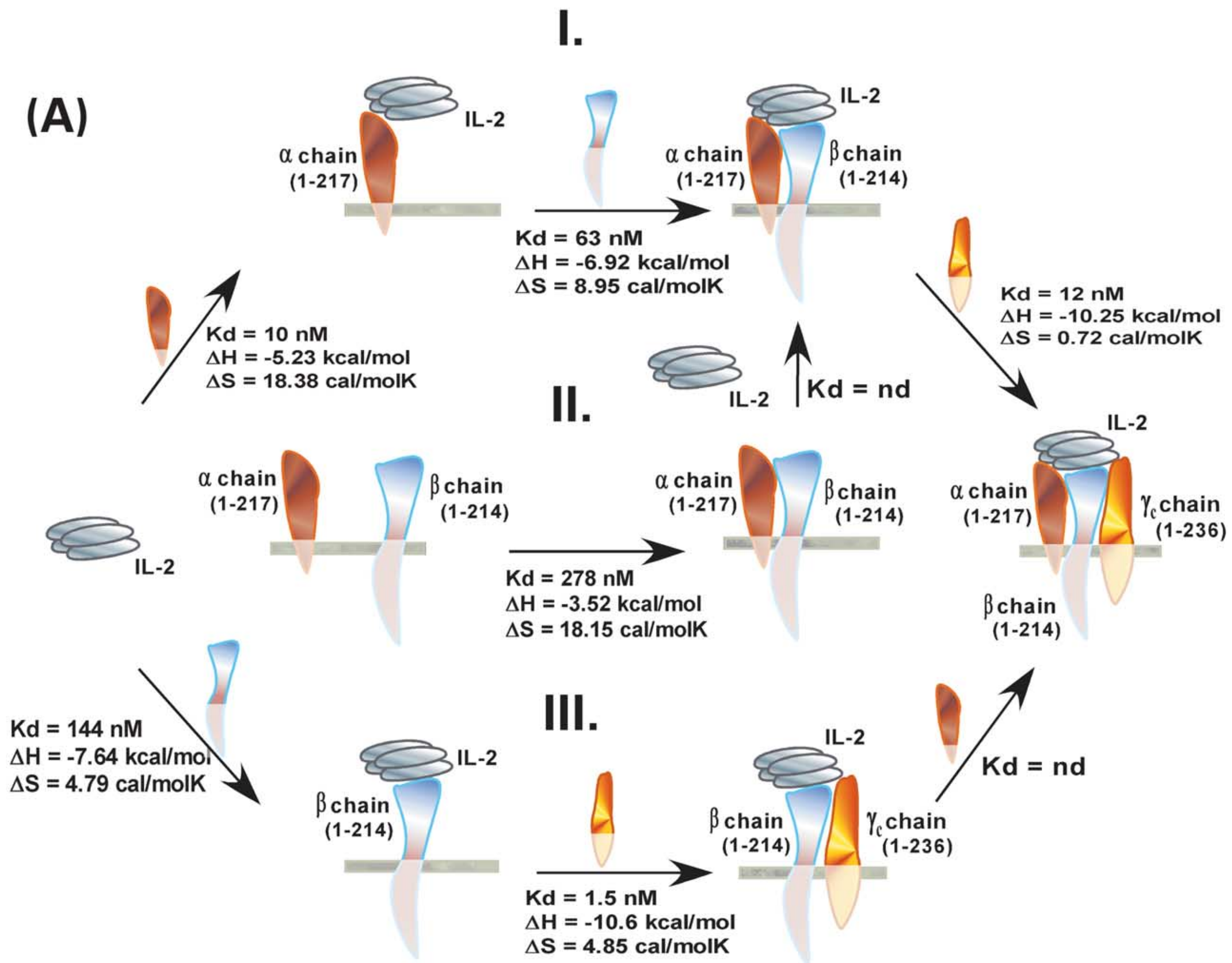
Using the complex crystal structure of human growth hormone (GH)/growth hormone receptor (GHR) as a template,[40] Bamborough *et al*.[36] generated a model of the ternary complex where IL-2 is sandwiched on opposite helical faces by IL2-Rβ and IL2-Rγ_c and capped at the tip of the four helix bundle by IL2-Rα. Based on this model, the overall buried surface area between IL-2 and the single N-terminal sushi domain of IL2-Rα is approximately 1500 Å^2, the second sushi domain of Rα is not thought to make a significant interaction with IL-2, although this has not been verified by a structure of the complex. Mutagenesis has demonstrated that the IL2-Rα contact epitope on IL-2 is formed by residue Phe44 that forms a central point of a broader surface completed by Phe42, Lys43, Tyr45, Glu62, Pro65 and Val69 located in the loop between helix A and B-B′ and in the B-helix of IL-2.[37,41] These observations fit well with our observations in two ways; first the

presence of a centralized hydrophobic core residue (Phe44) is consistent with a primarily entropy-driven interaction, and second, the incorporation of several charged residues at the interface matches well with the significant enthalpic contribution to binding. The use of apparently different chemical surfaces to mediate binding events has been observed recently in other cytokine receptor assemblies.[42] In addition to the entropic and enthalpic terms, we determined the heat capacity of the binding reaction between IL-2 and IL2-Rα, which is considered to be a more distinctive thermodynamic signature for the burial of hydrophobic surface, than enthalpy or entropy alone.[43,44] The value measured for ΔC_p of −519 cal/mol K is consistent with expected values for a typical protein–protein interaction driven by the hydrophobic effect,[45,46] but somewhat higher than might be expected for the IL2-Rα interaction based on predicted buried surface area. This may indicate the occurrence of some structural adaptation of flexible loops and side-chains within the IL-2/Rα interface. Such anomalously high ΔC_p values are known to be a thermodynamic signature of structural adaptations or distortion. This fits well with some known structural information on the Rα binding site of IL-2. Small molecules for antagonism of receptor binding have targeted this site. In crystal structures of some of these small molecules complexed with the Rα binding epitope of IL-2, the local area of the epitope is observed to undergo significant structural remodeling to bind to different drugs. Thus, the Rα binding site epitopes seem to possess the capacity to undergo ligand-induced rearrangements, as the large ΔC_p would suggest.

The energetics of the interaction between IL-2 with IL2-Rβ show significant differences compared to the IL-2/IL2-Rα binding. The most noticeable change is that the primary thermodynamic driving force is enthalpy with an associated weaker, but still moderately favorable entropic contribution indicating that enthalpy–entropy compensation mechanisms play a role during association. Residues on the face of IL-2 made up from helix A and helix C are known to interact with IL2-Rβ.[38,47] In the model, the IL-2/Rβ interface buries ~1700 Å, and Asp20 of IL-2, is within hydrogen-bonding distance of Tyr134 of IL2-Rβ, which is conserved between the human and mouse receptors. Additionally, Asn88, which is also important for binding of IL-2 to IL2-Rβ[41] is within hydrogen bond distance to Arg15 of IL2-Rβ.[36] The contributions of polar interactions in the IL-2/IL2-Rβ interface is consistent with our thermodynamic data that show a largely enthalpy driven interaction. The measured value for ΔC_p of −597 cal/mol K does not appear to correlate with the reduced entropic contribution. It is possible, however, that ordering of solvent at a protein–protein interface, which is known to contribute unfavorably to ΔS and favorably to ΔH,[42,48] as well as interface adaptation, may be responsible for the more pronounced ΔC_p than predicted from surface area measurements.[49,50]

Our thermodynamic data describing the IL-2 ligand receptor interactions support several possible pathways towards a functional IL-2 signaling complex (Figure 5). In the literature the assembly of the IL-2 signaling complex is discussed somewhat controversially with two main proposed mechanisms. Initially, the observation on activated human T-cells where an increasing number of IL2-Rα chains correlated with an increase in the rate of IL-2 association with high affinity IL2-Rα/IL2-Rβ binding sites lead to the "affinity conversion" or "stepwise binding" model for the high affinity IL-2 receptor.[51–53] This model proposes that IL2-Rα and IL2-Rβ subunits exist unassociated on the cell surface in the absence of ligand and that IL-2 first has to bind to IL2-Rα, because of its faster association rate. This complex would then stably assemble with the IL2-Rβ subunit[51-53] leading to a conversion of affinity for ligand. In contrast, the "pre-assembly" model proposes that IL2-Rα and IL2-Rβ subunits are associated in the absence of ligand. This model is supported by evidence of chemical cross-linking of mouse IL2-Rα and IL2-Rβ in the absence of ligand and by SPR data with soluble human receptor subunits.[23,54] Our ITC data suggest that theoretically both models are feasible on activated T-cells. The lower K_d value of the IL-2 interaction with IL2-Rα compared with IL2-Rβ would support the first model, assigning the role for IL2-Rα of concentrating IL-2 to the plasma membrane. On the other hand, our observations of cooperative binding of IL2-Rβ by a preformed complex of IL-2 and IL2-Rα favor the "pre-assembly" model of IL2-Rα and IL2-Rβ. Myszka *et al.*[9] found similar results when they determined cooperative binding of IL-2 by an IL2-Rα/Rβ complex, called the pseudo-high-affinity site.

From our isothermal titrations involving IL2-Rγ_c we determined that IL2-Rγ_c is capable of binding to IL2-Rβ only in the presence of IL-2. The IL2-Rα subunit does not appear to be involved in binding to IL2-Rγ_c in the signaling complex, though titration of IL2-Rγ_c into the preformed complexes of IL-2/IL2-Rα/IL2-Rβ and IL-2/IL2-Rβ does result in different thermodynamic profiles. Collectively we reason that IL2-Rγ_c requires a composite binding epitope formed by the binary complex of IL-2 and IL2-Rβ. This requirement then makes it difficult to compare data from point mutations to specific structural features of individual molecules. To date only Glu126 in the D-helix of IL-2 was determined to affect binding to IL2-Rγ_c.[39] The requirement for a composite binding site to recruit IL2-Rγ_c to the IL-2 signaling complex is most likely a regulatory mechanism to prevent stable biding of IL2-Rβ to IL2-Rγ_c in the absence of the ligand IL-2, since these two receptors have intracellular signaling domains. The highest affinity energetics of the quaternary complex are clearly not an additive function of each of the individual lower-order



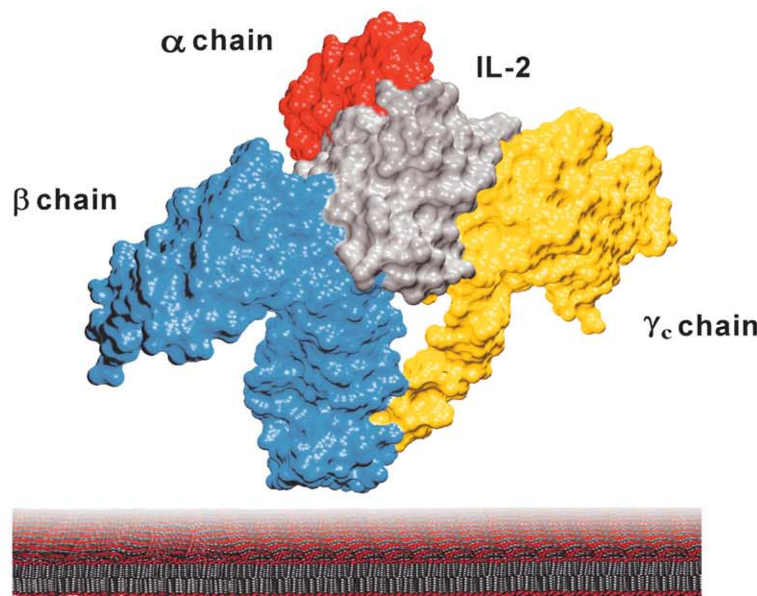


Figure 5. Redundancy in the assembly pathways of IL-2 with its receptors. (A) Alternate assembly pathways of the IL-2 quaternary signaling complex. On antigen activated T-cells complex formation might start with binding of IL-2 to IL2-Rα and proceed in a stepwise mode to finally build up the quaternary signaling complex (I). Alternatively, a second pathway (II) may integrate a preformed complex of IL2-Rα and IL2-Rβ (pseudo-high-affinity complex) to

reactions. Our measurements indicate that the structurally composite interfaces give rise to an attendant energetic cooperativity which serves to drive the ultimate formation of the higher-order signaling complex despite working against the entropic cost of fixation of four individual molecules.

The energetic mechanism utilized by γ_c to interact with IL-2, Rα and Rβ can be compared and contrasted with analogous studies carried out on a different shared signaling receptor, gp130. This gp130 is a highly cross-reactive shared cytokine signaling receptor for over ten different members of the IL-6/IL-12 family. In that system, highly favorable entropy of binding was observed between gp130 and four structurally variant cytokines (CNTF, LIF, IL-6, and OSM).[55] Thus, it appears that gp130 utilizes entropy, likely mediated through desolvation, as a structurally insensitive energetic mechanism to cross-react with diverse cytokines.[55] This stands in contrast to what we now observe for γ_c, where highly favorable enthalpy appears to surmount the increasingly unfavorable rigid body entropy of the system as a whole. Consistent with this observation, mutational studies of the γ_c cytokine binding site show that the critical energetic hotspots for different cytokines (e.g. IL-2, -4, -7 and -15) are focused on unique zones of an overlapping binding site,[56] thus minimizing the need for a common, structurally insensitive cross-reactivity mechanism. In contrast, the gp130 binding site is almost completely overlapping, necessitating the need for a large degree of thermodynamic plasticity to accommodate the unique surfaces, which it engages by the identical receptor amino acids. By comparison, significant non-overlapping regions of γ_c play a major energetic role in interactions with different cytokines, implying the formation of highly cytokine-specific interactions through charged and/or polar contacts with large enthalpic signatures. Thus, gp130 and γ_c have evolved unique energetic mechanisms for cytokine recognition. The generality of the γ_c recognition thermodynamics we see here for the IL-2 components remains to be determined for additional γ_c family cytokines.

In summary, we have employed ITC for the energetic analysis of the stepwise assembly of the IL-2 receptor complex. Throughout our ITC studies we used the recombinant soluble domains of the IL-2 receptor subunits, which is not completely reflecting the physiological situation of membrane-embedded receptor molecules. But the use of soluble receptor subunits is regarded as being a valuable methodology to amplify biophysical parameters of ligand–receptor interactions. The thermodynamic data of our study give an insight into the binding mechanisms between IL-2 and its individual receptor subunits and explain the energetics of the higher-order assembly of the complete IL-2 receptor complex. Our ITC study supports several possible pathways towards a functional signaling complex on activated T and NK-cells and suggests that a preformed complex of IL2-Rα and IL2-Rβ could serve as an initial high affinity site to bind IL-2. The energetic mechanism for γ_c cross-reactivity appears to contrast with that utilized by another shared cytokine receptor gp130. In combination with currently available structure–function studies, our thermodynamic analysis will lead to a more complete understanding of the structural biology of IL-2 signaling and further enhance efforts to obtain direct structural information.

Materials and Methods

Protein expression and purification

All proteins used in this study were expressed using the baculovirus system (Pharmingen). Briefly, insect *Spodoptera frugiperda* (Sf9) cells were used for generating high titer recombinant virus and were cultured at 28 °C using SF900 II SFM medium (Invitrogen). *Trichopulsia ni* (High-Five®) cells (Invitrogen) were used to express the recombinant protein and were grown in Insect Xpress medium (Cambrex) at 28 °C.[57,58]

Full length IL-2 (residues 1–133), the ectodomains of IL2-Rα (residues 1–217), IL2-Rβ (residues 1–214) and IL2-Rγ_c (residues 1–236) were cloned into the pAcgp67A vector (Pharmingen). The primers (available upon request) were designed to be in-frame with the gp67A signal sequence of the vector and included a C-terminal hexa-histidine tag. After infection of High-Five cells with recombinant virus, the supernatant was harvested through a two-step centrifugation process to remove cellular material, and reduced in volume using tangential flow concentration. Ni-NTA resin (Qiagen) was added to the concentrated supernatant and allowed to "batch" bind at 4 °C, and elution fractions from the Ni-NTA resin containing the protein of interest as determined by SDS-PAGE analysis were concentrated using Centricon (Millipore, Bedford, MA) spin concentrators and injected onto an FPLC gf200 Sephadex sizing column (Pharmacia).

Additional steps for protein production and modification

Wild-type IL2-Rα has a free cysteine residue at

bind IL-2[32] followed by the recruitment of IL2-Rγ_c. On certain NK-cells and monocytes, which lack IL2-Rα, pathway (III) likely represents the activation mechanism used to form the three-way signaling complex of IL-2/IL2-Rβ/IL2-Rγ_c. (B) Surface representation of a model of the IL-2 receptor signaling complex proposed by Bamborough *et al*.[36] Bamborough *et al*. made use of the Brookhaven Data Bank[61] structures IL-4 (entry name 1ITM),[62] IL-2 (3INK)[63] and human growth hormone complex (3HHR).[40] Raster3D[64] and VMD[65] were used to prepare the MSMS surface representation.[66] n.d., not determined.

position 192 of its ectodomain, which results in the formation of disulfide-linked homodimers during protein purification. To remove the fraction of disulfide-linked homodimers of IL2-Rα we reduced Cys192 in 10 mM cysteine (Sigma) and alkylated with 20 mM iodoacetamide for one hour at room temperature. As reported the alkylation, or removal of Cys192 has no influence on ligand binding.[59,60] The completeness of IL2-Rα-alkylation was monitored with reducing and non-reducing SDS-PAGE.

IL2-Rγ_c eluted of the FPLC gf200 sizing column in three fractions as observed.[31] These fractions include a high molecular mass irreversibly aggregated complex, a dissociable complex having an elution volume consistent with a trimer, and a lower molecular mass fraction having an elution volume consistent with a monomer. Both, the IL2-Rγ_c trimer and monomer fractions were shown to bind IL-2,[31] but for our ITC experiments we only used the IL2-Rγ_c monomer fraction.

Isothermal titration calorimetry

Calorimetric titrations were carried out on a VP-ITC calorimeter (MicroCal, Northhampton, MA) at 10 °C, 15 °C and 20 °C for the binary titrations and 20 °C for the higher-order complex. Prior to each titration, the protein samples were degassed for ten minutes. Data were processed with the MicroCal Origin 5.0 software. All experiments were carried out in the same buffer (10 mM Hepes (pH 7.5) supplemented with 150 mM sodium chloride) to control for heat of dilution effects.

Protein concentrations were determined using the BCA method (in duplicate or triplicate). The precision of the protein concentration determinations varies between different proteins (IL-2, Rα, Rβ and Rγ) by 15–20%, and appears to be affected by the heavy glycosylation content and reactivity of each protein with BCA. However, each ITC experiment was carried out multiple times, at different temperatures, at different protein concentrations, with very close agreement between the measured and calculated values, indicating consistency in protein concentration values. The n, or stoichiometry values determined by ITC can vary by ~25% (e.g. n values between 0.7 and 1.3 for an n value of 1.0), but the ultimate stoichiometries were assigned using n values confirmed by gel filtration and MALS of the various complexes after the ITC titration. Further, prior kinetic analysis using SPR, and our own preliminary crystallization results confirm that only one copy of each component is recruited into complexes in this system.

In the titrations of binary complexes (Figures 1 and 2), the titrand was used in the cell at a concentration of 3–4 μM, and the titrant at a seven- to tenfold higher concentration in the syringe. In the titrations of the higher-order trimeric complexes (Figure 3) involving IL-2 and IL2-Rα, a preformed and purified complex of IL-2 and IL2-Rα was used as the titrand in the cell and IL2-Rβ and IL2-Rγ_c as titrants in the syringe. When testing the complex made of IL-2 and IL2-Rβ and IL2-Rγ_c, a preformed complex of IL-2 and IL2-Rβ was put into the cell and saturated with IL2-Rγ_c. Studying the four-way heteromeric signaling complex (Figure 4) a preformed complex of IL-2 and IL2-Rα was used as the starting titrand and first saturated with IL2-Rβ followed by IL2-Rγ_c as the final titrant. As a last control, the content of the cell following each titration was injected onto the gf200 Sephadex sizing column on the FPLC to confirm stoichiometry by elution position of the complex, to ensure the proteins had not aggregated in the ITC cell and finally visualized on an SDS-PAGE gel to ensure the presence of all proteins in the final complex.

Multi-angle light scattering

A DAWN EOS (Wyatt Technology, Santa Barbara, CA) equipped with a K5 flow cell and a 30 mW linearly polarized GaAs laser of wavelength 690 nm was used in all experiments. All measurements were made in the in-line flow mode. A Jasco model PU-980 (Jasco Corp, Tokyo, Japan) pump was used to flow 0.1 μm filtered solvent (10 mM Hepes(pH 7.5), 200 mM NaCl) through a Shimadzu DGU-14A (Shimadzu Corp., Kyoto, Japan) degasser and into an HR 10/30 Superdex-200 (Amersham Biosciences, Piscataway, NJ) gel filtration column. The sample was at approximately 2 mg/ml (in the eluent buffer). Both the light scattering unit and the refractometer were calibrated as per the manufacturer's instructions. A value of 0.175 ml/g was assumed for the dn/dc ratio of the protein. Light scattering data were used from 11 detectors ranging from 50.0° to 134.0° (detectors 6 through 16). Measuring the signal from monomeric bovine serum albumin normalized the detector responses. The temperature of the light scattering unit was maintained at 25 °C and the temperature of the refractometer was kept at 35 °C. The column and all external connections were at ambient temperature (20–25 °C). The flow rate was maintained at 0.5 ml/minute throughout the experiments.

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References

1. Brandhuber, B. J., Boone, T., Kenney, W. C. & McKay, D. B. (1987). Three-dimensional structure of interleukin-2. *Science*, **238**, 1707–1709.
2. Parry, D. A., Minasian, E. & Leach, S. J. (1988). Conformational homologies among cytokines: interleukins and colony stimulating factors. *J. Mol. Recognit.* **1**, 107–110.
3. Bazan, J. F. (1989). A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor beta-chain. *Biochem. Biophys. Res. Commun.* **164**, 788–795.
4. Smith, K. A. (1988). Interleukin-2: inception, impact, and implications. *Science*, **240**, 1169–1176.
5. Caligiuri, M. A., Zmuidzinas, A., Manley, T. J., Levine, H., Smith, K. A. & Ritz, J. (1990). Functional consequences of interleukin 2 receptor expression on resting human lymphocytes. Identification of a novel natural killer cell subset with high affinity receptors. *J. Expt. Med.* **171**, 1509–1526.
6. Chada, S., Ramesh, R. & Mhashilkar, A. M. (2003). Cytokine- and chemokine-based gene therapy for cancer. *Curr. Opin. Mol. Ther.* **5**, 463–474.
7. Dutcher, J. (2002). Current status of interleukin-2

therapy for metastatic renal cell carcinoma and metastatic melanoma. *Oncology (Huntingt)*, **16**, 4–10.
8. Liu, K. D., Lai, S. Y., Goldsmith, M. A. & Greene, W. C. (1995). Identification of a variable region within the cytoplasmic tail of the IL-2 receptor beta chain that is required for growth signal transduction. *J. Biol. Chem.* **270**, 22176–22181.
9. Myszka, D. G., Arulanantham, P. R., Sana, T., Wu, Z., Morton, T. A. & Ciardelli, T. L. (1996). Kinetic analysis of ligand binding to interleukin-2 receptor complexes created on an optical biosensor surface. *Protein Sci.* **5**, 2468–2478.
10. Liparoto, S. F. & Ciardelli, T. L. (1999). Biosensor analysis of the interleukin-2 receptor complex. *J. Mol. Recognit.* **12**, 316–321.
11. Minami, Y., Kono, T., Miyazaki, T. & Taniguchi, T. (1993). The IL-2 receptor complex: its structure, function, and target genes. *Annu. Rev. Immunol.* **11**, 245–268.
12. Leonard, W. J., Depper, J. M., Crabtree, G. R., Rudikoff, S., Pumphrey, J., Robb, R. J. *et al.* (1984). Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. *Nature*, **311**, 626–631.
13. Willerford, D. M., Chen, J., Ferry, J. A., Davidson, L., Ma, A. & Alt, F. W. (1995). Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity*, **3**, 521–530.
14. Waldmann, T. A. (1993). The IL-2/IL-2 receptor system: a target for rational immune intervention. *Immunol. Today*, **14**, 264–270.
15. Dukovich, M., Wano, Y., Le thi Bich, T., Katz, P., Cullen, B. R., Kehrl, J. H. & Greene, W. C. (1987). A second human interleukin-2 binding protein that may be a component of high-affinity interleukin-2 receptors. *Nature*, **327**, 518–522.
16. Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N. *et al.* (1992). Cloning of the gamma chain of the human IL-2 receptor. *Science*, **257**, 379–382.
17. Theze, J., Alzari, P. M. & Bertoglio, J. (1996). Interleukin 2 and its receptors: recent advances and new immunological functions. *Immunol. Today*, **17**, 481–486.
18. Wu, Z., Johnson, K. W., Choi, Y. & Ciardelli, T. L. (1995). Ligand binding analysis of soluble interleukin-2 receptor complexes by surface plasmon resonance. *J. Biol. Chem.* **270**, 16045–16051.
19. Wang, H. M. & Smith, K. A. (1987). The interleukin 2 receptor. Functional consequences of its bimolecular structure. *J. Expt. Med.* **166**, 1055–1069.
20. Sana, T. R., Wu, Z., Smith, K. A. & Ciardelli, T. L. (1994). Expression and ligand binding characterization of the beta-subunit (p75) ectodomain of the interleukin-2 receptor. *Biochemistry*, **33**, 5838–5845.
21. Voss, S. D., Leary, T. P., Sondel, P. M. & Robb, R. J. (1993). Identification of a direct interaction between interleukin 2 and the p64 interleukin 2 receptor gamma chain. *Proc. Natl Acad. Sci. USA*, **90**, 2428–2432.
22. Waldmann, T. A. (1989). The multi-subunit interleukin-2 receptor. *Annu. Rev. Biochem.* **58**, 875–911.
23. Landgraf, B. E., Goldstein, B., Williams, D. P., Murphy, J. R., Sana, T. R., Smith, K. A. & Ciardelli, T. L. (1992). Recombinant interleukin-2 analogs. Dynamic probes for receptor structure. *J. Biol. Chem.* **267**, 18511–18519.
24. Wu, Z., Johnson, K. W., Goldstein, B., Choi, Y., Eaton, S. F., Laue, T. M. & Ciardelli, T. L. (1995). Solution assembly of a soluble, heteromeric, high affinity interleukin-2 receptor complex. *J. Biol. Chem.* **270**, 16039–16044.
25. Cassell, D. J., Choudhri, S., Humphrey, R., Martell, R. E., Reynolds, T. & Shanafelt, A. B. (2002). Therapeutic enhancement of IL-2 through molecular design. *Curr. Pharm. Des.* **8**, 2171–2183.
26. Murphy, K. P. & Freire, E. (1992). Thermodynamics of structural stability and cooperative folding behavior in proteins. *Advan. Protein Chem.* **43**, 313–361.
27. Lo Conte, L., Chothia, C. & Janin, J. (1999). The atomic structure of protein–protein recognition sites. *J. Mol. Biol.* **285**, 2177–2198.
28. Atwell, S., Ultsch, M., De Vos, A. M. & Wells, J. A. (1997). Structural plasticity in a remodeled protein–protein interface. *Science*, **278**, 1125–1128.
29. Balasubramanian, S., Chernov-Rogan, T., Davis, A. M., Whitehorn, E., Tate, E., Bell, M. P. *et al.* (1995). Ligand binding kinetics of IL-2 and IL-15 to heteromers formed by extracellular domains of the three IL-2 receptor subunits. *Int. Immunol.* **7**, 1839–1849.
30. Johnson, K., Choi, Y., Wu, Z., Ciardelli, T., Granzow, R., Whalen, C. *et al.* (1994). Soluble IL-2 receptor beta and gamma subunits: ligand binding and cooperativity. *Eur. Cytokine Netw.* **5**, 23–34.
31. Liparoto, S. F., Myszka, D. G., Wu, Z., Goldstein, B., Laue, T. M. & Ciardelli, T. L. (2002). Analysis of the role of the interleukin-2 receptor gamma chain in ligand binding. *Biochemistry*, **41**, 2543–2551.
32. Wu, Z., Goldstein, B., Laue, T. M., Liparoto, S. F., Nemeth, M. J. & Ciardelli, T. L. (1999). Solution assembly of the pseudo-high affinity and intermediate affinity interleukin-2 receptor complexes. *Protein Sci.* **8**, 482–489.
33. Murphy, K. P., Xie, D., Thompson, K. S., Amzel, L. M. & Freire, E. (1994). Entropy in biological binding processes: estimation of translational entropy loss. *Proteins: Struct. Funct. Genet.* **18**, 63–67.
34. Gomez, J., Hilser, V. J., Xie, D. & Freire, E. (1995). The heat capacity of proteins. *Proteins: Struct. Funct. Genet.* **22**, 404–412.
35. Smith, K. A. (1989). The interleukin 2 receptor. *Annu. Rev. Cell Biol.* **5**, 397–425.
36. Bamborough, P., Hedgecock, C. J. & Richards, W. G. (1994). The interleukin-2 and interleukin-4 receptors studied by molecular modelling. *Structure*, **2**, 839–851.
37. Sauve, K., Nachman, M., Spence, C., Bailon, P., Campbell, E., Tsien, W. H. *et al.* (1991). Localization in human interleukin 2 of the binding site to the alpha chain (p55) of the interleukin 2 receptor. *Proc. Natl Acad. Sci. USA*, **88**, 4636–4640.
38. Moreau, J. L., Bossus, M., de Groote, D., Francois, C., Jacques, Y., Tartar, A. & Theze, J. (1995). Characterization of a monoclonal antibody directed against the NH2 terminal area of interleukin-2 (IL-2) and inhibiting specifically the binding of IL-2 to IL-2 receptor beta chain (IL-2R beta). *Mol. Immunol.* **32**, 1047–1056.
39. Buchli, P. & Ciardelli, T. (1993). Structural and biologic properties of a human aspartic acid-126 interleukin-2 analog. *Arch. Biochem. Biophys.* **307**, 411–415.
40. de Vos, A. M., Ultsch, M. & Kossiakoff, A. A. (1992). Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science*, **255**, 306–312.
41. Zurawski, S. M., Vega, F., Jr, Doyle, E. L., Huyghe, B., Flaherty, K., McKay, D. B. & Zurawski, G. (1993).

Definition and spatial location of mouse interleukin-2 residues that interact with its heterotrimeric receptor. *EMBO J.* **12**, 5113–5119.
42. Boulanger, M. J., Chow, D. C., Brevnova, E. E. & Garcia, K. C. (2003). Hexameric structure and assembly of the interleukin-6/IL-6 alpha-receptor/gp130 complex. *Science*, **300**, 2101–2104.
43. Spolar, R. S., Livingstone, J. R. & Record, M. T., Jr (1992). Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of non-polar and polar surface from water. *Biochemistry*, **31**, 3947–3955.
44. Ross, P. D. & Rekharsky, M. V. (1996). Thermodynamics of hydrogen bond and hydrophobic interactions in cyclodextrin complexes. *Biophys. J.* **71**, 2144–2154.
45. Stites, W. E. (1997). Proteinminus signprotein interactions: interface structure, binding thermodynamics, and mutational analysis. *Chem. Rev.* **97**, 1233–1250.
46. Hibbits, K. A., Gill, D. S. & Willson, R. C. (1994). Isothermal titration calorimetric study of the association of hen egg lysozyme and the anti-lysozyme antibody HyHEL-5. *Biochemistry*, **33**, 3584–3590.
47. Collins, L., Tsien, W. H., Seals, C., Hakimi, J., Weber, D., Bailon, P. *et al.* (1988). Identification of specific residues of human interleukin 2 that affect binding to the 70-kDa subunit (p70) of the interleukin 2 receptor. *Proc. Natl Acad. Sci. USA*, **85**, 7709–7713.
48. Holdgate, G. A., Tunnicliffe, A., Ward, W. H., Weston, S. A., Rosenbrock, G., Barth, P. T. *et al.* (1997). The entropic penalty of ordered water accounts for weaker binding of the antibiotic novobiocin to a resistant mutant of DNA gyrase: a thermodynamic and crystallographic study. *Biochemistry*, **36**, 9663–9673.
49. Guinto, E. R. & Di Cera, E. (1996). Large heat capacity change in a protein–monovalent cation interaction. *Biochemistry*, **35**, 8800–8804.
50. Bhat, T. N., Bentley, G. A., Boulot, G., Greene, M. I., Tello, D., Dall'Acqua, W. *et al.* (1994). Bound water molecules and conformational stabilization help mediate an antigen-antibody association. *Proc. Natl. Acad. Sci., USA*, **91**, 1089–1093.
51. Kondo, S., Shimizu, A., Saito, Y., Kinoshita, M. & Honjo, T. (1986). Molecular basis for two different affinity states of the interleukin 2 receptor: affinity conversion model. *Proc. Natl Acad. Sci. USA*, **83**, 9026–9029.
52. Saito, Y., Sabe, H., Suzuki, N., Kondo, S., Ogura, T., Shimizu, A. & Honjo, T. (1988). A larger number of L chains (Tac) enhance the association rate of interleukin 2 to the high affinity site of the interleukin 2 receptor. *J. Expt. Med.* **168**, 1563–1572.
53. Saito, Y., Ogura, T., Kamio, M., Sabe, H., Uchiyama, T. & Honjo, T. (1990). Stepwise formation of the high-affinity complex of the interleukin 2 receptor. *Int. Immunol.* **2**, 1167–1177.
54. Yamaguchi, A., Ide, T., Hatakeyama, M., Doi, T., Kono, T., Uchiyama, T. *et al.* (1989). Structure of the functional interleukin-2 receptor. Evidence for the association of human p55 and murine p75 molecules in a mouse T cell line. *Int. Immunol.* **1**, 160–168.
55. Boulanger, M. J., Bankovich, A. J., Kortemme, T., Baker, D. & Garcia, K. C. (2003). Convergent mechanisms for recognition of divergent cytokines by the shared signaling receptor gp130. *Mol. Cell*, **12**, 577–589.
56. Olosz, F. & Malek, T. R. (2002). Structural basis for binding multiple ligands by the common cytokine receptor gamma-chain. *J. Biol. Chem.* **277**, 12047–12052.
57. Smith, G. E., Ju, G., Ericson, B. L., Moschera, J., Lahm, H. W., Chizzonite, R. & Summers, M. D. (1985). Modification and secretion of human interleukin 2 produced in insect cells by a baculovirus expression vector. *Proc. Natl Acad. Sci. USA*, **82**, 8404–8408.
58. Summers, M. D. S. G. (1987). A manual of methods for baculovirus vectors and insect cell culture procedures. *Tex Agric. Expt. Stn. Bull.* **1555**, 1–56.
59. Kato, K. & Smith, K. A. (1987). Tac antigen forms disulfide-linked homodimers. *Biochemistry*, **26**, 5359–5364.
60. Rusk, C. M., Neeper, M. P., Kuo, L. M., Kutny, R. M. & Robb, R. J. (1988). Structure–function relationships for the IL-2 receptor system. V. Structure–activity analysis of modified and truncated forms of the Tac receptor protein: site-specific mutagenesis of cysteine residues. *J. Immunol.* **140**, 2249–2259.
61. Bernstein, F. C., Koetzle, T. F., Williams, G. J., Meyer, E. F., Jr, Brice, M. D., Rodgers, J. R. *et al.* (1977). The Protein Data Bank: a computer-based archival file for macromolecular structures. *J. Mol. Biol.* **112**, 535–542.
62. Smith, L. J., Redfield, C., Boyd, J., Lawrence, G. M., Edwards, R. G., Smith, R. A. & Dobson, C. M. (1992). Human interleukin 4. The solution structure of a four-helix bundle protein. *J. Mol. Biol.* **224**, 899–904.
63. McKay, D. B. (1992). Reply to Bazan. J.F. Unraveling the structure of IL-2. *Science*, **257**, 412–413.
64. Merritt, E. A. & Murphy, E. P. M. (1994). Raster3D version 2.0 a program for photorealistic molecular graphics. *Acta. Crystallog. sect. D*, **50**, 869–873.
65. Humphrey, W., Dalke, A. & Schulten, K. (1996). VMD: visual molecular dynamics. *J. Mol. Graph.* **14**, 33–38. See also pp. 27–28.
66. Sanner, M. F., Olson, A. J. & Spehner, J. C. (1996). Reduced surface: an efficient way to compute molecular surfaces. *Biopolymers*, **38**, 305–320.

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