Kinetic analysis of ligand binding to interleukin-2 receptor complexes created on an optical biosensor surface

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Abstract

The interleukin-2 receptor (IL-2R) is composed of at least three cell surface subunits, IL-2R α , IL-2R β , and IL-2R γ . On activated T-cells, the α - and β -subunits exist as a preformed heterodimer that simultaneously captures the IL-2 ligand as the initial event in formation of the signaling complex. We used BIAcore™ to compare the binding of IL-2 to biosensor surfaces containing either the α -subunit, the β -subunit, or both subunits together. The receptor ectodomains were immobilized in an oriented fashion on the dextran matrix through unique solvent-exposed thiols. Equilibrium analysis of the binding data established IL-2 dissociation constants for the individual α - and β -subunits of 37 and 480 nM, respectively. Surfaces with both subunits immobilized, however, contained a receptor site of much higher affinity, suggesting the ligand was bound in a ternary complex with the α - and β -subunits, similar to that reported for the pseudo-high-affinity receptor on cells. Because the binding responses had the additional complexity of being mass transport limited, obtaining accurate estimates for the kinetic rate constants required global fitting of the data sets from multiple surface densities of the receptors. A detailed kinetic analysis indicated that the higher-affinity binding sites detected on surfaces containing both α - and β -subunits resulted from capture of IL-2 by a preformed complex of these subunits. Therefore, the biosensor analysis closely mimicked the recognition properties reported for these subunits on the cell surface, providing a convenient and powerful tool to assess the structure–function relationships of this and other multiple subunit receptor systems.

Keywords: BIAcore; interleukin-2; kinetics; mass transport; protein–protein interactions; receptor, surface plasmon resonance

Interleukin-2 and the interleukin-2 receptor complex is one of the most extensively studied ligand:receptor systems in the helical cytokine–hematopoietin receptor superfamily. The cytokines IL-2, interleukin-15, and ciliary neurotropic factor comprise a subfamily

whose receptor complexes are composed of three different cell surface subunits (Sato & Miyajima, 1994; Giri et al., 1995). The IL-2R α -subunit exhibits a dissociation constant for IL-2 of approximately 10 nM (Wang & Smith, 1987), the β -subunit's K_d is approximately 450 nM (Sana et al., 1994; Wu et al., 1994), and the γ -subunit interacts only weakly ($K_d > 50 \mu\text{M}$) (Voss et al., 1993; Johnson et al., 1994). The functional IL-2R, however, uses these subunits cooperatively to first bind IL-2 and then to assemble a signaling complex. The IL-2R α/β pseudo-high-affinity heterodimer exists, preformed, on the surface of activated T-cells (Landgraf et al., 1992). The higher affinity of this complex ($K_d \approx 0.1$ – 0.6 nM) facilitates ligand capture. Subsequent recruitment of the γ -subunit allows interaction of the cytoplasmic domains of the β - and γ -subunits, initiating signaling events (Nakamura et al., 1994). Thus, signaling is ligand dependent and can be observed in

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Abbreviations: IL-2, interleukin-2; IL-2R, interleukin-2 receptor; k_m , mass transport coefficient; k_a , association rate constant; k_d , dissociation rate constant; K_d , equilibrium dissociation constant; R_{max} , surface capacity; RU, resonance units; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide; BSA, bovine serum albumin; SPR, surface plasmon resonance.

the absence of the α -subunit on the majority of natural killer cells (Caligiuri et al., 1990).

As part of an effort to understand the recognition properties of the IL-2 receptor, we previously constructed fusion proteins containing coiled-coil (leucine zipper) recognition sequences attached to the extracellular domains of the IL-2R α - and β -subunits. A mixture of these fusion proteins formed a heteromeric pseudo-high-affinity complex (Wu et al., 1995b). We analyzed the interaction of this complex with IL-2 and a series of IL-2 mutants both in solution and on a surface plasmon resonance biosensor (Wu et al., 1995a, 1995b). The results of this study were remarkably similar to direct ligand-binding studies conducted on cell surface IL-2R complexes, lending support to the assumption that the IL-2R α - and β -subunits are precomplexed on the cell surface. However, we found no evidence of association of the extracellular domains of these two subunits in solution, either in the presence or absence of IL-2, when not fused to the coiled-coil recognition sequences (Wu et al., 1995b). This observation suggested that recognition between the IL-2R α - and β -subunits occurs either in the transmembrane or cytoplasmic domains of these proteins or both and is in accord with findings reported by others in cell transfection studies (Goldsmith et al., 1995).

In this report, we again apply biosensor technology to further study the role of the extracellular domains of the IL-2 receptor in complex formation. We compare the equilibrium and kinetic binding properties of the α - and β -subunit ectodomains immobilized on separate sensor surfaces and on the same sensor surface in different subunit ratios. Equilibrium studies demonstrate unequivocally that surfaces with both subunits immobilized bind IL-2 ligand with a significantly greater affinity than either of the individual receptor subunits. A detailed kinetic analysis was performed by globally fitting IL-2 response data from multiple surface densities of the receptor subunits and incorporating mass transport effects into the binding mechanisms. Results of the kinetic analysis on mixed receptor surfaces indicated that the IL-2 that is bound in the higher-affinity complex is captured simultaneously by the α - and β -subunits. This is the first evidence that the extracellular domains themselves may preassociate in the absence of ligand. The ability to assemble and determine binding constants for receptor complexes on the sensor surface provides a useful tool for the analysis of this and other multiple subunit receptor systems.

Results

Equilibrium analysis

The equilibrium binding constants for the extracellular domains of the IL-2R α - and β -subunits for IL-2 ligand were determined by immobilizing each subunit onto a dextran sensor surface using unique solvent-exposed thiols. Wild-type IL-2R α contained a free thiol that could be exploited without influencing ligand-binding properties (Kato & Smith, 1987; Rusk et al., 1988). The IL-2R β -subunit had no reactive Cys thiol, so we introduced one at Ser 111 via site-directed mutagenesis. Residue 111 was chosen because it should be membrane proximal and distal to the ligand interaction site, based on sequence homology with the growth hormone and interleukin-4 receptors and their known or predicted three-dimensional structures (de Vos et al., 1992; Gustchina et al., 1995). Serial dilutions of IL-2 were injected over surfaces immobilized with either the IL-2R α - or the IL-2R β -ectodomains and the responses were allowed to reach equilibrium (Fig. 1A,B). The re-

Fig. 1. Sensorgram overlays for IL-2 binding to different IL-2 receptor surfaces. **A:** IL-2R α immobilized at 1200 RU. **B:** IL-2R β immobilized at 2200 RU. **C:** IL-2R α and IL-2R β immobilized at 620 and 1570 RU. Concentrations of IL-2 (bottom to top sensorgrams) in 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.005% surfactant P-20, and 100 μ g/mL BSA were: (A) 60, 70, 80, 90, 100, 150, and 200 nM; (B) 62.5–2,000 nM in twofold dilutions; (C) 3.9–2,000 nM in twofold dilutions injected over each surface at a flow rate of 10 μ L/min.

fractive index contributions from bulk solvent and nonspecific binding of IL-2 to the dextran matrix were determined from injections of the same series of samples over a mock surface. The corrected response values at equilibrium for each surface were fit to a single-site binding isotherm to determine the respective equilibrium dissociation constants (Fig. 2). The individual IL-2R α - and IL-2R β -ectodomains bound IL-2 with affinities of 37 ± 2 nM and 480 ± 30 nM, respectively. These results are consistent with the affinities determined previously by BIAcore™ for the α - and β -subunit coiled-coil complexes (30 nM and 410 nM, respectively) (Wu et al., 1995a), as well as for those obtained in solution-binding assays (15 nM and 300 nM, respectively) (Wu et al., 1995b). Therefore, the thiol-maleimide coupling procedure used to orient the attachment of the receptors to the dextran surface did not alter ligand recognition.

To investigate the binding properties of a mixed receptor surface, known quantities of IL-2R α and IL-2R β were coupled sequentially to the same sensor surface. One co-surface was prepared with an excess of the β -subunit in a 1:3.4 molar ratio (590 RU α :1,600 RU β). The equilibrium binding data obtained for a series of IL-2 injections (2 μ M–4 nM) are shown in Figure 1C and were

Fig. 2. Equilibrium response data for IL-2 binding to the different IL-2 receptor surfaces. Equilibrium data from the IL-2R α surface (●) and the IL-2R β surface (■) were fit separately to a single site binding isotherm. Data for the IL-2R α / β surface (▲) were fit to a two-site binding model.

fit well by a two-site binding model (Fig. 2). The lower-affinity site yielded a K_d of 420 ± 50 nM, which corresponds to the value determined previously for the β -subunit alone. This was anticipated because the β -subunit was in excess on this surface. However, the higher-affinity site gave a K_d of 1.0 ± 0.3 nM, which is much greater than expected for binding to the α -subunit alone and is close to the values reported for the preformed cell surface IL-2R α / β pseudo-high-affinity receptor (0.2–0.6 nM) (Wu et al., 1995b, loc. cit.). When a different surface of approximately the same total receptor density but with an excess of α -subunit was examined ($\alpha:\beta \approx 2:1$), the equilibrium data again fit a two site model (data not shown). In this case, the lower-affinity site ($K_d = 52 \pm 18$ nM) was also reflective of the subunit present in excess on the sensor surface (IL-2R α). Similarly, the second site had an affinity ($K_d = 3.0 \pm 0.7$ nM) that was higher than either of the subunits alone.

Although it is clear that a higher-affinity site for IL-2 exists on the co-surfaces, it is not possible to determine the mechanism for

its formation from equilibrium data. IL-2 either may be simultaneously captured by a pre-associated IL-2R α / β complex, as occurs on the surface of cells (Fig. 3A), or the ligand may first bind to the IL-2R α (due to its higher affinity) and then recruit the β -subunit to form the complex (Fig. 3B). This second model resembles a previous proposal for IL-2 cell surface binding termed “affinity conversion” (Saito et al., 1988). In an attempt to differentiate between these two possible mechanisms, we conducted a detailed kinetic analysis of IL-2 binding to biosensor surfaces prepared from each individual receptor as well as from both subunits.

Kinetic analysis of individual receptor surfaces

Resolving the kinetic constants for the IL-2 interactions recorded on the sensor required that we optimize the experimental conditions by lowering the surface density and increasing the flow rate. Using these conditions, sensor data were collected for two different surface densities of each receptor alone and the injections of IL-2 were repeated to determine total experimental noise in the system. The α -subunit was immobilized at 400 RU and 160 RU, whereas the β -subunit was immobilized at 900 RU and 240 RU. Four concentrations of IL-2 representing threefold serial dilutions (225–8.3 nM for the α -surface, 675–25 nM for the β -surface) in addition to a buffer blank were injected separately at a high flow rate (50 μ L/min) over each surface and repeated four times. Between each injection, the signal was allowed to decay to baseline so that no regeneration step was required. All of the data obtained at each IL-2 concentration are displayed in Figure 4A, B, C, and D after normalizing the injection times and baselines and correcting for the bulk refractive index change during the association phase. The reproducibility of the BIAcore™ is evident from how well each separate run overlays the others at the same concentration. In addition, the lack of signal drift and injection noise in the blank samples confirms that instrument artifacts do not affect the binding response significantly.

If the binding reactions recorded on the sensor followed a simple bimolecular mechanism, then the responses obtained from the high- and low-density surfaces, as plotted in Figure 4, should look identical. However, the binding responses from the lower-density surfaces for both receptor subunits (Fig. 4B,D) appear to decay

Fig. 3. Model for IL-2 ligand binding mechanisms that lead to the formation of a high-affinity receptor complex. **A:** IL-2 is captured simultaneously by a preassociated IL-2R α / β complex. **B:** IL-2 is captured initially by IL-2R α (step 1), which subsequently recruits the IL-2R β (step 2), forming the receptor complex.

Fig. 4. Kinetic response data for IL-2 binding to surfaces containing the individual receptor subunit ectodomains. Experimental data (black dots) represent four repeated injections of each IL-2 concentration (see the Materials and methods) over surfaces containing (A) 400 RU and (B) 160 RU of IL-2R α and (C) 900 RU and (D) 240 RU of IL-2R β at a flow rate of 50 μ L/min. A global fit of these data to a bimolecular mass transport limited binding reaction [Mechanism 1] is shown by the red lines. Residual plots are shown at the top of each data set.

back to baseline faster than the higher-density surfaces (Fig. 4A,C). This indicates that these binding reactions are influenced by mass transport. Lowering the surface capacity lowers the flux of analyte near the sensor surface and reduces the effects of mass transport (Fisher et al., 1994; Karlsson et al., 1994). This is also consistent with the observation that altering the flow rate also altered the binding responses (data not shown). It was therefore not surprising to find that a simple bimolecular reaction model failed to describe the binding of IL-2 to any of the receptor surfaces, even when applied within a single data set. The standard deviation in the residuals for a simple bimolecular interaction fit to either the α - or β -subunit data (not shown) were at least five times the replication STD of 1.00 RU. The response data from all four different receptor surfaces, however, fit a model incorporating mass transport limited binding by the addition of one floating parameter [Mechanism 1] (see Materials and methods). The red lines (Fig. 4A,B,C,D) represent the best global fit and essentially intersect the response data over the entire time course of binding for each of the data sets. The quality of fit was excellent as judged by the plots of the residuals, which were low and evenly distributed (Fig. 4A,B,C,D). The highest residuals occur at the junctions of the sample plugs, due to noise caused by the injections and mixing effects. In contrast to the fit to a simple bimolecular interaction, the residual standard deviation of the fit incorporating a transport coefficient was 1.02 RU. This value is essentially equivalent to the experimental replication standard deviation of 1.00 RU, which is a model-independent assessment of the total experimental noise.

The returned values for the parameters that were floated during the fitting procedures are displayed in Table 1. We used statistical profiling to confirm that the error space was essentially linear within a standard deviation of each parameter value (Watts, 1994), so their variance and correlation are reported directly from the linear approximation statistics in the covariance matrix. Overall, the errors in the parameters were low (averaging around 1.5%) because the their values were highly constrained by globally fitting data from multiple surface densities for each receptor subunit. The correlations for most of the parameters were also very low, except for the association and dissociation rate constants. It is not surprising that these parameters were highly correlated due to their relationship in the reaction model and yet, even with this level of correlation, unique values for these parameters were obtained.

Based on the biosensor kinetic analysis, the α -subunit bound IL-2 with an association rate ($7.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) that is six times

faster than that of the β -subunit ($1.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) and with a dissociation rate about three times slower (0.24 s^{-1} versus 0.66 s^{-1}). In all cases, these rate constants were significantly faster than those obtained previously from SPR analysis of IL-2 binding to equivalent surfaces prepared from coiled-coil IL-2R complexes of the same subunit ectodomains (Wu et al., 1995a). It is unlikely that these differences are a result of the methods used for complex formation or immobilization. It is more likely that the previous values were underestimated because the experiments were performed at slower flow rates, over higher surface densities, and analyzed without accounting for mass transport limitations. The ratio of the rate constants (k_d/k_a) determined from the kinetic analysis, predicts IL-2 equilibrium dissociation constants for IL-2R α and IL-2R β of $31 \pm 0.7 \text{ nM}$ and $500 \pm 10 \text{ nM}$, respectively. Within standard error, these values are the same as those obtained from the direct equilibrium binding experiments despite being conducted under different experimental conditions ($K_d = 37 \pm 2 \text{ nM}$ for IL-2R α and $480 \pm 30 \text{ nM}$ for IL-2R β).

Kinetic analysis of mixed receptor surfaces

In an attempt to resolve the binding mechanism for the formation of the higher-affinity site observed on the co-surfaces, a mixed surface with excess β -subunits was used. A ratio of IL-2R α to IL-2R β of 1:2.5 was chosen to provide a surplus of sites for complete interaction with the α -subunits. As shown in the previous equilibrium binding studies, the difference in affinities between the higher-affinity site and the excess receptor site was greatest with this stoichiometry. Thus, this ratio would make it easier to identify the kinetic mechanism for the formation of the higher-affinity site.

Six concentrations of IL-2, representing threefold serial dilutions (675–2.7 nM), and a buffer blank were injected in triplicate over a surface containing 140 RU of IL-2R α and 280 RU IL-2R β (molar ratio 1:2.5). Data were collected as described previously and the sensorgrams are depicted in Figure 5. Visual inspection of the data indicates that a significant fraction of the captured IL-2 dissociates much more slowly from the co-surface than from either of the homogeneous receptor surfaces at equivalent ligand concentrations. Because the dissociation was not complete within a reasonable time, the remaining captured ligand was removed by a brief pulse (5 μL) of 10 mM HCl. Despite this regeneration step,

Table 1. Values, standard deviations, and correlations for the parameters from the mass transport limited model [Mechanism 1], globally fit to the IL-2 ligand binding data from individual IL-2R α and IL-2R β receptor surfaces

Parameter	Value	Parameter correlations							
		k_m	R_{max1}^α	R_{max2}^α	k_a^α	k_d^α	R_{max1}^β	R_{max2}^β	k_a^β
k_m (m/s)	$9.78(7)^a \times 10^{-6}$								
R_{max1}^α (RU)	34.7(1)	−0.16							
R_{max2}^α (RU)	97.7(1)	−0.09	0.24						
k_a^α ($\text{M}^{-1} \text{s}^{-1}$)	$7.8(1) \times 10^6$	−0.79	0.08	−0.06					
k_d^α (s^{-1})	0.235(4)	−0.85	0.15	0.09	0.97				
R_{max1}^β (RU)	81.0(3)	−0.01	0.00	0.00	0.01	0.01			
R_{max2}^β (RU)	339.1(8)	−0.02	0.00	0.00	0.02	0.02	0.65		
k_a^β ($\text{M}^{-1} \text{s}^{-1}$)	$1.27(2) \times 10^6$	−0.86	0.13	0.07	0.68	0.73	−0.17	−0.24	
k_d^β (s^{-1})	0.66(1)	−0.89	0.14	0.08	0.71	0.76	0.01	0.03	0.96

^aValues in parentheses represent the standard deviation in the final significant digit.

Fig. 5. Kinetic response data for IL-2 binding to a co-surface containing both the IL-2 receptor α - and β -ectodomains. Experimental data (black dots) represent three repeated injections of each IL-2 concentration (see Materials and methods) over a surface containing 140 RU of IL-2R α and 280 RU of IL-2R β . Red lines are the global fit to (A) a simultaneous capture model [Mechanism 2] and (B) an affinity conversion model [Mechanism 3]. Residual plots are shown at the top of each data set.

the sensorgrams obtained from replicate injections remained highly reproducible.

The response data obtained from the co-surface were tested against different models for the formation of the pseudo-high-affinity receptor complex, including those depicted in Figure 3. In the one model, IL-2 is captured simultaneously by a preformed IL-2R α / β receptor complex [Mechanism 2]. In another model, IL-2 is captured initially by the IL-2R α -subunit, subsequently recruiting the β -subunit to form the high-affinity complex [Mechanism 3]. Although both of these mechanisms appear rather complex, several parameters in each reaction could be fixed during the fitting procedure because their values were determined previously from the experiments conducted on homogeneous surfaces. The kinetic parameters for all IL-2R α or β single-site interactions, as well as the mass transport coefficient (because the same ligand and flow rates were employed), were fixed to the values determined previously. The populations of each available IL-2 site for both models were floated, along with the rate constants for binding to the preformed IL-2R α / β complex or the affinity conversion step.

The nonlinear least-squares best fit of the co-surface data to a simultaneous capture model and affinity conversion model are shown by the red lines in Figure 5A and B, respectively. The simultaneous capture model provides a very good fit to the data for all the IL-2 concentrations across the entire time course of the experiment. The residuals were low and evenly distributed, as shown in Figure 5A. The residual standard deviation for this model is 1.05 RU, which is very close to the experimental replication standard deviation of 1.04 RU. In contrast, the affinity conversion model provided a poor fit to the data. Although it resembled the shapes of the slower dissociation phase observed on the co-surface, it failed to accurately describe the association phase and the position of the data for several of the IL-2 concentrations (see Fig. 5B) and resulted in a 50% greater residual standard deviation (1.54 RU). This emphasizes the benefits of global fitting, which takes into account both the shapes and positions of the progress curves in relationship to each other. Other variations of the affinity conversion model, for example, where the binding of IL-2 was again limited to the association rate for the IL-2R α but which allowed the complex to break down directly to free receptors, failed to improve the fit.

The parameter values determined for the fit of the simultaneous capture model to the co-surface data are shown in Table 2. As with the data from the homogeneous surfaces, the error spaces within a standard deviation of the parameter values were found to be linear

by statistical profiling. The maximum capacity determined for the IL-2R α and IL-2R α / β sites suggests that 80% of the immobilized α -subunits bound IL-2 as a complex with the β -subunit. The association rate constant to this IL-2R α / β complex ($k_a = 2.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) is threefold faster than the association rate for IL-2R α alone and 18-fold faster than binding to IL-2R β , suggesting the two subunits participate to capture IL-2 simultaneously. More significantly, the dissociation rate constant of the complex ($k_d = 0.018 \text{ s}^{-1}$) is 13-fold slower than IL-2R α alone. Together these rate constants predict an equilibrium dissociation constant of $0.78 \pm 0.01 \text{ nM}$, which is indistinguishable from the value obtained directly from equilibrium binding analysis ($1.0 \pm 0.3 \text{ nM}$).

Discussion

We employed biosensor technology to measure the IL-2 binding constants for the individual IL-2R α - and β -subunits and for an IL-2R α / β complex created on the sensor surface. The equilibrium binding data obtained using the receptor co-surfaces clearly indicated that the IL-2R α - and β -ectodomains could cooperate to bind IL-2 with an affinity that was significantly greater than the affinity of either of the individual subunits. In previous solution-binding studies, we were unable to detect any higher-affinity species in subunit mixtures (Wu et al., 1995b). In those studies, however, the concentrations of receptor ectodomains never exceeded $2 \mu\text{M}$. On the biosensor, the receptors are attached within the contained volume of the dextran matrix, resulting in high local concentrations. Under the experimental conditions employed in this study, the concentrations of receptor subunits often exceeded $300 \mu\text{M}$. Although covalently linked, macromolecules attached to the dextran matrix remain highly mobile and retain solution-binding properties. The ability of immobilized receptors to interact with each other within the dextran matrix has been demonstrated before. In a report employing a similar oriented coupling method, growth hormone receptors were attached to the dextran in different known orientations and a comparison was made of the ability of ligands to induce receptor homodimerization on the surface (Cunningham & Wells, 1993).

In an effort to understand the mechanism involved in the formation of the higher-affinity site observed on surfaces with both IL-2R α - and β -subunits immobilized, we performed a detailed kinetic analysis of IL-2 binding to the homogeneous and mixed receptor surfaces. In order to discriminate between possible complex binding mechanisms, we used nonlinear least squares analysis with simultaneous fitting of multiple ligand concentrations (Morton et al., 1995). This, in turn, places a high demand on the quality of the sensor data. The use of thiol-maleimide coupling was ideal because it produced a stable, oriented, and homogenous reacting surface, while allowing precise control of immobilization levels. We lowered the surface density to reduce the influence of mass transport and the likelihood of steric occlusion of receptor sites. In addition, the flow rate was increased to $50 \mu\text{L}/\text{min}$ to minimize both diffusion of the sample plug during injection and mass transport effects. Data were collected from multiple surface densities to provide information about the mass transport coefficient, and help to constrain parameter values during the fitting procedure. Finally, the binding experiments were replicated to determine a model-independent estimate of the total experimental noise in the system, a requirement in order to assess if the models described the data adequately. Together, these improvements in the experimental design generated sensor data that were suitable for a detailed kinetic

Table 2. Values, standard deviations, and correlations for the parameters from the simultaneous capture model [Mechanism 2] fit to the IL-2 ligand binding data from the coreceptor surface

Parameter	Value	Parameter correlations			
		$R_{\max}^{\alpha}$	$R_{\max}^{\beta}$	$R_{\max}^{\alpha/\beta}$	$k_a^{\alpha/\beta}$
$R_{\max}^{\alpha}$ (RU)	8.5(3) ^a				
$R_{\max}^{\beta}$ (RU)	91.9(5)	-0.87			
$R_{\max}^{\alpha/\beta}$ (RU)	33.6(1)	-0.89	0.62		
$k_a^{\alpha/\beta}$ ($\text{M}^{-1} \text{ s}^{-1}$)	$2.32(3) \times 10^7$	-0.13	0.08	0.15	
$k_d^{\alpha/\beta}$ (s^{-1})	0.0183(3)	-0.63	0.40	0.75	0.72

^aValues in parentheses represent the standard deviation in the final significant digit.

analysis. Nonlinear least-squares global curve fitting was essential to discriminate between possible binding mechanisms to the IL-2R α and β -subunits. The higher association rate observed on these surfaces suggested the IL-2 ligand was captured by both subunits, simultaneously.

Simultaneous capture of the IL-2 ligand by a pre-associated IL-2R α / β complex is consistent with the accepted model of ligand binding to the cell surface IL-2R α / β pseudo-high-affinity site (Landgraf et al., 1992; Goldsmith et al., 1995). This is the first evidence, however, that the extracellular domains of these subunits are all that is required for the formation of the complex in the absence of ligand. Although the surface densities employed in the kinetic analyses were low, the lowest actual molar concentration exceeded 30 μ M for the α -subunit. This is a concentration that could be achievable on the surface of cells at reasonable receptor densities where the proteins are confined by the two-dimensional surface of the membrane (Grasberger et al., 1986). It is likely that the cytoplasmic and transmembrane domains also play a role in the stabilization of the IL-2R α / β complex. Others have reported that, when both α and β extracellular domains are expressed on the cell surface in the form of chimeric or truncated fusion proteins, sometimes the pseudo-high-affinity site is not observable (Goldsmith et al., 1995). This indicates that the formation of this site is easily susceptible to inhibition by intracellular components; however, our studies demonstrate that, under physiological conditions, the recognition elements present on the extracellular domains are sufficient to stabilize association.

The influence of mass transport on rate constants measured by biosensor methods is well known (Karlsson et al., 1994). Recent reports illustrate that it is quite easy to simulate conditions that emphasize mass transport phenomena both above and within the dextran matrix (Hall et al., 1996; Schuck, 1996). Although we took experimental precautions to minimize mass transport effects, they could not be eliminated entirely because the binding rates for the receptor surfaces were so fast. Therefore, the application of a two-compartment, mass transport limited model to represent diffusion-limited access to the receptors was a key feature of our approach to data analysis and allowed us to derive accurate estimates for the intrinsic reaction rate constants whose values are an order of magnitude beyond the limitations suggested by the manufacturer (BIAcore™ operations manual). Because a single ligand was employed for all binding experiments and the flow rate and buffer conditions remained constant, only one parameter was used to describe mass transport to and from the different receptor surfaces. The validity of employing a single parameter to depict the mass transport event is supported by the observation that it described simultaneously the response data over a 250-fold concentration range of ligand and across two different densities of two different receptor subunits. A similar approach was successful in describing the mass transport limited binding responses observed for a protein antigen across three different surface densities of an immobilized antibody (Myszka et al., 1996).

Recently, others have reported the analysis of IL-2R complexes using SPR (Balasubramanian et al., 1996). In that study, the authors claim assembly of heteromeric complexes via antibody capture of epitope tagged receptor subunits. Although the results of the study resemble our findings qualitatively, it is clear from our results that antibody-assisted complexation is not required in order to observe higher-affinity sites on the biosensor surface and their existence does not confirm a direct role of the antibody in their formation. It is also clear from the results reported here that the

kinetic binding constants for the IL-2R α - and β -subunits are within the range in which mass transport effects have a significant influence on biosensor measurements even at the lowest of surface densities. Because the results reported by these authors were obtained by fitting only portions of single sensorgrams without consideration of mass transport effects, it is not surprising that they report significantly slower off-rate constants as a result of rebinding of ligand to the biosensor surface. Although it has been demonstrated recently that a simple bimolecular interaction model is adequate to describe some macromolecular interactions recorded on the biosensor (Rodén & Myszka, 1996), for many studies reported in the literature (including our own previous study, Wu et al., 1995a), the accuracy and interpretation of the data are compromised to varying degrees by mass transport limitations. Although the method remains valuable and the results useful as long as this limitation is recognized, the approach employed in this study demonstrates that the accuracy in the determination of kinetic binding constants can be extended considerably by optimizing the experimental conditions and improving data analysis methods.

Receptor subunit complexation is a hallmark of cytokine receptor systems. Receptor sites with widely divergent affinities often exist for a single cytokine as a result of different subunit compositions. The equilibrium dissociation constants measured in this study compare favorably with previous studies performed on biosensors, in solution and on the surface of cells. Comparison of our results with values reported previously for the kinetic binding constants show more deviation. Others have attempted to study the kinetics of IL-2 binding to the IL-2R α - and β -subunits and the pseudo-high-affinity receptor on the surface of cells (Ringheim et al., 1991; Matsuoka et al., 1993). The greatly divergent kinetic parameters reported in these studies demonstrate the difficulty associated with cell-based measurements and the inability to acquire data in the critical early stages of rapidly associating and dissociating systems. These reports cannot be compared directly to our study because they were conducted at 4 °C; however, even at this lower temperature, some of the kinetic constants were too rapid to be measured in cell-based assays. In this study, we have shown the advantages of an oriented and stable attachment of ectodomains with controlled stoichiometry to the biosensor surface (avoiding the background dissociation and regeneration problems found in antibody or metal chelate mediated immobilization that preclude kinetic analysis). We believe that, in combination with the acquisition of high-quality data and global data analysis, this approach should prove extremely valuable for the determination of kinetic parameters for cytokines and cytokine analogues in these complex systems.

Materials and methods

Protein expression and purification

The IL-2 receptor subunit extracellular domains (ectodomains) were expressed in insect cells via recombinant baculovirus infection and immunoaffinity purified as described previously (Sana et al., 1994; Wu et al., 1994, 1995b). For the purposes of oriented thiol-mediated coupling to activated biosensor surfaces, the free thiol group of the Cys 192 residue in the wild-type IL-2R α ectodomain was exploited. This group participates in the formation of disulfide-linked homodimers and may be replaced without influencing ligand binding (Kato & Smith, 1987; Rusk et al., 1988). Because the IL-2R β -subunit has no reactive Cys thiol, we introduced one via

mutagenesis at serine 111 (Sana, 1995), based on sequence homology with the growth hormone and interleukin-4 receptors and their known or predicted three-dimensional structures (de Vos et al., 1992; Gustchina et al., 1995). The (Cys 111) IL-2R β protein was expressed and purified in a fashion similar to the wild-type receptor ectodomain. Nondenaturing SDS-PAGE and reverse-phase HPLC revealed that the isolated protein was a mixture of monomer and disulfide-linked homodimer (not shown). The ratio of these components varied depending upon expression levels and length of storage. Because only the monomeric form is reactive in thiol-mediated coupling to the activated biosensor surface, the mixtures were used directly for surface preparation.

Recombinant IL-2 was expressed in *Escherichia coli*, refolded and purified as described (Landgraf et al., 1991, 1992). All protein preparations were judged to be >95% pure as monitored by reverse phase HPLC. Protein concentrations were determined from $A_{280\text{nm}}$ values measured in 6 M guanidine HCl (Johnson, 1990).

Biosensor reagents and surface preparation

BIAcore™ biosensor, CM5 sensor chips, and amine coupling reagents containing NHS/EDC and ethanolamine·HCl were obtained from Pharmacia Biosensor (Piscataway, New Jersey). Ethylenediamine was obtained from Sigma Chemical Co. (St. Louis, Missouri) and *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sulfo-MBS) was purchased from Pierce Chemical Co. (Chicago, Illinois).

The IL-2R α - and β -ectodomains were covalently linked to biosensor surfaces through a single cysteine thiol group employing maleimide-mediated coupling (O'Shannessy et al., 1992). The carboxymethyl dextran surface on the sensor chips was first activated with NHS/EDC (15 μ L) as described in the BIAcore™ systems manual. A 1 M aqueous solution of ethylenediamine (15 μ L) was then injected and the amino functionalized dextran was allowed to react with a solution of Sulfo-MBS (50 mM in NaHCO₃, pH 8.5, 20 μ L) to form a reactive maleimide surface. Purified IL-2R ectodomains were diluted to a concentration of <25 nM in NaOAc buffer (10 mM, pH 5.0) and injected in 5- μ L aliquots until desired surface densities had been achieved. To prepare the heterogeneous receptor surfaces, solutions of individual subunits were injected sequentially so that accurate estimates of the relative surface densities of each protein could be determined. The remaining activated groups were blocked by injection of a solution of cysteine (100 mM, pH 4.0, 25 μ L).

Equilibrium data analysis

IL-2 samples to be injected were first dialyzed against phosphate buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl), the protein concentrations determined, and the samples were then diluted to the desired concentrations in the same buffer containing 0.005% surfactant P-20 and 100 μ g/mL BSA.

To determine the equilibrium dissociation constants for the individual receptor ectodomains, the α - and β -subunits were immobilized onto separate surfaces at 1,200 and 2,200 RU, respectively. Two different co-surfaces were created that contained an excess of either receptor. Based on their molecular weights and the measured response after immobilization, one surface contained the α - and β -subunits in a 1:3.4 molar ratio (620:1,570 RU) and the other contained a 2:1 molar ratio (1,570:630 RU). Twofold serial dilutions of IL-2, as well as a sample buffer blank, were injected over

these surfaces at a flow rate of 10 μ L/min of running buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.005% surfactant P-20). An equivalent set of samples for each receptor surface was injected over a mock derivatized surface (prepared using the same protocol as for receptor derivatization, but without injection of receptor protein) for subtraction of bulk refractive index values and nonspecific binding. Regeneration of the receptor surfaces was achieved by 5- μ L injection of 10 mM HCl.

The equilibrium value for each injection was determined by first subtracting the buffer blank from each data set and then subtracting the response obtained over the mock surface from that obtained over the receptor surface at each concentration. The dissociation constants were determined from nonlinear least-squares curve fitting of the data to single or two site binding model using the program EBDA/RADLIG (version 4, BIOSOFT) (McPherson, 1985).

Kinetic data generation

To determine the kinetic rate constants for the IL-2 binding reactions, surfaces with lower receptor densities were employed (IL-2R α , 400 and 160 RU; IL-2R β , 900 and 240 RU; IL-2R α/β , 140 RU α /280 RU β). IL-2 was injected over each surface at the following concentrations: 675 nM (IL-2R β and IL-2R α/β only); 225 nM, 75 nM, 25 nM, 8.3 nM (IL-2R α and IL-2R α/β only); 2.7 nM (IL-2R α/β only); 0 nM. Samples were injected at a flow rate of 50 μ L/min to reduce sample diffusion and to minimize mass transport effects. BSA (100 μ g/mL) was added to the running buffer to reduce the bulk refractive index difference between sample and running buffer. No regeneration was required with the surfaces prepared from single receptor subunits. The dissociation phase was monitored until the signal returned to baseline values. Due to the decrease in dissociation rate on the IL-2R α/β co-surface, regenerations were performed using a 5- μ L pulse of 10 mM HCl. Each sample injection was repeated 3 or 4 times over each receptor surface. Binding data were prepared for nonlinear regression analysis by normalizing the time and baseline response prior to each injection.

Mass transport limited model

Because the receptors are immobilized within a dextran layer on a surface, the IL-2 ligand in the bulk flow must first be transported through the unstirred solvent layer that is set up over the sensor surface, in order to bind to the receptor in the dextran environment (Karlsson et al., 1994). We used a two-compartment model, similar to the one described by Fisher et al. (1994), to represent this diffusion-limited binding [see Mechanism 1]. The model assumes there are two homogeneous compartments, one representing the analyte in the bulk flow and the second containing the immobilized ligand in a stagnant buffer layer. During the association phase, the concentration of IL-2 in the outer compartment ([IL-2]_o) is taken to be equal to the bulk injected concentration. In the inner compartment, the concentration of IL-2 is zero initially and changes with time according to the transport rate, the concentration of free and complexed receptor sites, and the rate constants for the binding reaction. During the dissociation phase, the concentration of IL-2_o is set to zero; however, the concentration of IL-2 in the unstirred layer depends on the same transport rate, concentration of free and complexed receptor sites, and the reaction rate constants. We average any variation in k_m in the direction of flow and take this

parameter to be a constant. Because a single ligand was employed for all of the binding experiments and the flow rate and buffer conditions were the same throughout the kinetic analysis, only one parameter was used to describe mass transport to and from the different receptor surfaces.

Binding mechanisms

The rate constants that appear in boxes were the parameters whose values were searched for (floated) during the fitting procedure. Simultaneous fitting of Mechanism 1 to all of the data from both IL-2R α - and IL-2R β -subunit surfaces allowed the use of one parameter, k_m , to describe the mass transport rate:

The rate constants for each receptor subunit (k_a^α , k_d^α , k_a^β , and k_d^β) were applied to the data sets corresponding to each receptor surface. Four additional parameters were used to describe the maximum capacity of each surface (R_{max}), which were modeled in response units. The bulk refractive index changes present during the association phase of every injection were fit by a simple step function and subtracted from the displayed data.

IL-2 binding data obtained from the surfaces containing both receptor subunits were fit to a simultaneous capture Mechanism 2, where $\alpha:\beta$ represents a distinct binding site composed of a pre-associated receptor subunit complex:

This model allows IL-2 to bind to the co-surface with a faster association rate and assumes the populations of the different IL-2 sites are not changed by ligand binding. The mass transport coefficient and the rate constants for binding to the individual α - and β -subunits were fixed at the values determined previously from the individual surfaces. Three parameters that describe the maximum capacity of each site (R_{max}) were floated, along with a step function for refractive index changes.

The same co-surface data were also tested against an "affinity conversion" Mechanism 3, in which IL-2 was initially bound by the α -subunit, which subsequently recruits the β -subunit to form the higher-affinity complex:

In this model, two parameters were floated that described the affinity conversion step (recruitment of the β -subunit), but the maximum initial capture rate of IL-2 was restricted to the association rate for IL-2R α only. Again, the mass transport coefficient and rate constants for binding to the α - and β -subunits were fixed at the values determined previously. Four parameters were floated to describe the population of each site and a step function was used for refractive index changes.

Nonlinear regression analysis

Modeled data for each mechanism were generated by numerical integration of the rate of change of each species according to the model being fit using the Bader and Deufhard semi-implicit extrapolation method (Press et al., 1992). To perform the nonlinear least-squares analysis, the values of the unknown parameters were adjusted by the Levenberg-Marquardt method (Press et al., 1992) to minimize the difference between the modeled and experimental data. In each case, the fitting procedures were repeated 100 times with different starting parameters to assure that a true minimum had been achieved. The computational routines used in the kinetic analysis were described previously (Morton et al., 1995) and were run on a 100 MHz pentium™ PC. Statistical profiling techniques (Watts, 1994) were employed to assess the error space for the parameters in the nonlinear models as described previously (Myszka et al., 1996). The replication standard deviation, which represents a model-independent estimate of the total experimental noise, was determined from the average responses of the replicated IL-2 injections.

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