Use of a Biosensor with Surface Plasmon Resonance Detection for the Determination of Binding Constants: Measurement of Interleukin-6 Binding to the Soluble Interleukin-6 Receptor†

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ABSTRACT: The interaction of recombinant human interleukin-6 (IL-6) with the soluble extracellular form of its receptor (sIL-6R) has been characterized by the application of expressions developed for quantitative affinity chromatography to results obtained with a biosensor based on surface plasmon resonance detection. First, the interaction of sIL-6R with IL-6 covalently attached to the biosensor-chip was characterized from the dependence of the surface plasmon resonance response upon the concentration of receptor injected into the biosensor. A binding constant for the interaction between sIL-6R and IL-6 was then determined from the biosensor response observed for mixtures of IL-6 and receptor—a procedure that is shown to provide unequivocal characterization of the competing reaction, irrespective of the model used to describe the biphasic interaction between partitioning receptor and immobilized IL-6. A binding constant of 5×10^7 M^{-1} has been obtained for the interaction of sIL-6R with two equivalent and independent sites on an essentially dimeric IL-6 preparation produced using the pUC vector system, and also for the interaction of sIL-6R with a monomeric IL-6 preparation that was univalent in its interaction with receptor.

The commercial availability of a biosensor with surface plasmon resonance detection has invoked considerable interest in its potential for the characterization of high-affinity interactions such as those between antigens and their elicited monoclonal antibodies (Karlsson et al., 1991; Chaiken et al., 1991; Altschuh et al., 1992; Zeder-Lutz et al., 1993; O'Shannessy et al., 1993). However, those determinations of equilibrium constants as ratios of apparent kinetic rate constants for the adsorption and desorption of antibody to/from a surface coated with antigen incorporate the combined relative errors of the two rate constants into the uncertainty of the estimated binding constant. Furthermore, in the particular instance that the antigen is immobilized (Karlsson et al., 1991; Altschuh et al., 1992; Zeder-Lutz et al., 1993), the reliance upon kinetic expressions based on 1:1 stoichiometry for the interaction between antibody and immobilized antigen may well render the method of questionable validity in light of the effect that solute bivalency has on dissociation kinetics (Hogg et al., 1987b; Posner et al., 1991).

Little attention seems to have been given to the fact that the analogous problem has already been addressed in the context of quantitative affinity chromatography (Nichol et al., 1974, 1981; Hogg & Winzor, 1984; Hogg et al., 1991; Winzor et al., 1992). Indeed, there is an extremely close correspondence between the experimental setups in the biosensor methodology and simple partition experiments (Kuter et al., 1983, Harris & Winzor, 1989) or the recycling partition variant thereof (Nichol et al., 1981; Hogg et al., 1991). Whereas the concentration of partitioning solute bound to the solid phase is determined as the difference between total and liquid-phase concentrations in the latter methods, the biosensor resonse is a direct measure of the difference. In this investigation, the interaction between human interleukin-6 (IL-6)[1] and the soluble form of the 80-kDa human IL-6 receptor (sIL-6R) is characterized by means of a biosensor instrument utilizing surface plasmon resonance detection.

Interleukin-6 is a pleiotropic cytokine which regulates the growth and differentiation of various cell types. Activities attributed to IL-6 include the ability to promote megakaryocyte maturation, immunoglobulin production in B cells, expression of acute-phase proteins in liver, and the growth and differentiation of T cells [see reviews by Hirano et al. (1990), Van Snick (1990), and Taga and Kishimoto (1992)]. Interleukin-6 acts by binding to a cell membrane receptor

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[1] Abbreviations: Gdn-HCl, guanidine hydrochloride; IL-6, interleukin-6; sIL-6R, extracellular or "soluble" form of human IL-6 receptor; PBS, phosphate-buffered saline; RP-HPLC, reversed-phase high-performance liquid chromatography; SPR, surface plasmon resonance; TFA, trifluoroacetic acid.

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complex composed of two transmembrane proteins referred to as the 80-kDa IL-6 receptor (IL-6R) and the 130-kDa glycoprotein gp-130 (Taga & Kishimoto, 1992). Fortunately, the biological potency of IL-6 is unaffected by removal of the cytoplasmic domain of the IL-6R, and hence the extracellular domain (sIL-6R) affords a convenient soluble form of the receptor for the characterization of its interaction with IL-6.

We have initiated a study to probe the specific amino acids of IL-6 involved in its binding to both IL-6R and gp-130. Fundamental to these studies is a quantitative understanding of the protein−protein interactions responsible for signal transduction and high-affinity binding. Here we explore application of the existing theoretical expressions for quantitative affinity chromatography (Winzor, 1992) to the characterization of the sIL-6R/IL-6 interaction by means of the BIAcore instrument.

EXPERIMENTAL PROCEDURES

Materials. Ultrapure Gdn-HCl (8 M aqueous stock solution), trifluoroacetic acid (Sequenal grade), and Tween-20 were Pierce products. Ethanolamine, *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide, *N*-hydroxysuccinimide, and CNBr-activated Sepharose 4B were obtained from Pharmacia. Centricon-30 microconcentrators were supplied by Amicon, and the nitrocellulose membrane was obtained from Schleicher & Schüll (Dassel, Germany).

Construction of IL-6 Expression Vectors. Human interleukin-6 cDNA in a pGex expression vector, kindly provided by Dr. T. Wilson (Walter and Eliza Hall Institute, Melbourne), was digested with *Bam*HI and *Hin*dIII and subcloned into pUC8 for expression.

An N-terminally truncated form of IL-6 commencing at Thr20 [numbering according to Simpson et al. (1988a)] was obtained by polymerase chain reaction (PCR) using IL-6 in pUC8 as template. After subcloning into pCDM8, the cDNA corresponding to the N-terminally truncated IL-6 was subjected to site-directed mutagenesis (Kunkel et al., 1987) to substitute an asparagine residue for a lysine residue at position 63 and introduce an *Afl*II restriction endonuclease site. Clones containing this mutation, (N63/K)IL-6, were identified by restriction endonuclease mapping and subcloned into *Eco*RI/*Bam*HI-digested pUC8. Prior to expression, all constructs were verified by DNA sequencing.

Expression and Purification of IL-6. Full-length IL-6 in pUC8 and (N63/K)IL-6 were expressed in *Escherichia coli* (strain NM522) as fusion proteins with β-galactosidase. The nine N-terminal amino acid residues of recombinant IL-6 (Thr-Met-Ile-Thr-Asn-Ser-Arg-Gly-Ser) and the six N-terminal residues of (N63/K)IL-6 (Thr-Met-Ile-Thr-Asn-Ser) are derived from β-galactosidase and the polylinker region of pUC8. These recombinant proteins were isolated from urea-washed inclusion bodies by size-exclusion chromatography and RP-HPLC (Zhang et al., 1992), their purity being assessed as >95% by N-terminal sequence analysis and analytical RP-HPLC. The molecular masses of IL-6 (21 854 Da) and (N63/K)IL-6 (19 575 Da), determined by electrospray mass spectrometry, were within 0.02% of the theoretical values—a finding which indicates that no chemical modification or proteolytic clipping occurred during the purification procedure.

Another recombinant IL-6 preparation (Yasukawa & Saito, 1990) was expressed as a growth hormone fusion protein before purification and cleavage with thrombin to remove the growth hormone moiety (Yasukawa & Saito, 1990). This IL-6 was also full-length but possessed an additional alanine residue at the N-terminus, a feature confirmed by N-terminal sequence analysis and electrospray mass spectrometric analysis. Size-exclusion chromatography on a column of Superose 12 (Pharmacia) was used to remove a small amount (<5%) of high-molecular-weight material from this preparation.

Preparative Chromatographic Procedures. Reversed-phase HPLC on a 4.6 × 100 mm Brownlee RP-300 cartridge (Applied Biosystems) and size-exclusion chromatography on a 9.4 × 250 mm Zorbax GP-450 column were performed using a Hewlett-Packard 1090A liquid chromatograph fitted with a Model 1040A diode-array detector (Simpson et al., 1988b). For the purification of sIL-6R, an IL-6 affinity column (10 × 35 mm) was prepared by covalently attaching (N63/K)IL-6 to CNBr-activated Sepharose 4B (Pharmacia). Noncovalently bound IL-6 was removed by treatment of the matrix with 0.1 M Tris/HCl (pH 8.0) containing 6 M Gdn-HCl, a medium in which immobilized IL-6 is relatively stable (Ward et al., 1993a).

Expression and Purification of sIL-6R. Recombinant sIL-6R (truncated at amino acid 344) was purified from the conditioned medium of CHO cells transfected with a construct (pECdhfr344) which expresses the extracellular binding domain of the IL-6R (Yasukawa et al., 1990). The stably-transfected CHO cell clone H26 (Yasukawa et al., 1990) was grown to confluency in nucleoside-deficient αMEM with 5% fetal calf serum medium containing glutamine (1 mM), penicillin (60 μg/mL), and streptomycin (100 μg/mL) at 37 °C and 5% CO_2. Conditioned medium was collected and loaded directly, at a flow rate of 4 mL/min, onto the (N63/K)IL-6 affinity column equilibrated with phosphate-buffered saline (PBS). After removal of unbound material by washing the affinity matrix with a further 10 column volumes of PBS, the bound sIL-6R was eluted with 0.1 M glycine-HCl buffer, pH 2.5, at 2 mL/min and neutralized immediately by the addition of 50 μL of 1 M Tris-HCl buffer, pH 8.0, per milliliter of column eluate. Soluble IL-6R-containing fractions were pooled and loaded directly onto a Brownlee RP-300 column, which was developed with a 60-min linear gradient in which the proportion of acetonitrile in 0.1% (v/v) aqueous TFA increased from 0 to 60% (by volume). Fractions containing sIL-6R were pooled and then equilibrated with PBS by dialysis and concentrated using a Centricon-30 microconcentrator. These concentrated fractions were then chromatographed at a flow rate of 1 mL/min on a Zorbax GP-450 column equilibrated with PBS, after which fractions containing sIL-6R were identified by SDS/PAGE and Western blotting, as detailed below.

Electrophoretic Techniques. SDS/PAGE was performed using a Bio-Rad Mini Protean II apparatus and the Laemmli buffer system (Laemmli, 1970), after which protein bands were detected either by silver staining (Bio-Rad Plus silver stain kit) or by immunostaining after electrotransfer to a nitrocellulose membrane by means of a mini two-dimensional gel system (Bio-Rad). The first probe was rabbit polyclonal sera (Yasukawa et al., 1990) directed against the sIL-6R, and the second probe was goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad).

Characterization of Recombinant Proteins. The concentrations of purified sIL-6R and IL-6 were determined by amino acid analysis on a Beckman Model 6300 amino acid analyzer (Simpson et al., 1986). N-Terminal sequence analysis, electrospray mass spectrometry, and the 7TD1 hybridoma growth factor assay were performed as described previously (Ward et al., 1993a).

Sedimentation Equilibrium. The molecular weights of IL-6 and sIL-6R were determined by subjecting dialyzed samples (approximately 0.1 mg/mL) of the two proteins to sedimentation equilibrium in a Beckman XL-A analytical ultracentrifuge operated at 20 °C. Respective angular velocities of 8000 and 12 000 rpm for IL-6 and sIL-6R were used to generate low-speed sedimentation equilibrium distributions (Van Holde & Baldwin, 1958), which were recorded spectrophotometrically at 230 nm and analyzed by the standard linear transform of the sedimentation equilibrium distribution for a single solute. The partial specific volumes of the protein component of the sIL-6R and IL-6 were both calculated as 0.72 mL/g from their amino acid compositions (Cohn & Edsall, 1943).

Studies of the IL-6/sIL-6R Interaction. The interaction between IL-6 and sIL-6R was monitored by surface plasmon resonance (SPR) detection using a BIAcore instrument (Pharmacia Biosensor, Uppsala, Sweden). For these experiments, IL-6 was covalently attached to the carboxymethylated dextran matrix coating the gold sensor chip (Ward et al., 1993a). After the derivatization step, any noncovalently bound IL-6 was removed from the sensor surface by treatment with 6 M Gdn-HCl for 3 min. Binding of sIL-6R to immobilized IL-6 was monitored by passing aliquots (35 μL) of the receptor (9−137 nM) in buffer [10 mM Hepes, pH 7.4, containing 0.15 M NaCl, 3.4 mM EDTA, and 0.005% (w/v) Tween-20] across the sensor plate at a flow rate of 1 μL/min. Between assays, the surface was regenerated by treatment of the sensor surface with 10 mM HCl for 3 min.

Analysis of Equilibrium Data. In accordance with the procedure devised for quantitative affinity chromatography (Nichol et al., 1981; Hogg & Winzor, 1984, 1985), the interaction of the receptor (A) with immobilized IL-6 (matrix-bound sites, X) has been characterized by means of the expression:

$$(\bar{\bar{C}}_A^{1/f} - \bar{C}_A^{1/f})/\bar{C}_A^{1/f} = K_{AX}\bar{\bar{C}}_X - fK_{AX}\bar{C}_A^{(f-1)/f}(\bar{\bar{C}}_A^{1/f} - \bar{C}_A^{1/f}) \quad (1)$$

where $\bar{\bar{C}}_A$ denotes the total molar concentration of sIL-6R in a situation where the affinity matrix is in equilibrium with a liquid-phase concentration $\bar{C}_A$ of receptor. The interaction is described in terms of an intrinsic binding constant (Klotz, 1946), K_{AX}, and an effective total concentration, $\bar{\bar{C}}_X$, of immobilized IL-6 sites. Because the sIL-6R is univalent ($f = 1$), eq 1 becomes

$$(\bar{\bar{C}}_A - \bar{C}_A)/\bar{C}_A = K_{AX}\bar{\bar{C}}_X - K_{AX}(\bar{\bar{C}}_A - \bar{C}_A) \quad (2)$$

which is a form of the conventional Scatchard relationship (Scatchard, 1949). On the grounds that the biosensor plateau response, R_p (see Results), is proportional to the amount of bound receptor and hence related to the difference between total and ligand-phase concentrations of sIL-6R, eq 2 may be rewritten as

$$R_p/\bar{C}_A = BK_{AX}\bar{\bar{C}}_X - K_{AX}R_p \quad (3)$$

where B is the proportionality constant in the expression $R_p = B(\bar{\bar{C}}_A - \bar{C}_A)$. The receptor−matrix binding constant (K_{AX}) may therefore be obtained from the slope of the plot of $R_p/\bar{C}_A$ *versus* R_p.

The equilibrium constant for the interaction between IL-6 and sIL-6R in solution has been obtained from competitive binding studies in which the receptor solution flowing across the sensor plate was supplemented with known total concentrations of IL-6, thereby decreasing the free concentration of receptor available for interaction with the immobilized IL-6 on the sensor plate, and hence decreasing R_p. The results have been analyzed in terms of eq 3, but with K_{AX} replaced by $\bar{K}_{AX}$, a constitutive binding constant related to K_{AX} by the expression (Winzor, 1992; Winzor & Jackson, 1993):

$$\bar{K}_{AX} = K_{AX}/(1 + K_{AS}C_S) \quad (4)$$

where, in the event that S is multivalent (valence q), C_S denotes the equilibrium concentration of competing IL-6 sites, assumed equivalent and independent in their interaction with receptor (A) governed by intrinsic binding constant K_{AS}. On the grounds that the ratio of experimental constants for the receptor−matrix interaction, $Q = K_{AX}/\bar{K}_{AX}$, is described in terms of the total concentration of competing ligand, $\bar{C}_S$, by (Hogg et al., 1991; Winzor et al., 1992; Winzor & Jackson, 1993)

$$Q = 1 + K_{AS}\{q\bar{C}_S - [(Q - 1)/Q]\bar{C}_A\} \quad (5)$$

K_{AS} has been evaluated from the slope of a plot of Q *versus* $\{q\bar{C}_S - [(Q - 1)/Q]\bar{C}_A\}$ (Hogg et al., 1991).

Analysis of Kinetic Data. Association and dissociation rate constants were determined from the progress curves for adsorption and desorption of receptor on the basis of the integrated form of the relevant rate equation for pseudo-first-order kinetics (O'Shannessy et al., 1993). These nonlinear regression analyses were achieved via the SigmaPlot program, which employs the Levenberg−Marquardt algorithm (Marquardt, 1963) for iterative curve-fitting.

RESULTS

Purification of sIL-6R. Recombinant sIL-6R was purified from CHO cell supernatant by a combination of affinity chromatography and RP-HPLC. The mutant (N63/K)IL-6 was used as the immobilized ligand for affinity chromatography because it could be prepared in much higher yield (16 mg/L) as opposed to 3 mg/L for wild-type recombinant IL-6 when using the pUC8 expression vector. The bioactivity of the mutant material is comparable with that of IL-6 in the 7TD1 hybridoma growth factor assay (data not shown).

After fractionation of the CHO cell supernatant by affinity chromatography, the partially purified sIL-6R preparation was subjected to RP-HPLC on a Brownlee RP-300 column. The major peak eluting from this column was identified by N-terminal sequence analysis as sIL-6R (Figure 1): the minor peak was bovine serum albumin. The purity of the sIL-6R was greater than 95% as judged by silver staining and immunoblotting of SDS/PAGE (inset, Figure 1). However, size-exclusion chromatography on Zorbax GP-450 yielded

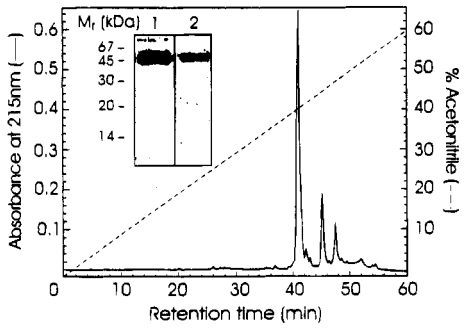

FIGURE 1: Fractionation of recombinant sIL-6R, after affinity chromatography on immobilized (N63/K)IL-6, by RP-HPLC on a Brownlee RP-300 column with a linear gradient of 0−60% acetonitrile in 0.1% aqueous TFA. Inset: SDS/PAGE analysis of the purified receptor using Western blotting (lane 1) and silver staining (lane 2) to detect the sIL-6R.

two fractions (molecular weights of 50 000 and >100 000), both of which corresponded to sIL-6R on the basis of their identical SDS/PAGE and Western-blotting behavior (data not shown). Since rechromatography of the isolated fractions precluded the existence of an association equilibrium between the major component and an aggregate thereof, the smaller form was used for all subsequent studies. The apparent molecular weight of 50 000 for this component that is determined by SDS/PAGE and size-exclusion chromatography is considerably higher than the value of 36 370 calculated from the amino acid sequence. This difference in molecular weight presumably reflects glycosylation of the CHO cell-derived sIL-6R, a concept consistent with the relative broadness of the band observed in SDS/PAGE (Figure 1). The purified sIL-6R was bioactive as determined by its ability, in the presence of IL-6, to prevent differentiation of embryonic stem cells (data not shown).

Molecular Weights of IL-6 and sIL-6R in Solution. Analysis of the sIL-6R by sedimentation equilibrium indicated homogeneity with respect to molecular weight as judged by the essentially linear dependence of the logarithm of absorbance upon the square of radial distance (Figure 2A). The slope of this plot signifies a value of 1.75 (±0.05) × 10^4 for $M(1 - \bar{\nu}\varrho_s)$, the product of the molecular weight (M) and the buoyancy factor in which ϱ_s is the buffer density (Wills et al., 1993) and $\bar{\nu}$ the partial specific volume: a value of 63 000 for M results from assigning $\bar{\nu}$ the value of 0.72 mL/g inferred from amino acid composition. However, as aready noted, the isolated receptor is believed to be glycosylated, in which case $\bar{\nu}$ is an appropriately weighted average of the partial specific volumes of the protein and carbohydrate (approximately 0.60 mL/g) components. For a 36 370 kDa polypeptide chain, we note that magnitudes of 53 000 for M and 0.67 mL/g for $\bar{\nu}$ satisfy the requirement that $\bar{\nu} = [0.72(36370) + 0.60(M - 36370)]/M$. Although a more definitive estimate of the molecular weight by sedimentation equilibrium must clearly await the experimental determination of the partial specific volume of sIL-6R, the exact value is not crucial to the binding studies which follow, because the concentrations of receptor are based on amino acid composition. The important feature to emerge from Figure 2A is that the molecular weight of the native sIL-6R agrees well with the value obtained by SDS/PAGE, and that the receptor therefore exhibits no quaternary structure. A valence of unity ($f = 1$) was therefore assigned to sIL-6R in its interaction with IL-6.

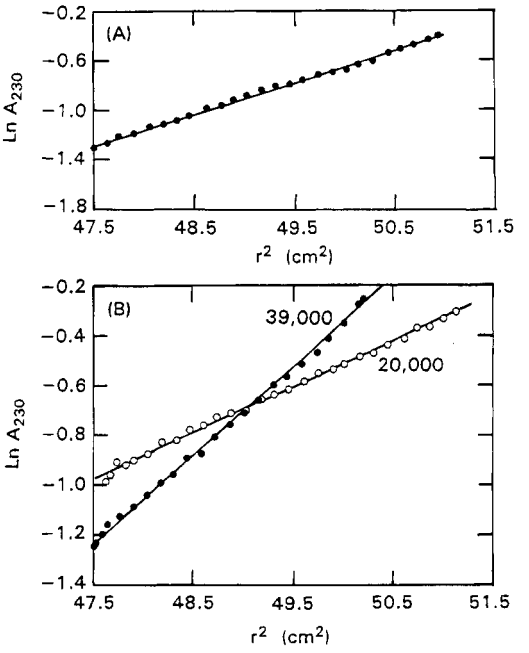

FIGURE 2: Determination of the molecular sizes of recombinant sIL-6R and recombinant IL-6 by sedimentation equilibrium (pH 7.4, I 0.176). (A) Linear transform of the equilibrium distribution obtained for sIL-6R at 8000 rpm. (B) Corresponding plots of data obtained at 12 000 rpm for the two IL-6 preparations, together with the theoretical relationships for single species with $\bar{\nu} = 0.72$ mL/g and the indicated molecular weights.

The molecular weights of the two preparations of recombinant IL-6 have also been determined by sedimentation equilibrium (Figure 2B). For the preparation expressed using the pUC8 vector, the sedimentation equilibrium distribution (●) is reasonably described by that of a single species with $M = 39\,000$ (±1000). On the grounds that the polypeptide chain corresponds to a 21 000 kDa species, we conclude that this preparation of IL-6 is predominantly a dimer with two sites for sIL-6R. The other recombinant receptor preparation (○, Figure 2B) exhibited the sedimentation equilibrium characteristics of a monomer ($M = 20\,200 \pm 800$), which should be univalent in its interaction with sIL-6R.

Binding of IL-6 to Soluble IL-6 Receptor. Before subjecting results obtained by the SPR technique to thermodynamic analysis, it was necessary to ascertain whether the measured responses reflected equilibrium between bound and free sIL-6R states. Specifically, the point at issue was whether the experimental traces in Figure 3 approach a limiting signal (dependent on the receptor concentration flowing across the sensor plate) before decay in response to the onset of an automatic washing step at the conclusion of the 35 μL injection. It is possible to inject up to 50 μL of sample into the BIAcore instrument, but gradual decreases in the response signal were observed during the final stages of the application (presumably a consequence of sample dilution due to mixing with the trailing buffer). Because application was terminated at 35 μL to circumvent this problem, it was necessary to confirm that the plateau response obtained with a 35 μL injection volume reflected equilibrium between adsorbed receptor and the concentration of free sIL-6R present in the injected sample. The first application was therefore followed immediately by a second 35 μL injection of the same solution. Unfortunately, the design of the instrument is such that sample application is terminated by injection of buffer, a phenomenon that is reflected in the resultant sensorgram

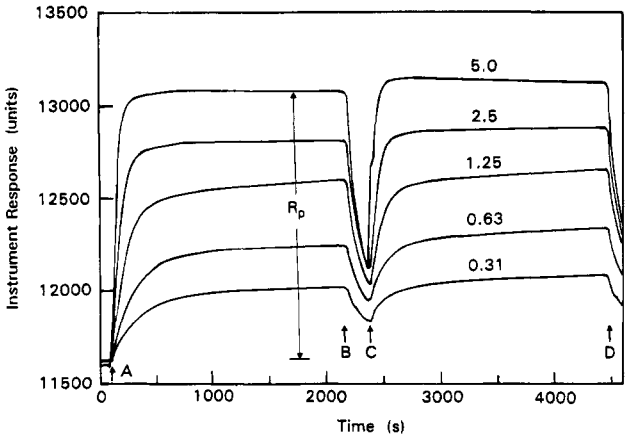

FIGURE 3: Concentration dependence of the binding of sIL-6R to immobilized IL-6 as monitored by surface plasmon resonance. Solutions (35 μL) with varying concentrations (μg/mL) of sIL-6R were injected at a flow rate of 1 μL/min. Arrows A and B denote the commencement and end of the first injection, and arrows C and D are the corresponding points relating to injection of a second sample of the same solution. R_p signifies a typical plateau response used in the application of eq 3 to characterize the interaction between receptor and immobilized IL-6.

as a decreased response before the effects of sample re-injection are manifested (Figure 3). On the grounds that the plateau responses are reasonably similar, we concluded that the plateau response for a single 35 μL injection of sIL-6R could be combined with the composition of the injected solution for thermodynamic characterization of the receptor–matrix interaction.

Figure 4a presents the Scatchard plot (eq 3) of results obtained by flowing a range of concentrations of sIL-6R across a biosensor chip to which hIL-6 had been attached covalently. The slope signifies an intrinsic binding constant (K_{AX}) of 2.4 ($\pm$0.2) $\times$ 10^7 M^{-1} (a dissociation constant of 42 nM), whereas the abscissa intercept implies a binding capacity ($B\bar{C}_X$) of 2200 ($\pm$100) response units. These parameters emanate from nonlinear regression analysis of the untransformed results in terms of the rectangular hyperbolic dependence of R_p upon sIL-6R concentration. Although an equilibrium constant for the interaction of receptor with IL-6 has been determined by the above means, the magnitude of K_{AX} does not necessarily reflect the affinity constant for the interaction between these two species in solution. First, K_{AX} refers to the interaction of sIL-6R with an immobilized and therefore chemically modified form of IL-6; and secondly, it is only an effective thermodynamic parameter based on the premise that matrix sites are distributed uniformly throughout the volume accessible to partitioning receptor (Nichol et al., 1974). A comparable value (6×10^7 M^{-1}) for K_{AX} is calculated from the ratio of association and dissociation rate constants [4.5 ($\pm$0.5) $\times$ 10^5 M^{-1} s^{-1} and 8.0 ($\pm$0.8) $\times$ 10^{-3} s^{-1}, respectively].

The required equilibrium constant for the interaction between IL-6 and recombinant receptor in solution must be obtained from competitive binding studies in which the receptor solution injected into the biosensor instrument is supplemented with known total concentrations of IL-6. This aspect of the characterization is summarized in Figure 4b, where the results for the dimeric IL-6 (●) are plotted according to eq 5 with $\bar{C}_S$ based on M = 42 000 and q, the valence of the competing ligand, taken as 2 on the bais of the dimeric nature of the IL-6 preparation (Figure 2B): an

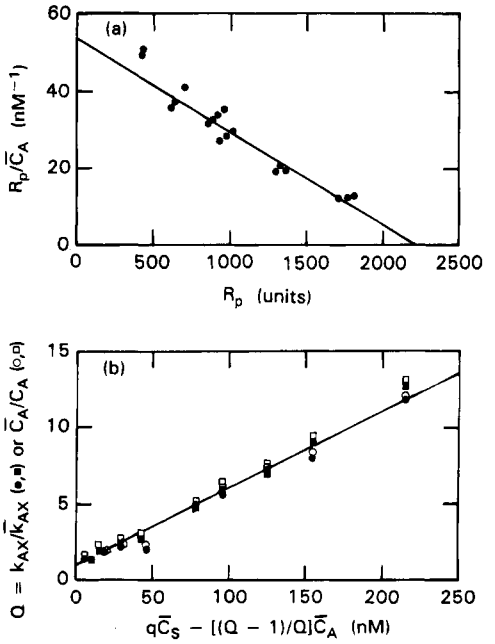

FIGURE 4: Characterization of the interaction between IL-6 and sIL-6R by means of SPR and a biosensor chip with IL-6 as immobilized affinity ligand. (a) Evaluation of the binding constant for the interaction of sIL-6R with the immobilized IL-6 (K_{AX}) by analyzing results (Figure 3) in terms of eq 3. (b) Determination of the binding constant for the interaction between sIL-6R and IL-6 in solution (K_{AS}) by including the latter component with the receptor in the injected sample. Results for dimeric IL-6 (●) and monomeric IL-6 (■) preparations are plotted according to eq 5 with $f = 2$ and 1, respectively, and with Q taken as $K_{AX}/\bar{K}_{AX}$. Open symbols refer to an alternative analysis based on a calibration plot (Figure 5) and recognition of Q as $\bar{C}_A/C_A$ (see text). The solid line is the theoretical relationship for $K_{AS} = 5.0 \times 10^7$ M^{-1}, the mean of values for the two IL-6 preparations.

intrinsic binding constant (K_{AS}) of 4.8 ($\pm$0.3) $\times$ 10^7 M^{-1} was obtained from the slope. An essentially identical estimate, 5.2 ($\pm$0.3) $\times$ 10^7 M^{-1}, was obtained from a corresponding experiment (■) with the monomeric IL-6 preparation, for which M = 21 000 and $q = 1$. These binding constants characterize unequivocally the interactions between the IL-6 preparations and sIL-6R in solution, and are not merely relative values that are dependent upon the correctness of the model used for characterizing the receptor–matrix interaction—a misconception that we shall now endeavor to dispel.

On the basis that the use of eq 5 to obtain K_{AS} requires values of K_{AX} and $\bar{K}_{AX}$, it may appear, at first sight, that the method is invalid if the model of the receptor–matrix interaction were to provide an inappropriate physical description of the absorption/desorption phenomenon. However, irrespective of any inadequacies on physical grounds, these two constants in conjunction with $\bar{C}_X$ (or $B\bar{C}_X$) do provide a valid thermodynamic description of the distribution of sIL-6R (the partitioning solute, A) between solution and adsorbed (matrix-bound) states. Indeed, it is readily shown that the ratio $Q = k_{AX}/\bar{k}_{AX}$ is measuring the ratio $\bar{C}_A/C_A$, which can also be measured (for a univalent partitioning solute) without resort to any model of the affinity chromatographic process.

Instead of using the results obtained in the absence of competing IL-6 to define the magnitude of K_{AX} (Figure 4a), we now choose to regard the experimentally determined dependence of biosensor response upon sIL-6R concentration

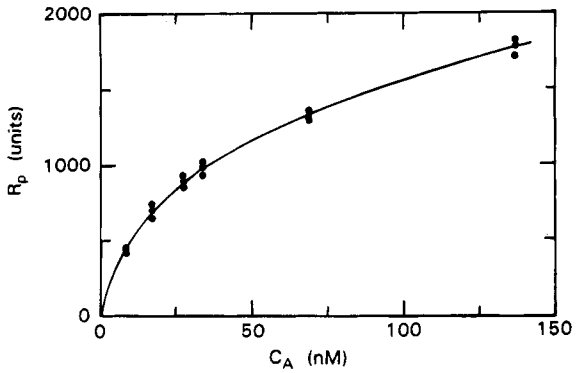

FIGURE 5: Alternative presentation of the results contained in Figures 3 and 4a as a calibration plot for evaluating the free concentration of sIL-6R (C_A) in IL-6−receptor mixtures from the magnitude of the biosensor response (R_p) as a function of sIL-6R concentration (C_A). The value of C_A so obtained is then combined with the total receptor concentration ($\bar{C}_A$) to obtain Q as $\bar{C}_A/C_A$ for analysis of results in terms of eq 5 (open symbols in Figure 4b).

(Figure 5) as a calibration plot whereby the concentration of free receptor in equilibrium with the immobilized IL-6 affinity matrix may be assessed from the magnitude of the biosensor response. Combination of the consequent value of C_A with $\bar{C}_A$, the total concentration of sIL-6R in any injected mixture of receptor and IL-6, yields Q and hence allows the interaction to be characterized as before via eq 5. As required, interpretation of the present results by this method yields a plot of Q *versus* $\{q\bar{C}_S - [(Q - 1)/Q]\bar{C}_A\}$ (open symbols, Figure 4b) that is indistinguishable from that used for characterization of the interaction via thermodynamic analysis of the competition between free and immobilized forms of IL-6 for receptor.

DISCUSSION

The use of existing quantitative affinity chromatography theory (Nichol et al., 1974, 1981; Hogg & Winzor, 1984; Hogg et al., 1991; Winzor et al., 1992) to characterize the interaction of IL-6 with sIL-6R has served to emphasize its relevance to the measurement of binding constants from the information provided by biosensor technology. This method of analysis is superior to that based on kinetic analysis (Karlsson et al., 1991; Chaiken et al., 1991; Altschuh et al., 1992; Zeder-Lutz et al., 1993; O'Shannessy et al., 1993), because the kinetic measurements only yield K_{AX}, which does not necessarily provide a reliable estimate of the affinity between solute and ligand in solution. By performing the competition experiment with IL-6 as inhibitor of the interaction between sIL-6R and immobilized IL-6, it has been possible to measure K_{AS} for the competing interaction, which is the equilibrium constant of greater interest in the biological context.

Furthermore, an alternative method of analyzing the experimental results has emphasized that affinity chromatography, either as such or in the guise of a solid-phase ELISA (Hogg et al., 1987a), can afford an unequivocal means of characterizing a competing interaction in the liquid phase. For the particular system examined, it transpires that the equilibrium constant for the competitive interaction in solution (K_{AS}) is of similar magnitude to that determined for the adsorption phenomenon (K_{AX}) either thermodynamically or kinetically. However, because such agreement between estimates of K_{AX} and K_{AS} is likely to be the exception rather than the norm, the competitive binding segment should be included in biosensor-based studies of ligand binding to eliminate the assumption that the thermodynamics of the biphasic absorption phenomenon necessarily pertain to the corresponding reaction in the solution phase.

The interaction of IL-6 with either the IL-6R in cells (Taga et al., 1987, 1989; Gearing et al., 1992) or the sIL-6R in solution has been studied in a number of laboratories. Both high- and low-affinity binding sites for IL-6 have been detected on a variety of cells (Coulie et al., 1987, 1989), the former being associated with the IL-6R/gp-130 complex, and the low-affinity sites with IL-6R (Taga & Kishimoto, 1992). Dissociation constants ranging from 0.7 nM (Yamasaki et al., 1988) to 35 nM (Gearing et al., 1992) have been reported for the low-affinity binding site on cells, whereas values of 1 nM (Taga et al., 1989) to 5 nM (Yasukawa et al., 1990) have been measured for the interaction between IL-6 and sIL-6R in solution. These values are of the same order of magnitude as, but smaller than, the value of 20 nM measured in this study. This discrepancy may, in part, be explained by the fact that most investigations of sIL-6R/IL-6 binding have employed solid-phase assays with sIL-6R immobilized to the plate surface (Yasukawa et al., 1990). Consequently, the lower value of the dissociation constant inferred from those solid-phase assays may reflect failure to consider the question of IL-6 valence in its reaction with receptor. As shown in Figure 2B, IL-6 produced using the pUC vector system is predominantly a dimer and hence possesses two sites for interaction with the sIL-6R. The dimeric nature of that preparation of IL-6 (but not the other) could reflect self-association through the nine additional N-terminal amino acids derived from β-galactosidase and the polylinker region of pUC8. Alternatively, because the purification of both constructs involved solubilization of "inclusion body" material with denaturants, subtle changes in refolding conditions may have influenced the pathway of refolding. In this regard, it may be relevant that pUC9-derived murine IL-6 which had been purified (Zhang et al., 1992) by an identical procedure to that adopted for human IL-6 also exhibited a tendency to aggregate under conditions of moderate ionic strength (Ward et al., 1993b). Although murine IL-6 is monomeric at low ionic strength ($I < 0.01$), human IL-6 is essentially dimeric under all conditions (pH 4−7.4, I = 0.005−0.20), even at concentrations in the vicinity of 10 μg/mL: relatively strong interactions are thus responsible for the quaternary structure of human IL-6. The subunit interactions responsible for dimer formation do not affect significantly the binding of IL-6 to sIL-6R, for which the two equivalent and independent sites on dimeric IL-6 exhibit an intrinsic binding constant comparable with that exhibited by the single site on the monomeric preparation of IL-6 (Figure 4b).

Multimeric forms of a natural fibroblast-derived IL-6 have been reported by May et al. (1991), who observed an 85-kDa oligomer as well as minor amounts of 45- and 65-kDa forms: no monomeric form of IL-6 was detected. Furthermore, the interaction of IL-6 dimers with IL-6R on cells has been observed by standard covalent cross-linking techniques—a finding taken to imply that ligand dimerization may play a role in the IL-6 signalling mechanism by cross-linking IL-6R molecules on the cell surface (Rose-John et al., 1990). Studies are therefore under way to establish whether ligand dimerization plays any role in the formation of the high-

affinity complex between IL-6 and the IL-6R/gp-130 complex. In that regard, the availability of the ligand in monomeric and dimeric forms clearly provides a convenient starting point for characterizing the cellular binding phenomena.

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