Published in final edited form as:

Biochemistry. 2010 June 29; 49(25): 5117–5133. doi:10.1021/bi100041f.

Inter-molecular interactions in a 44 kDa interferon-receptor complex detected by asymmetric reverse-protonation and 2D NOESY[†]

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Abstract

Type I Interferons (IFNs) are a family of homologous helical cytokines initiating strong anti-viral and anti-proliferative activity. All type I IFNs bind to a common cell surface receptor consisting of two subunits, IFNAR1 and IFNAR2, associating upon binding of interferon. We studied inter-molecular interactions between IFNAR2-EC and IFN α 2 using asymmetric reverse-protonation of the different complex components and 2D homonuclear NOESY. This new approach revealed with excellent signal-to-noise ratio 24 new intermolecular NOEs between the two molecules despite the low concentration of the complex (0.25 mM) and its high molecular weight (44 kDa). Sequential and side-chain assignment of IFNAR2-EC and IFN α 2 in their binary complex helped assign the inter-molecular NOEs to the corresponding protons. A docking model of the IFNAR2-EC/IFN α 2 complex was calculated based on the inter-molecular interactions found in the present study as well as four double mutant cycle constraints, previously observed NOEs between a single pair of residues and the NMR mapping of the binding sites on IFNAR2-EC and IFN α 2. Our docking model doubles the buried surface area of the previous model and significantly increases the number of inter-molecular hydrogen bonds, salt bridges and Van der-Waals interactions. Furthermore, the current model reveals participation of several new regions in the binding site such as the N-terminus and A-helix of IFN α 2 and the C-domain of IFNAR2-EC. As a result of these additions, the orientation of IFNAR2-EC relative to IFN α 2 has changed by 30° in comparison with a previously calculated model that was based on NMR mapping of the binding sites and double mutant cycle constraints. In addition, the new model strongly supports the recently proposed allosteric changes in IFN α 2 upon IFNAR1-EC binding to the binary IFN α 2/IFNAR2-EC complex.

Type I IFNs are a major component of the innate immune system protecting against viral infection. They provide an early line of defense, hours to days ahead of the adaptive immune

[†]This study was supported by the Israel Science Foundation, NIH Grant GM53329 and the Kimmelman Center. J.A. is the Dr. Joseph and Ruth Owades Professor of Chemistry.

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Supporting Information Available

Figure showing the elimination of very slowly-exchanging amide protons from the 2D NOESY spectra of asymmetrically labeled IFNAR2/IFN α 2 complexes.

This material is available free of charge via the Internet at <http://pubs.acs.org>.

response, and are essential for the survival of higher vertebrates (1–3). In addition to a strong anti-viral activity, type I IFNs are also attributed anti-proliferative and immunomodulatory properties (4,5).

All human type I IFNs elicit their activity through the same cell surface receptor consisting of two trans-membranal protein subunits, IFNAR1 and IFNAR2 (6,7). IFNAR2 is the major ligand binding component and has nM affinity to IFNs without the presence of IFNAR1. The affinity of the IFNAR1 subunit to IFNs is much lower and the dissociation constant is in the μ M range (8). The IFN signaling process begins with IFN driven dimerization of the receptor subunits which initiates a tyrosine phosphorylation cascade inside the cell resulting in transcription stimulation of genes responsible for the anti-viral and anti-proliferative response (9–14).

The three-dimensional structures of several IFNs namely, human IFN α 2a (15), IFN α 2b (16) and IFN β (17) were solved. The structure of the 25 kDa extra-cellular domain of IFNAR2, was solved in our group using multidimensional NMR techniques (18). Mutagenesis, immunoblocking and various NMR techniques were used to obtain information about residues in IFN α 2 and IFNAR2-EC (from here on referred to as IFNAR2) which participate in the binding (19–23). The binding site on IFNAR2 was mapped to three loops in the N domain and the hinge region of the receptor. The binding site on IFN α 2 was mapped to the A and E helices as well as the AB loop. The binding affinity of the IFN α 2/IFNAR2 complex is approximately 1 nM (24).

Several models of the IFNAR2/IFN α 2 complex have been computed over the recent years. Roisman *et al.* calculated a docking model of IFN α 2 with modeled structure of IFNAR2-EC using site-directed-mutagenesis mapping of the binding sites as well as 5 double mutant cycle (DMC) distance constraints (25). When the NMR structure of IFNAR2 has become available, Chill *et al.* calculated another docking model based on the 4 most significant out of the 5 previously identified DMC constraints (18). The fifth DMC interaction, between R²Y43 on IFNAR2 and α ²F27 on IFN α 2, was omitted from the calculation because it was incompatible with the IFNAR2 NMR structure (18). The latest docking model of the IFNAR2/IFN α 2 complex was derived by Quadt-Akabayov *et al.* based on the NMR mapping of the binding sites on IFNAR2 and IFN α 2, 4 DMC constraints and a single inter-molecular NOE (26). The DMC data provided qualitative information about interactions that could be translated to distance constraints only very roughly. The DMC constraints, together with the observed NOEs between a single pair of residues and the NMR mapping of the binding sites are not sufficient to define accurately the orientation of the two molecules in the complex.

In the present study we conducted high resolution NMR investigations of the IFNAR2/IFN α 2 complex in order to detect a larger number of inter-molecular NOE interactions to better define the structure of the IFNAR2/IFN α 2 complex. Acquiring such information about the 44 kDa IFNAR2/IFN α 2 complex by NMR has presented considerable challenges due to its size and the conditions in which it is stable.

The size of biomolecules studied by NMR is limited by the enhanced transverse relaxation of carbon and hydrogen nuclei and increasing spectral complexity. In the past 10–15 years several techniques have been introduced to help overcome these limitations. ¹³C and ¹⁵N labeling, higher dimensionality spectra (27) as well as uniform deuteration of non-exchangeable protons (28,29), and TROSY-based triple-resonance experiments (30) have enabled sequential assignment of backbone nuclei of large proteins and macromolecular complexes. Backbone (HN, C α and C β) resonance assignment of uniformly ²H, ¹³C, ¹⁵N labeled proteins up to a molecular weight of 100 kDa has been achieved (31,32). However,

uniform deuteration of non-labile protons, while vastly contributing to the increased signal-to-noise ratio in NMR spectra, eliminates all the proton probes giving rise to NOE interactions, except for the labile amide protons. Reverse protonation of uniformly deuterated proteins has been applied to regain distance information in high molecular weight systems (32–34). Several approaches have been used to selectively reverse-label protons in proteins. One way is to supplement the D₂O based bacterial growth medium with the desired ¹H- or ¹H/¹⁵N- or ¹H/¹⁵N/¹³C-labeled amino acids. Reverse-protonation of a combination of aliphatic and aromatic residues such as ILVIFY has been used in several NMR studies of large proteins and protein complexes to detect intra-molecular aromatic-methyl NOEs instrumental to their structure determination as well as to the backbone and side chain assignment (33–42). An additional method for selective reverse-protonation is ¹H/¹³C labeling of the methyl-groups of isoleucine, leucine and valine by adding specifically labeled biosynthetic precursors to the growth medium (31,43,44). Low resolution structures of large proteins can be obtained using this approach as was demonstrated by the determination of the global fold of the monomeric 82 kDa protein malate synthase G (45).

Despite all recent advances, high resolution structure determination by NMR of proteins larger than 35 kDa is very challenging. Out of 6,124 different protein structures determined by NMR currently deposited in the PDB, only 14 are atomic resolution structures of systems larger than 35 kDa. Four of these are proteins and protein complexes smaller than 37 kDa (40,42,46,47), 9 are multimeric proteins (35,48–55), and the remaining one is the extensively studied by crystallography and NMR 42 kDa maltose binding protein (MBP) (55). To date, the 65 kDa hemoglobin tetramer is the highest molecular weight protein complex the high-resolution structure of which was solved by NMR (55). This structure, along with the previously mentioned MBP structure were recently re-determined by Yang and coworkers using their approach for the structure determination of fully protonated large proteins (55). This method is based on measuring a combination of 4D-¹³C, ¹⁵N separated NOESY, 3D TROSY-HNCA and MQ-CCH-TOCSY (56) spectra on a uniformly ¹³C, ¹⁵N labeled protein without deuteration. The 4D-¹³C, ¹⁵N separated NOESY experiment when applied to large unlabeled proteins suffers from poor signal-to-noise ratio especially in the low protein concentration used in the present study (0.25 mM). Without this 4D spectrum, it is impossible to implement the above method as it was originally developed. However, several basic concepts from it have been used in this study.

Structure determination of protein complexes at atomic resolution relies on obtaining a large number of inter-molecular NOEs. Obtaining inter-molecular NOEs is a challenging process since it is difficult to distinguish between strong intra-molecular and a few weak inter-molecular interactions, a problem which is aggravated in large molecules. One way to overcome the obstacle in identifying inter-molecular interactions is to label one of the components with ¹⁵N and/or ¹³C while the second component remains unlabeled. Using isotope filtered-edited experiments, it is possible to identify NOEs across interfaces between two protons, where only one is bound to a labeled heteroatom (57–61). However, with increasing size of protein complexes, these experiments become much less sensitive and require partial or complete deuteration of non-exchangeable protons to achieve a good S/N ratio.

A different approach for the detection of inter-molecular NOEs in large protein complexes was used in several studies recently. It is based on selective ¹H/¹³C labeling of the I(δ1)LV methyl groups on a deuterated background of one complex component while the other component remains unlabeled or reverse-protonated with several aromatic and/or aliphatic amino acids. The inter-molecular NOEs are obtained from a 3D ¹³C-edited NOESY spectrum by observing the NOEs involving the I(δ1)LV methyl protons (46,62–64).

High resolution NMR structural studies of the IFNAR2/IFN α 2 interactions required further development in methodology, not only due to the size of the complex, but also since it is stable only at concentrations no higher than 0.25 mM. In this study we present a novel approach for determination of inter-molecular side chain – side chain NOEs. Our method is based upon “asymmetric” reverse-protonation of the two complex components on a deuterated background. In one molecule, amino acids with aliphatic side chains are reverse-protonated and in the other molecule aromatic amino acids are reverse-protonated. As a result, the aromatic-aliphatic section of the 2D homonuclear NOESY spectrum measured in D₂O shows in principle only cross-peaks originating from inter-molecular interactions. The high sensitivity of the 2D NOESY spectrum, even for large proteins at concentrations as low as 0.25 mM, enables the detection of these interactions with high signal to noise ratio. Using this technique we have been able to obtain 24 inter-molecular NOEs which led to a much improved model of the IFNAR2/IFN α 2 complex. The model contains a considerable amount of new structural information and reveals a 30° change in the orientation of IFNAR2 relative to IFN α 2 in comparison with the previously published model (26) as well as a direct involvement of the C-domain of IFNAR2 in IFN α 2 binding.

Experimental Procedures

Expression of IFNAR2 and IFN α 2

Unlabeled and uniformly ¹⁵N, ¹³C labeled IFNAR2 and IFN α 2 were obtained following previously published expression and purification protocols (19,26,65). Selectively reverse-protonated samples were obtained by adding 4-fold excess of the selected unlabeled amino acids to otherwise deuterated, ¹⁵N-labeled Celtone medium (Spectra Stable Isotopes). The 4-fold excess was calculated with respect to the amount of the amino acids present in the deuterated, ¹⁵N-labeled Celtone medium. Specifically, unlabeled amino acids His (1.35 mg/ml), Tyr (1.2 mg/ml), Phe (2.4 mg/ml) and Trp (0.61 mg/ml) were used for samples with protonated aromatic residues and Ile (2.9 mg/ml), Val (3.36 mg/ml), Leu (4.57 mg/ml), Met (1.19 mg/ml), Ala (2.9 mg/ml), Lys (4.24 mg/ml), and Thr (2.44 mg/ml) for samples with protonated aliphatic residues. Otherwise, the protocols for expression and purification were used as mentioned above.

NMR sample preparation

IFNAR2/IFN α 2 complex as well as the free IFNAR2 is stable at pH 8, 25 mM Tris buffer, 0.02% NaN₃. These measurement conditions are the result of an optimization study which was previously carried out in our group (19). IFN α 2 is stable at pH 8 only in at least 500 mM NaCl solution. In order to make the 1:1 complex (in 10% excess for the unlabeled component), 5 ml of concentrated IFN α 2 (500 mM NaCl) was slowly added to 145 ml of dilute IFNAR2 (25 mM NaCl). The volumes and salt concentrations of each component were calculated such that the final salt concentration did not exceed 50 mM. After incubation for 1–2 h at room temperature the dilute complex was concentrated using Amicon Ultra tubes (Millipore, MW cutoff 10 kDa). The buffer was exchanged by repeated dilution/concentration cycles to 25 mM d₁₁-Tris pH 8, 0.02% NaN₃, 5% D₂O or to 25 mM d₁₁-Tris pH 8, 0.02% NaN₃, 98% D₂O. Formation of the complex was verified using an analytical Superdex 75 size exclusion column (Pharmacia). The complex elutes at a volume corresponding to an approximately 44 kDa protein. The final concentration of the complex in all samples was 0.2–0.3 mM. Under these conditions the NMR samples are stable for 2–3 months at 35 °C, with negligible aggregation or denaturation (19).

Deuterium exchange for several IFNAR2/IFN α 2 samples was achieved by partial unfolding in 4M d₄-urea. For this procedure the complex was dissolved in a D₂O based unfolding buffer (25 mM tris pH 8.0, 4M d₄-urea, 0.1% protease inhibitor cocktail (Sigma) and 50

mM Glycylglycine) to a final concentration of 0.1 mg/ml, incubated for 4 hrs at room temperature while shaking gently and refolded by dialyzing twice against a 25-fold volume of D₂O based refolding buffer (25 mM tris pH 8.0, 0.1% protease inhibitor cocktail (Sigma) and 50 mM Glycylglycine). In order to check that the structural integrity of the complex is not compromised by the partial unfolding and refolding procedures, these were performed first on ¹⁵N-IFNAR2/IFNα2 and ¹⁵N-IFNα2/IFNAR2 samples in H₂O and the structural integrity was checked by measuring the 2D TROSY [¹H,¹⁵N] HSQC spectra before and after the treatment as well as by size exclusion chromatography.

NMR measurements

All NMR spectra were acquired at 305 K on a Bruker DRX 800 MHz spectrometer equipped with a 5 mm triple resonance TCI CryoProbe with z gradients and a 5 mm z-gradient triple resonance TXI probe. Data were processed and analyzed using the NMRDraw/NMRPipe (66) and NMRView (67) software packages.

The 2D TROSY [¹H¹⁵N] HSQC spectra of the free and bound IFNAR2 (free and bound IFNα2) were acquired using 256 (256) *t*₁ increments with a sweep width of 1979 (1622) Hz and 1280 (1024) *t*₂ points with a sweep width of 11,161 (10,417) Hz. The TROSY [¹H¹⁵N] HSQC pulse sequence (68) was modified in order to obtain greater sensitivity by suppressing the anti-TROSY component without lengthening the pulse sequence or introducing a spin-state selective filter (69). The following experiments were carried out for the bound IFNAR2 (numbers in parenthesis indicate the number of complex points and sweep width in Hz for each dimension): CT-[¹H¹³C] HSQC (H: 1280/10,417; C: 512/14,085), 3D TROSY HNCA (55,68,70) (H: 1024/11,161; C: 50/4024; N: 106/2108), 3D TROSY HNCOC (68,70) (H: 1280/11,161; C: 50/4024; N: 100/1979), 3D ¹⁵N-edited NOESY (H: 1280/11,161; H: 148/8803; N: 78/1979), 3D MQ-(H)CCH-TOCSY (71) (H: 1280/11,161; C: 68/4427; C: 182/13,280), 3D MQ-(H)CCmHm-TOCSY (72) (H: 1280/8013; C: 122/2516; C: 140/8299), 3D (H)CCmHm-TOCSY (71) (H: 1280/7003; C: 122/2516; C: 140/7003). The following experiments were carried out for the free IFNAR2: CT-[¹H¹³C] HSQC (H: 2048/10,417; C: 512/14,085), 3D ¹⁵N-edited NOESY (H: 768/11,161; H: 160/8237; N: 72/1946), 3D MQ-(H)CCmHm-TOCSY (72) (H: 1280/8013; C: 120/2516; C: 136/8299). The following experiments were carried out for the bound and free IFNα2: 3D ¹⁵N-edited NOESY (bound IFNα2: H: 1280/11,161; H: 146/8803; N: 78/1622; free IFNα2: H: 1024/9615; H: 140/8001; N: 72/1622). 2D NOESY experiments for all samples were acquired using 400 *t*₁ increments with a sweep width of 10,417 Hz and 1024 *t*₂ points with a sweep width of 10,417 Hz.

An inter-scan delay of 1.5 s was applied in experiments performed on fully protonated samples and a delay of 2 s was used for partially deuterated samples. The 3D ¹⁵N-edited NOESY experiments were acquired with a mixing time of 100 ms while the 2D NOESY experiments were measured with mixing times of 25–200 ms. Water suppression was achieved with the use of flip-back and gradient pulses (73,74). Residual water magnetization was suppressed using the WATERGATE sequence in the final INEPT transfer to protons immediately before data acquisition (75). All experiments were optimized for large proteins by applying minimum delays for transverse ¹H magnetization and optimal water flip-back pulses. Carrier positions for all experiments were 118.5 ppm for ¹⁵N, 174 ppm for ¹³CO, 54 ppm for ¹³Ca, 43 ppm for ¹³Ca/¹³Cβ and 4.7 ppm for ¹H. Proton decoupling was achieved using WALTZ-16 (76) or GARP (77) train pulses. Quadrature detection was obtained by acquiring the data in States-TPPI mode (78) or Echo-Antiecho (79,80).

Docking

The docking of the IFNAR2/IFN α 2 complex was performed using the software HADDOCK2.0 (81) which utilizes CNS (82,83). The docking was based on the chemical shift perturbation data for IFNAR2, the cross saturation data for IFN α 2, NOE interactions and mutagenesis data (18,19,25,26). Starting structures for the docking were the published structure of IFNAR2 (PDB entry 1N6U) and IFN α 2 (PDB entry 1ITF (15)). Active and passive residues in IFN α 2 and IFNAR2 were selected using the strategy outlined by Dominguez *et al.* (81) based on the binding sites mapped in previous studies in our group (19,26). Solvent accessibility was calculated using the program NACCESS (84,85). Additional pairwise distance restraints were defined based on double mutant cycle analysis data found by Roisman *et al.* (25). All ambiguous distance restraints were defined with a maximum effective distance of 2 Å. A total of 1000 structures were calculated in the rigid body minimization. Semi-flexible simulated annealing followed by refinement in explicit water was performed for the best 200 solutions based on the haddock score (weighted sum of all the energy terms and the buried surface area). Violation analysis of the final 200 structures showed that all the unambiguous distance restraints were maintained for 99.5% of the structures. Solutions were clustered using a 7.5 Å interface RMSD cut-off. 197 out of 200 structures were included in the 7 clusters found. Cluster analysis was performed on the 4 best structures in each cluster to remove the dependency of cluster averages upon their size. The cluster with the lowest average HADDOCK score was considered to be the best solution.

Structure Analysis

The structure of the IFNAR2/IFN α 2 complex and the interface were analyzed with PISA (Protein interfaces, surfaces and assemblies service at European Bioinformatics Institute) (86), CMA (Contact Map Analysis from SPACE - Tools for protein Structure Prediction and Analysis based on Complementarity and Environment) (87) and MOLMOL (Analysis and display of molecules) (88). All molecular pictures were created with Pymol (89).

Results

Inter-molecular NOEs between IFNAR2 and IFN α 2 in the complex

Aromatic amino acids play an important role in protein-protein interactions. The region of the proton NMR spectrum showing aromatic protons resonances is usually less crowded and better dispersed in comparison to the aliphatic region of the spectrum. Of special interest is the region of the NOESY spectrum showing interactions between aromatic and aliphatic protons. Specific amino acid labeling can be used to assign inter-molecular interactions involving aromatic amino acids in large protein complexes as was demonstrated by us in the past (90–94). In these experiments we took advantage of the high sensitivity of 2D homonuclear NOESY even for large protein complexes.

The region of a 2D NOESY spectrum of a complex showing aromatic-aliphatic proton interactions can be considerably simplified to show only inter-molecular interactions by using asymmetric reverse labeling of the two proteins forming the complex. If one protein is reverse-protonated in the aromatic amino acids on a per-deuterated background and the other protein is reverse-protonated in selected aliphatic amino acids on a per-deuterated background, the upper left quarter of the complex 2D NOESY spectrum in D₂O will show, in principle, inter-molecular interactions between aromatic and aliphatic amino acids as well as intra-residue interactions between the aromatic protons and the α and β -protons of these amino acids. If preliminary data about the interface of the complex is available, the aliphatic amino acids can be reverse-labeled according to the expected interactions in order to reduce costs and the number of samples to be prepared.

The aromatic amino acids at the mapped binding site of IFNAR2 include residues ^{R2}H76, ^{R2}Y79, ^{R2}H97, ^{R2}F99 and ^{R2}W100, while those at the binding site of IFN α 2 include residues ^{α 2}F27, ^{α 2}F36, ^{α 2}F38, ^{α 2}W140 and ^{α 2}F151. Aromatic amino acids are usually involved in hydrophobic interactions and sometimes also in cation- π interactions with arginine and lysine residues. On the basis of previous mapping of the IFN α 2 and IFNAR2 binding sites (19,26), we prepared two samples of the IFN α 2/IFNAR2 complex. One sample was reverse-protonated with the unlabeled aromatic amino acids histidine, phenylalanine and tryptophan for IFNAR2 and reverse-protonated with the unlabeled aliphatic amino acids lysine, arginine, leucine, alanine and methionine for IFN α 2 (this sample will be referred to from here on as IFNAR2(HFW)/IFN α 2(KRLAM)). The second sample was reverse-protonated with the unlabeled aliphatic amino acids isoleucine, valine, leucine, threonine, methionine, alanine and lysine for IFNAR2 and reverse-protonated with the unlabeled aromatic amino acids histidine, phenylalanine and tryptophan for IFN α 2 (IFNAR2(IVLTMAK)/IFN α 2(HFWY)). The reverse-protonation of all proteins was done on a ¹⁵N labeled, deuterated background.

The labeling scheme was verified using ¹⁵N HSQC and 2D NOESY spectra performed on the free proteins prior to formation of the complex. No significant signs of scrambling were observed in these spectra which we attribute to the expression of the proteins in rich media in which all amino acids are essentially already present therefore their de-novo production is minimized.

2D NOESY spectra of the asymmetrically labeled samples in D₂O were measured and a large number of cross-peaks was observed in the aromatic-aliphatic region, many more than were expected based on the previous model of the complex (26) (Figure 1 and Figure 1S in the Supporting Information). The initial assumption was that many of the observed spin systems originate from amide protons that did not exchange with the solvent because of being buried. These amide protons could interact with the reverse-labeled aliphatic protons of the same protein and contribute to the observed signal in the asymmetrically labeled complex. In order to rid the aromatic region of the 2D NOESY spectrum of NOEs originating from amide protons, a rigorous D₂O exchange protocol was used. Both asymmetrically labeled samples (IFNAR2(HFW)/IFN α 2(KRLAM) and IFNAR2(IVLTMAK)/IFN α 2(HFWY)) were partially unfolded in 4M d₄-urea in D₂O solution and then refolded by dialysis against the D₂O NMR buffer. Structural integrity of the samples after refolding was confirmed by size exclusion chromatography and 2D TROSY [¹H, ¹⁵N] HSQC spectra (see Experimental Procedures) as well as by comparison of the 2D NOESY spectra before and after the urea treatment (Figure 1S in the Supporting Information). 2D NOESY spectra of IFNAR2(HFW)/IFN α 2(KRLAM) and IFNAR2(IVLTMAK)/IFN α 2(HFWY) were measured before and after the urea treatment, revealing that numerous NOE cross-peaks with amide protons disappeared (see Figure 1S in the Supporting Information).

However, despite the complete elimination of amide protons cross-peaks, several unexplained spin systems with narrow line width still remained in the 2D NOESY spectra along with spin systems which could originate from aromatic protons (Figure 1). Consequently, 2D NOESY spectra of each of the reverse-labeled complex components in their free form were measured to examine whether these narrow cross-peaks could arise from intra-molecular interactions. Surprisingly, several cross-peaks in the aromatic-aliphatic region were observed (Figure 1). As can be seen from the overlay of 2D NOESY spectra of the IFNAR2(HFW)/IFN α 2(KRLAM) complex and the free IFN α 2(KRLAM) (Figure 1A), two spin systems which appear in the aromatic-aliphatic region of both spectra, obviously result from intra-molecular NOEs of IFN α 2 and not inter-molecular NOEs between IFN α 2 and IFNAR2. These spin systems have a distinct narrow line width in comparison to other,

much broader peaks in the same spectral region. This might indicate that these NOE peaks result from isolated ^1H spins not surrounded by protons of the same residue. We postulate that while the majority of protein molecules in the IFN α 2(KRLAM) sample were ^1H labeled only as designed (i.e. only in aliphatic amino acids), a small percentage of ^1H labels were randomly incorporated in all amino acid types. This might be due to the incomplete ^2H labeling of the commercially available expression medium (97% instead of 100%). In a highly deuterated environment, even a small amount of protons will give an observable signal, simply because their transverse relaxation times are much longer and the ensuing signal is considerably higher due to its narrow line width. A second possibility is scrambling resulting from the unlabeled aliphatic amino acids which were added to the growth medium.

Following the replacement of the slowly exchanging amide protons by deuterium using urea induced partial denaturation, several new spin systems appeared in the 2D NOESY spectra of the urea treated samples (marked by light blue boxes in figure 1). A possible explanation for these cross-peaks is a minor chemical modification as a consequence of the urea induced unfolding, for example a carbamylation of lysine residues by the cyanate ion resulting from urea decomposition. Since the ^{15}N HSQC spectra of the IFN α 2/IFNAR2 complex remained the same before and after the urea treatment and so have the other cross-peaks in the 2D NOESY spectra, this modification did not interfere to any significant extent with the structure of the complex and the binding of the two proteins.

The few spin systems which remain in the aromatic–aliphatic region of the 2D NOESY spectra of the asymmetrically labeled samples after eliminating the intra-molecular NOEs and the cross-peaks resulting from the urea induced modification, are the inter-molecular NOEs between aromatic protons of IFN α 2 and aliphatic protons of IFNAR2 and vice versa. Using these NOE cross-peaks as distance restraints requires the identification of the specific protons involved in the interaction, i.e. obtaining side chain protons assignment of the IFNAR2/IFN α 2 complex. The side chain and backbone assignment of the IFNAR2/IFN α 2 complex will be discussed in the following sections.

Backbone assignment of IFNAR2 in complex with IFN α 2

The side-chain proton assignment process is not trivial for a complex of 44 kDa and is further complicated by the low concentration and high pH in which the complex is stable. While backbone assignment for proteins this size is usually obtained with per-deuterated samples, we used non-deuterated samples applying several basic principles from the assignment method recently developed by Yang and co-workers for fully protonated large proteins (55).

The assignment process began with using the 3D TROSY-HNCA and 3D TROSY-HN(CO)CA spectra to find candidates to be HN(i–1) by matching the inter-residual $\text{C}\alpha$ chemical shift of HN(i) to intra-residue $\text{C}\alpha$ chemical shifts of other amide pairs. Since the ^{13}C chemical shifts of the $\text{C}\alpha$ atoms suffer from low dispersion, there are likely to be several such candidates. This problem is resolved by using the 3D ^{15}N -edited NOESY spectrum. Inter-proton distance statistics indicate that amides i and j are more likely to have a sequential relationship when amide i shares a larger number of common NOEs with amide j than with other amides. The probability that two non-adjacent amides share the largest number of common NOEs, and have matching $\text{C}\alpha$ chemical shifts in their HNCA correlations as well, is very low (55). Following the above observation, an NOE score is calculated for each of the candidates by comparing their 3D ^{15}N -edited NOESY strips with that of HN(i) and summing up the number of NOE cross-peaks which have the same ^1H chemical shifts within a specified tolerance. The candidate with the highest NOE score is considered to be HN(i–1).

By using the above strategy, we confirmed the previously achieved 85% of backbone amide assignment for IFNAR2 in complex with IFN α 2 (19) and added the assignment of 87% of the C α resonances (of the fully protonated bound IFNAR2).

Side chain assignment of IFNAR2 in the IFNAR2/IFN α 2 complex using ^{15}N -edited NOESY

Side-chain proton assignment relied on the ^{15}N -edited NOESY spectrum of the complex and its similarity to the same spectrum measured for free IFNAR2 (Figure 2).

For residues not found in the binding site for IFN α 2, it is quite simple to transfer ^1H assignments from the free to the bound IFNAR2. As for residues which are part of the binding surface, a transfer of assignment is also possible for a number of reasons. First, H α and H β protons do not change their chemical shift significantly since the interactions in the binding site usually involve side chain protons other than the α and β protons. Second, since these residues are mainly situated in loops (19), the number of intra-molecular non-sequential inter-residual cross-peaks in their ^{15}N -NOESY strips is very small. Third, not many intermolecular NOEs originating from amides of IFNAR2 are expected to appear in the spectrum since those are usually located further away from the binding interface which is created primarily by side chains. Therefore, most NOESY strips of interface amides consist of intra-residual NOEs and sequential inter-residual NOEs that did not significantly change their chemical shift upon binding, making the assignment transfer fairly easy as can be seen from figure 2 for IFNAR2 residue W100 which is located in the binding interface. In summary, more than 50% side chain protons were assigned for 87% of the residues in bound IFNAR2 using the above method. Only 10% of residues in bound IFNAR2 do not have any side chain ^1H assignment.

Side chain assignment for methyl containing residues of IFNAR2 in the IFNAR2/IFN α 2 complex

MQ-(H)CC $_m$ H $_m$ -TOCSY, MQ-(H)CCH-TOCSY and H(C)C $_m$ H $_m$ -TOCSY (71) experiments were used to assign side-chain ^{13}C and ^1H resonances in methyl-containing residues of IFNAR2 in complex with IFN α 2. MQ-(H)CC $_m$ H $_m$ -TOCSY and H(C)C $_m$ H $_m$ -TOCSY experiments correlate chemical shifts of methyl carbons and protons with chemical shifts of all side chain carbons or protons in the same residue. Hence, side chain assignment of methyl containing residues is possible if assignments for the methyl protons and carbons are available, i.e. the CT- ^{13}C -HSQC spectrum of the methyl region is assigned. Comparison of the CT- ^{13}C -HSQC of the free ^{13}C , ^{15}N -IFNAR2 with that of ^{13}C , ^{15}N -IFNAR2 bound to unlabeled IFN α 2 showed that ~85% of the methyl group cross-peaks did not change their chemical shift upon IFN α 2 binding to any significant extent (Figure 3A). Cross-peaks showing considerable chemical shift changes originated from methyl groups of residues previously identified as situated in the binding site (19). Consequently, many resonance assignments could be transferred from the methyl groups of free IFNAR2 to the methyl groups of bound IFNAR2. For methyl groups located at the binding interface, it was in most cases possible to identify the shifted peaks in the CT- ^{13}C -HSQC of the bound IFNAR2 (Figure 3A). All assignments were validated by verifying that the corresponding MQ-(H)CC $_m$ H $_m$ -TOCSY strip showed the appropriate spin system type pattern and the C α chemical shift matched the one found from the HNCA spectrum (see backbone assignment section). Furthermore, for Ile, Leu and Val residues a match was confirmed between the strips originating from each of the two methyl groups.

Once methyl protons were identified, the other carbon or proton resonances of the spin system could be assigned using the MQ-(H)CC $_m$ H $_m$ -TOCSY and H(C)C $_m$ H $_m$ -TOCSY spectra (Figure 3B). The assignment was facilitated by the MQ-(H)CCH-TOCSY spectrum in cases of weak signals in the MQ-(H)CC $_m$ H $_m$ -TOCSY. The non-CT MQ-(H)CCH-

TOCSY experiment has lower resolution in comparison with the CT MQ-(H)CC_mH_m-TOCSY experiment, however, the former is significantly more sensitive (71). This difference in sensitivity is especially pronounced for leucine residues with strong scalar coupling interactions which give rise to low S/N in the MQ-(H)CC_mH_m-TOCSY because their ¹³C magnetization cannot be refocused completely during the CT period (71). Assignment of side chain carbons was achieved for more than 90% of methyl containing residues.

Side chain protons were assigned using the H(C)C_mH_m-TOCSY spectrum and was in agreement with the assignment based on ¹⁵N-edited NOESY for all methyl containing residues for which it was available. In most cases we were able to complete the side chain proton assignment of methyl containing residues.

Side-chain assignment of IFN α 2 in the IFNAR2/IFN α 2 complex

Backbone assignment of bound IFN α 2 was accomplished previously in our group using uniform ¹³C, ¹⁵N and ²H labeling and TROSY multidimensional NMR spectra (26).

Side chain proton assignment of the bound IFN α 2 was carried out by comparing ¹⁵N-edited NOESY strips of free IFN α 2 to those of the bound IFN α 2 in a similar manner as described for the bound IFNAR2 (Figure 2). This comparison resulted in >50% proton side chain assignment for 80% of bound IFN α 2 residues despite the fact that the spectra of the free and bound forms were not measured under the same pH conditions. The NMR spectra were measured under different conditions since free IFN α 2 is stable at acidic pH 3.5 and the IFNAR2/IFN α 2 complex is stable at slightly basic pH 8.0. Therefore, the amide resonances, which are strongly influenced by pH, radically change their positions and the ¹⁵N HSQC spectra of the free and bound IFN α 2 are very different. However, aliphatic protons are not greatly influenced by changes in the solution pH, making it possible to compare their positions at different pH conditions. Since the amide backbone assignment for both free and bound IFN α 2 is available from previous studies (15,26), strips of the ¹⁵N-edited NOESY of each form of IFN α 2 were prepared according to their individual HN sequential assignment and the chemical shifts of the aliphatic protons in each HN strip were compared between the free and the bound IFN α 2.

Assignment of inter-molecular NOEs between IFNAR2 and IFN α 2

Assignment of the cross peaks in the 2D NOESY spectra of the asymmetrically labeled samples (Figure 1) was enabled by the proton side chain assignment of the IFN α 2/IFNAR2 complex described above and greatly facilitated by the docking model of the complex previously calculated by Quadt-Akabayov *et al.* (26).

For the 2D NOESY spectrum of IFNAR2(IVLTMAK)/IFN α 2(HFWY) (Figure 1B) the assignment was quite straightforward since there are cross-peaks of only two aromatic protons in this spectrum and both interact with the same aliphatic protons. These two aromatic protons give NOEs to each other, indicating that they belong to the same aromatic residue. The aromatic residue was identified as α^2 F27 in IFN α 2 on the basis of the cross-peaks with H β and H α for which sequential assignment has been obtained. The aliphatic protons were assigned to the methyl protons of R²V80, R²V82, R²L52 and R²T44 in IFNAR2 according to their chemical shifts.

Assignment of the inter-molecular NOEs in the 2D NOESY spectrum of IFNAR2(HFW)/IFN α 2(KRLAM) (Figure 1A) was more complicated due to incomplete side chain assignment for aromatic residues of IFNAR2 and aliphatic residues of IFN α 2. Another asymmetrically labeled sample was prepared to assist the assignment process. This sample contained deuterated IFN α 2 reverse-labeled with protonated leucine residues bound to

deuterated IFNAR2 reverse-labeled with protonated tryptophan and histidine residues (IFNAR2(WH)/IFN α 2(L)). Figure 4 shows an overlay of the 2D NOESY spectra of IFNAR2(HFW)/IFN α 2(KRLAM) (black) and IFNAR2(WH)/IFN α 2(L) (red). All but two cross-peaks in the aromatic–aliphatic region of the IFNAR2(HFW)/IFN α 2(KRLAM) spectrum could be superimposed with cross-peaks in the IFNAR2(WH)/IFN α 2(L) spectrum (Figure 4). The cross-peaks appearing in both spectra represent NOEs which originate from a R^2W/α^2L and/or R^2H/α^2L interactions and cross-peaks appearing only in the IFNAR2(HFW)/IFN α 2(KRLAM) spectrum represent NOEs involving either phenylalanine, lysine, arginine, alanine or methionine residues.

According to the previous IFN α 2/IFNAR2 docking model, the best candidates for interactions between IFNAR2 tryptophan and/or histidine residue and IFN- α 2 leucine residues ($R^2W/R^2H/\alpha^2L$) are R^2W100 and R^2H76 in IFNAR2 and α^2L15 and α^2L153 in IFN α 2. Four spin systems out of the six common to both the IFNAR2(HFW)/IFN α 2(KRLAM) and the IFNAR2(WH)/IFN α 2(L) spectra were assigned to the aromatic protons of R^2W100 by matching the $H\alpha$, $H\beta$ and $H\delta$ chemical shifts to the previously obtained proton side chain assignment of bound IFNAR2. All of these interact with a single leucine residue as can be seen from the spectrum (Figure 1A). However, it was impossible to assign which Leu residue it is, since neither α^2L15 nor α^2L153 were assigned. Two of the remaining R^2W/α^2L and/or R^2H/α^2L interactions were assigned to R^2H76 by elimination, since R^2H76 also lacks assignment. In order to decide which aromatic residue (R^2H76 or R^2W100) interacts with which Leu residue (α^2L15 or α^2L153), two docking models of IFN α 2/IFNAR2 (see next section) were calculated using HADDOCK (81): one with distance restraints between R^2W100 and α^2L15 and between R^2H76 and α^2L153 and the other with distance restraints between R^2W100 and α^2L153 and between R^2H76 and α^2L15 . A comparison between the two models revealed that the R^2W100/α^2L15 and R^2H76/α^2L153 configuration of interactions is much more energetically favorable (inter-molecular energy of -379.29 kcal/mol as opposed to -136.49 kcal/mol for the second option). Furthermore, when these docking models were analyzed, it was quite clear that R^2H76 interacts both with α^2L153 and with α^2L15 using one of its two aromatic protons for each respective interaction (i.e. $H\delta$ interacts with α^2L153 and $H\epsilon$ interacts with α^2L15) (Figures 4 and 5B). This observation was also found to be supported by the spectrum (Figure 1A).

On the basis of the new docking model which included all the interactions assigned so far, we were able to identify the two remaining inter-molecular NOE cross-peaks in the IFNAR2(HFW)/IFN α 2(KRLAM) spectrum which were not derived from a R^2W/α^2L or R^2H/α^2L interaction (Figure 4). One of these is assigned to interaction between $H\epsilon$ of R^2H76 and $H\gamma$ of α^2R12 based on the model and ambiguous proton chemical shift assignment from the ^{15}N -edited NOESY spectrum. According to the docking model, the only possible interaction which could give rise to the last unassigned cross-peak in the IFNAR2(HFW)/IFN α 2(KRLAM) spectrum is between $H\epsilon$ of R^2H187 and $H\delta$ of α^2R12 and hence was assigned as such (Figure 4).

Stereo specific assignment of the cross-peaks originating from the methyl groups of Leu and Val residues was obtained from the docking model containing the ambiguous restraints by assigning the stronger NOE cross-peaks to the proton pairs situated in closer proximity to each other.

Docking model of the IFNAR2/IFN α 2 complex

A docking model of the IFN α 2/IFNAR2 was calculated previously in our group based on the NMR mapping of the binding sites on both proteins, double mutant cycle restraints and a single inter-molecular NOE (26). Here, our goal was to use the 24 inter-molecular NOEs found in this study to improve the existing model on the basis of a much larger number of

inter-molecular NOE interactions. A reliable model of a complex can be calculated based on the structures of its free components, provided that these do not undergo major structural changes as a result of binding. Both IFNAR2 and IFN α 2 satisfy this condition since their global and secondary structures are maintained upon binding and any conformational changes which do occur are restricted to the binding site region (19,26).

In silico docking of IFNAR2 and IFN α 2 was performed using the program HADDOCK which allows the input of experimental restraints to drive the docking calculation (81). The 21 active and 22 passive residues in IFNAR2 (Table 1) as well as the 18 active and 20 passive residues in IFN α 2 (Table 1) were selected based on the strategy outlined by Dominguez *et al.* (81) from the previous NMR mapping of the binding sites (19,26). The experimental inter-molecular distance restraints employed in the calculation include all the restraints used in the previous model (26), with the addition of all the inter-molecular NOEs found in this study (Table 1). A total of 1000 structures were calculated in the rigid body minimization. Semi-flexible simulated annealing followed by refinement in explicit water was performed for the best 200 solutions based on the haddock score (weighted sum of all the energy terms and the buried surface area). Violation analysis of the final 200 structures showed that all the NOE/DMC distance-restraints were maintained for 99.5% of the structures. Solutions were clustered using a 7.5 Å interface RMSD cut-off. 197 out of 200 structures were included in the 7 clusters found. Cluster analysis was performed on the 4 best structures in each cluster to remove the dependency of cluster averages upon their size. The cluster with the lowest average HADDOCK score was considered to be the best solution. The RMSD of the ensemble of the 10 best structures of the best cluster is 0.8 ± 0.1 Å for the backbone atoms and 1.1 ± 0.1 Å for all heavy atoms (Figure 5A). The average inter-molecular energy of this ensemble is -391 ± 45 kcal/mol and the average buried surface area is 3060 ± 129 Å² (Table 2). A close up view of the binding interface shows the side chain orientation of all residues involved in the NOE/DMC distance restraints used in the docking calculation of the current model (Figure 5B).

As shown in figures 6 and 7 and in table 3, the area of the binding sites on IFN α 2 and IFNAR2 has increased by about two fold, compared to the previous two models (Chill *et al.* (18) and Quadt-Akabayov *et al.* (26)). Most importantly, the present study reveals that the binding site on each protein is composed of two major parts, each contributing roughly half of the binding surface area. The upper section of the binding site in the two proteins which was revealed in previous studies (18, 26) consists of a striated motif of matching hydrophobic patches flanked by two stripes of polar residues arranged in an alternating charge pattern creating complementary electrostatic interactions. The lower section of the binding site, revealed in the present study, consists almost exclusively of an electrostatically complementary mosaic pattern of charged and polar residues. The upper section of the binding site incorporates residues from the A- and E-helices and the AB loop of IFN α 2 and from the S3–S4 and S5–S6 loops, the inter-domain loop (S7–S8) as well as small parts of the S3 and S6 strands of IFNAR2 (Figures 6, 7). The lower section of the interface consists of the N-terminus and beginning of A-helix as well as the C-terminus of IFN α 2 and residues from several loops (S9–S10, S11–S12 and S13–S14) from the C-domain of IFNAR2 (Figures 6, 7).

Most of the charged or polar residues situated in the binding interface participate in hydrogen bonds or salt bridges with residues from the other protein. 11 inter-molecular salt bridges and 12 hydrogen bonds are formed between IFNAR2 and IFN α 2 in at least 5 out of the 10 structures in the ensemble (Table 4).

Discussion

Determination of inter-molecular NOEs between IFNAR2 and IFN α 2 in the complex

The main focus in structural studies of protein complexes are the inter-molecular interactions that are formed when the two molecules bind each other. Hetero-nuclear experiments used to detect inter-molecular interactions suffer from poor signal-to-noise ratio when applied to large protein complexes. However, homo-nuclear 2D NOESY spectra retain considerable sensitivity even for large protein complexes. Difference spectra along with specific deuteration of proteins can be used to simplify the crowded and unresolved 2D spectra and obtain information about inter-molecular interactions even for large protein complexes (>50 kDa). These difference spectra, aimed to obtain structural information about the binding interface, are usually very well resolved (90,94–96) despite the size of the proteins, however they suffer from subtraction-artifacts as a result of measurements carried out with two different samples.

In the present study a novel approach for the observation of inter-molecular side chain–side chain NOE interactions in large protein complexes has been applied. This method is based on asymmetric reverse-protonation of the complex components and exploits the sensitivity of 2D-homonuclear NOESY experiments.

A major limitation in using the 2D approaches mentioned above for studying intermolecular interactions is the assignment of the observed inter-molecular interactions to the corresponding protons in the complex constituents. In the past the assignment had to rely on modeling the complex structure and the availability of biochemical data on the binding interface combined with specific amino acid labeling. In the present study we demonstrate that the analysis of the inter-molecular interactions can be considerably aided by the sequential and side-chain assignment procedures recently developed by Yang and co-workers (55,56). Nevertheless, the assignment of some inter-molecular interactions still had to rely on preliminary models and labeling schemes.

As demonstrated in this study, the section of the NOESY spectrum showing interactions between aromatic protons and aliphatic proton with resonances upfield of 2.5 ppm can be simplified to show almost exclusively cross-peaks due to inter-molecular interactions. Despite the simplification of the spectra, some contributions from amide protons that resisted exchange with D₂O have been observed. In addition, some cross-peaks due to less than 100% deuteration of the commercial growth medium and/or scrambling have been observed. Most of these contributions can be identified by measuring the NOESY spectra of the free proteins.

In summary, 24 new inter-molecular NOEs were identified using the reverse-labeling method developed in this study. These NOEs were introduced as distance restraints into a docking calculation of the IFNAR2/IFN α 2 complex which yielded a considerably improved model revealing new information about the structure of the binding interface.

Comparison with previously calculated docking models of the IFNAR2/IFN α 2 complex

Inclusion of a much larger number of inter-molecular NOEs substantially improved the quality of the model for the IFNAR2/IFN α 2 complex and increased the surface of the binding site by approximately two fold for both molecules compared with the previous models (Table 3). The high quality of the new model is also evident from the substantial increase in the number of possible hydrogen bonds and salt bridges in comparison to the Chill *et al.* and Quadt-Akabayov *et al.* models (Table 3) (18,26). The present study reveals a second large surface in the binding site of the two molecules that is made of a mosaic of

positively and negatively charged residues as well as some polar residues. The contribution of hydrophobic residues to this section is very minor.

The NOE constraints used in the docking are well dispersed over the entire binding surface, connecting most of the various determinants situated in the binding site (A-helix, AB-loop and E-helix in IFN α 2 as well as loops S3–S4, S5–S6, S7–S8 and S13–S14 in IFNAR2; see Figures 6, 7 and figure 2S in the Supporting Information). The N-terminus and A-helix of IFN α 2 were not found to be a part of the binding site in the NMR mapping done by Quadt-Akabayov *et al.* since cross-peaks belonging to the amide pairs of these residues were missing in the cross saturation ^{15}N HSQC spectrum of the bound IFN α 2 (26). These cross peaks were not observed due to the low sample concentration and a further 10-fold reduction in the amide cross-peaks intensity caused by the use of 10% $\text{H}_2\text{O}/90\%$ D_2O solution required to prevent spin-diffusion (26). The 4 DMC restraints and the single NOE used in the calculation of the Quadt-Akabayov *et al.* docking model connected only a subset of regions found to be involved in binding by previous mutagenesis and NMR studies (22, 23, 25) (Table 1). This subset did not include the A-helix of IFN α 2 as well. All of the above resulted in exclusion of the A-helix of IFN α 2 from the interface and a 30° difference in the orientation of IFNAR2 relative to IFN α 2 in comparison with the new model (Figure 8). Chill *et al.* used the same 4 DMC restraints in the docking of their model as were used by Quadt-Akabayov *et al.* However, due to lack of experimental data, no emphasis on a binding interface that excludes the A-helix was imposed in the calculation (18), resulting in incorporation of the A-helix in the interface and orientation of IFNAR2 relative to IFN α 2 much closer to that found in this study (Figures 6, 8). The input data for the docking model presented in this study consisted of the same binding site mapping and distance restraints used for the calculation of the Quadt-Akabayov *et al.* model with the addition of the 24 new NOEs found in this work. Therefore, in view of the limitations of binding site mapping techniques it can be concluded that the incorporation of a high number of experimental intermolecular distance constraints well dispersed over most of the binding site surface is essential for the calculation of a docking model which reveals the entire binding interface and the correct orientation of complex components.

An attempt was made to incorporate residual dipolar couplings (RDCs) in the structure calculation. Several alignment media were tested for the measurement of RDCs of the IFNAR2/IFN α 2 complex and most of these were found not to be suitable. The sample was stable in polyacrilamide gel doped with negative charges (97), however the relevant spectra exhibited a low signal-to-noise ratio, which allowed obtaining only a small number of RDCs. Most of these RDCs were for residues situated in flexible regions making their contribution to structure calculation very limited (98).

Structural analysis of the Chill *et al.* and Quadt-Akabayov *et al.* models shows that the regions of IFN α 2 and IFNAR2 added to the binding surface were not far apart in the previous models. Inclusion of a high number of inter-molecular NOEs in the new model brings the two proteins much closer together, revealing the participation of the above regions in the binding interface.

The significant increase in the binding area on IFN α 2 is mostly attributed to the addition of the N-terminus, A-helix and part of the C-terminus (Figures 6, 7). While the A-helix is connected with IFNAR2 by 12 inter-molecular NOEs (Table 1, Figure 7) ($\alpha^2\text{L15} - \text{R}^2\text{W100}$; $\alpha^2\text{L15} - \text{R}^2\text{H76}$; $\alpha^2\text{R12} - \text{R}^2\text{H76}$; $\alpha^2\text{R12} - \text{R}^2\text{H187}$), NOEs involving the N-terminal and C-terminal residues of IFN α 2 ($\alpha^2\text{Q5}$, $\alpha^2\text{T6}$, $\alpha^2\text{H7}$, $\alpha^2\text{L9}$, $\alpha^2\text{E159}$, $\alpha^2\text{L161}$, $\alpha^2\text{R162}$, $\alpha^2\text{S163}$ and $\alpha^2\text{K164}$) were not detected since mostly these interactions do not involve aromatic-aliphatic proton interactions. Six of these nine residues were not found to participate in IFNAR2 binding by the NMR cross-saturation studies due to lack of information: the HN resonances

of four are unassigned ($^2\text{T6}$, $^2\text{H7}$, $^2\text{L9}$ and $^2\text{S163}$) and the HN cross-peaks of the remaining two ($^2\text{Q5}$ and $^2\text{L161}$) were missing from the cross saturation ^{15}N HSQC spectrum of the bound IFN α 2 due to their low intensity as was mentioned in the previous paragraphs (26). The remaining three residues ($^2\text{E159}$, $^2\text{R162}$ and $^2\text{K164}$) did not show a significant decrease in their HN cross-peaks intensity upon IFNAR2 binding. However, residues from the C-terminus of IFN α 2 ($^2\text{R162}$ and $^2\text{E165}$) were found to be part of the binding site in the Quadts-Akabayov *et al.* docking model (Figure 6), while residues $^2\text{E159}$ and $^2\text{L161}$ are situated less than 8 Å from IFNAR2. Residues 5–9 from the C-terminal tail of IFN α 2 are less than 14 Å from IFNAR2 due to the more distant position of the N-terminal part of IFN α 2 from IFNAR2 in the Quadts-Akabayov *et al.* model (26). In the Chill *et al.* model the entire C-terminal tail of IFN α 2 (residues 157–165) was excluded from the calculation due to difficulties in docking such a flexible segment (Figure 6) (18). Residues $^2\text{T6}$, $^2\text{H7}$, $^2\text{L9}$ as well as residue $^2\text{Q5}$ from the N-terminal tail of IFN α 2 are situated less than 5 and 8 Å from IFNAR2, respectively, in the Chill *et al.* model (18). It is important to point out that the N-terminal tail of IFN α 2 features a disulfide bond connecting the first N-terminal cysteine with another cysteine in the C-helix (C98). As a result of this covalent bond formation, the mobility of the N-terminus of IFN α 2 is restricted as manifested by positive H-N NOE in the range 0.4–0.6 in comparison with the negative NOEs measured for the flexible C-terminal (15). Moreover, three residues in the N-terminus of IFN α 2 ($^2\text{C1}$, $^2\text{L3}$ and $^2\text{L9}$) are absolutely conserved among 35 IFN α species (15) suggesting possible involvement of the later two in IFNAR2 or IFNAR1 binding.

The binding site on IFNAR2 has increased compared to the previous models mostly due to the addition of charged or polar residues from the C-domain of the protein. One inter-molecular NOE connects the S13–S14 loop of the C-domain of IFNAR2 with IFN α 2 ($^2\text{R12}$ – $^2\text{H187}$), confirming the participation of this domain in the binding interface (Table 1, Figure 7). Residue $^2\text{L139}$ displays a change in the chemical shift of one of its methyl groups as can be seen in Figure 3, placing it and its direct neighbors ($^2\text{S140}$ and $^2\text{D138}$) in the binding site. However, all the other residues in the C-domain of IFNAR2 revealed by the new model as a part of the interface ($^2\text{E132}$ – $^2\text{Q136}$, $^2\text{K159}$) are not connected by NOEs to IFN α 2 and do not show any change in the position of their HN cross-peaks upon IFN α 2 binding (19). Several of these residues have been suggested by mutagenesis studies to be involved in IFN α 2 binding (24) as will be elaborated in the following section. In the Quadts-Akabayov *et al.* model residues 138–140, 159, 186 and 187 from the C-domain of IFNAR2 were found to be part of the interface (Figure 6) while residues 132–137 are situated less than 8 Å from IFN α 2 (26). Analysis of the inter-molecular distances in the Chill *et al.* model (18) shows that residues 136–140, 159 and 186–189 are situated less than 4.5 Å from the interfacial residues of IFN α 2. In addition, a significant attenuation of signal was observed in the HN cross-peaks of residues in the S13–S14 loop of the C-domain of IFNAR2 (residues 187–198) upon IFN α 2 binding.

In summary, the new model adds to the binding surface residues from both IFNAR2 and IFN α 2 which were not shown by previous NMR mapping studies to be affected by binding. Previous mapping of the binding sites were performed by examining changes in the position or intensity of the HN cross-peaks in the ^{15}N HSQC spectra of bound IFNAR2 and IFN α 2 (18,26). However, NMR experiments showing backbone resonances are in most cases unable to detect changes in cross-peaks of residues participating in the binding through their side-chain and not backbone atoms. Therefore, we believe that residues not found to be in the interface by ^{15}N HSQC type NMR mapping could still be a part of the binding surface provided they interact through their side-chains, which is very probable in case of the charged and polar residues such as the ones added to the interface in the new model. It is important to mention that NOE is the most reliable and informative NMR parameter for gaining intermolecular structural information. Therefore, we believe that the 24 NOEs

incorporated into the docking of the new model, being well dispersed over the binding surface, serve as anchoring points for the entire set of interface contacts including areas not directly connected by NOEs. The participation of these new regions in the binding site is an immediate consequence of the input of these NOE distance constraints into the docking calculation.

In order to assess the robustness of the model determined by the large number of intermolecular NOE constraints, a HADDOCK run was performed with the newly found intermolecular NOEs only. The two models were very similar in terms of RMSD (backbone and heavy atom RMSD 1.07 Å and 1.21 Å, respectively). However, the energetic parameters of the docking calculation were significantly improved with the inclusion of the DMC and the previously obtained single NOE restraints. Another docking calculation was performed using only ~70 % of the inter-molecular NOEs found in this study. In this calculation, NOEs between $R^2L52 - \alpha^2F27$, $R^2H76 - \alpha^2L15$, $R^2H76 - \alpha^2R12$ and $R^2H187 - \alpha^2R12$ as well as the single NOE obtained previously by Quadt-Akabayov *et al.* (26) were removed from the distance restraints list, however the DMC distance restraints were kept (Figure 2S in the Supporting Information). The resulting model was also very similar to the model containing all the distance restraints both in terms of the overall RMSD and in terms of the relative orientation between IFNAR2 and IFN α 2 (backbone and heavy atom RMSD 1.53 Å and 1.66 Å, respectively), further supporting the robustness of the proposed model and the correct assignment of the inter-molecular NOEs used to obtain it.

Comparison with the mutagenesis data about the IFNAR2/IFN α 2 complex

The individual binding sites on IFNAR2 and IFN α 2, as determined by our docking model, are in good agreement with the mutational data available (Figure 7). Residues found by mutagenesis to be important for IFNAR2/IFN α 2 binding form a subset of the interface residues found in the new model. It is important to point out that IFN α 2 residues α^2R12 , α^2L15 from the A-helix and α^2L153 from the E-helix, which were not found by previous NMR studies to participate in the binding due to lack of assignment, were found in the present study to give rise to inter-molecular NOEs. These residues were found by mutagenesis studies to have a significant effect on IFN α 2 binding to IFNAR2, strongly supporting the inter-molecular NOEs obtained for them.

Several regions in the interface of the new model were not subjected to mutational analysis or did not have a pronounced effect on binding affinity upon mutation. On IFN α 2, these areas include the N- and C-termini, the first of which was not studied by mutagenesis so far while the latter might participate in the binding as can be concluded from the two-fold decrease in the binding affinity upon removal of 5 C-terminal residues (α^2L161 - α^2E165) (23,24). Contribution of the C-domain of IFNAR2 to the binding has been considered small, mainly since mutagenesis studies revealed minor effects on binding affinity of IFNAR2 to IFN α 2 upon mutation of C-terminal domain residues or removal of the entire C-domain of IFNAR2 (99). However, in this study two NOEs are connecting the S13–S14 loop of IFNAR2 (specifically R^2H187) with the A-helix of IFN α 2, demonstrating a clear involvement of the C-domain in the binding site. In addition, the previously mentioned chemical shift change in the resonance of a methyl group of R^2L139 further supports the participation of this domain in the binding interface. Areas from the C-domain of IFNAR2 are situated in very close proximity to the binding interface in all the docking models of the IFNAR2/IFN α 2 complex obtained over the years, beginning with the model calculated by Roisman et al. and ending with the current model (18,25,26). Single mutations of residues R^2E132 , R^2E133 , R^2E134 and R^2Q136 as well as multiple alanine substitutions in the S11–S12 loop of IFNAR2 (R^2E157 -IKG- N161 to $^{157}AIAGN^{161}$) had a very minor effect on binding affinity (20,22). However, a triple alanine substitution of R^2E132 - R^2E134 decreased binding affinity two-fold (24). In addition, recently, an interaction between the

IFN C-terminus and a negatively charged loop in the IFNAR2 C-domain (^{R2}E132-^{R2}E134) has been suggested to play a role in the differential signaling of the various type I IFNs by causing up to 20-fold difference in binding affinities to IFNAR2 upon insertion of C-terminal tails from different alpha IFNs into an IFN α 2 scaffold (24). Slutzky *et al.* docked the C-terminal tail of the IFN α 2 mutant containing IFN α 8 C-terminal tail and found it to gain a specific structure in the bound state, binding to a groove below the ^{R2}E132-^{R2}E134 loop in IFNAR2 (24). On the basis of this study, it is plausible to suggest that the entropic cost of ordering the unstructured C-terminal tail of IFN α 2 upon binding could cause only small and insignificant change in ΔG upon complex formation for mutants preventing this interaction (such as those lacking the C-tail of IFN α 2 and/or a negative charge in the ^{R2}E132-^{R2}E134 loop of IFNAR2). It has been shown previously for the complex between the human growth hormone and its receptor that while two-thirds of the interface residues had little impact on the binding affinity, large and compensating changes were observed in the enthalpy and entropy of binding (100). This study further supports the claim that residues not found to have an effect on binding kinetics or affinity can still be part of a binding interface (100).

Allostery in the binding of IFNAR1-EC to the IFN α 2/IFNAR2 complex

Recently, we found that IFNAR1-EC binding to the IFN α 2/IFNAR2 complex resulted in the significant chemical shift changes or disappearance of HSQC cross-peaks of residues on the face of IFN α 2 containing the binding site for IFNAR2 (101). However, only three of these residues (^{α 2}F27, ^{α 2}R149 and ^{α 2}S152) were implicated in IFNAR2 binding by NMR cross-saturation studies (26). As shown in Figure 9, the present study demonstrates that also residues ^{α 2}Q5, ^{α 2}N156, ^{α 2}E159 and ^{α 2}S160 and ^{α 2}K164 whose HSQC cross peaks disappeared upon IFNAR1 binding, are located in IFN α 2 binding site for IFNAR2. Moreover, ^{α 2}Q20 and ^{α 2}R22 which changed their chemical shift upon IFNAR1 binding are also in the binding site for IFNAR2 in the present model. Thus the present model more than doubles the number of IFN α 2 residues in the binding site for IFNAR2 whose HSQC cross peaks were affected by IFNAR1 binding, strongly supporting the proposed allosteric changes in IFN α 2/IFNAR2 complex upon IFNAR1 binding. Unfortunately, the exact structural nature of this allostery is not known since the atomic resolution structure of the ternary IFNAR1/IFN α 2/IFNAR2 complex has not been elucidated thus far.

Conclusions

In this study we present a novel approach for obtaining inter-molecular side chain - side chain NOEs in large protein complexes using asymmetric reverse-protonation and 2D homonuclear NOESY spectra. Applying this technique to the 44 kDa IFN α 2/IFNAR2 complex yielded 24 new inter-molecular NOEs which were used as distance restraints in the docking calculation of a model of the complex. This model doubles the binding site surface on both IFN α 2 and IFNAR2 by adding a new section in the lower part of IFN α 2 and IFNAR2 binding sites that is formed by a mosaic of charged and polar residues. A significant 30° change was observed in the orientation of IFNAR2 relative to IFN α 2 in comparison with the Quadt-Akabayov *et al.* model (26) as a result of the addition of the newly found inter-molecular NOEs which makes it much more similar to the model previously suggested by Chill *et al.* (18). Moreover, the present study demonstrates that practically the entire IFN α 2 binding site for IFNAR2 undergoes conformational changes upon IFNAR1 binding, strongly supporting a mechanism of allosteric changes in IFN α 2 caused by IFNAR1 binding to the IFN α 2/IFNAR2 complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are most grateful to Dr. Naama Kessler for help with protein expression and purification. We also thank Dr. Lewis Kay, Dr. Tali Scherf, Dr. Osnat Rosen and Dr. Jordan Chill for helpful suggestions and discussions.

Abbreviations

2D, 3D, 4D	two-, three- and four-dimensional, respectively
AIR	ambiguous interaction restraints
CNS	Crystallography & NMR System
CT	constant time
D₂O	deuterium oxide
DMC	double mutant cycle
HCRII	class II helical cytokine receptor family
HSQC	hetero-nuclear single quantum coherence
IFN	interferon
IFNAR1	the first subunit of the receptor for class 1 interferons
IFNAR2	the second subunit of the receptor for class 1 interferons
IFNAR2-EC	extra-cellular part of IFNAR2
INEPT	Insensitive Nuclei Enhanced by Polarization Transfer
MBP	maltose binding protein
MQ	multiple quantum
MW	molecular weight
NMR	nuclear magnetic resonance
NOE	Nuclear Overhauser enhancement
NOESY	Nuclear Overhauser enhancement spectroscopy
PDB	protein data bank
R²X and α²Y	IFNAR2-EC and IFNα2 residues are labeled by R2 and α2 superscripts, respectively
RDC	residual dipolar coupling
RMSD	root mean square deviation
S/N	signal-to-noise ratio
TOCSY	total correlation spectroscopy
TROSY	transverse relaxation optimized spectroscopy

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Figure 1. Inter-molecular NOE interactions in the IFNAR2/IFNα2 complex detected in asymmetrically labeled complex

(A) Overlay of 2D NOESY spectra in D₂O of IFNAR2(HFW)/IFNα2(KRLAM) (black) and IFNα2(KRLAM) (red). (B) Overlay of 2D NOESY spectra in D₂O of IFNAR2(IVLTMAK)/IFNα2(HFWY) (black) and IFNAR2(IVLTMAK) (red). Red cross-peaks originate from intra-molecular interactions. Arrows indicate minute changes in the positions of the intra-molecular cross-peaks between the spectra of the free molecule and the complex. Black cross-peaks not overlaid with red cross-peaks and not marked by arrows originate from inter-molecular interactions and are labeled according to the assignment of the aliphatic proton. Vertical lines indicate spin systems of cross-peaks originating from the same aromatic proton and are labeled according to the assignment of the specific proton. Light blue boxes indicate cross peaks which appeared after the urea induced partial denaturation.

Figure 2. $^1\text{H}^{15}\text{N}$ strips from 3D ^{15}N -edited NOESY of free and bound IFNAR2 and free and bound IFN α 2

NOESY strips of $\text{R}^2\text{W100}$ and of $\alpha^2\text{K133}$ for which the side chain assignment was based on the side chain assignment of free IFNAR2 and free IFN α 2. Bound r2 and free r2 stand for bound and free IFNAR2, respectively, while bound a2 and free a2 stand for bound and free IFN α 2, respectively. The strips are marked with the chemical shift of the amide nitrogen.

Figure 3. Side chain assignment of methyl containing residues of the bound IFNAR2
(A) Overlay of CT-¹³C-HSQC spectra of free IFNAR2 (black) and IFNAR2 bound to unlabeled IFNα2 (red). (B) MQ-(H)CCmHm-TOCSY strips of IFNAR2 in complex with IFNα2. Representative strips of five methyl containing residues: Ile, Val, Thr, Ala and Leu are shown. The strips are marked with the chemical shift of the methyl carbon.

Figure 4. Assignment of inter-molecular interactions involving IFNAR2 histidine residues
 Overlay of 2D NOESY spectra in D₂O of IFNAR2(HFW)/IFNα2(KRLAM) and IFNAR2(WH)/IFNα2(L) (red). Black boxes indicate cross peaks which do not originate from an NOE between aromatic protons of Trp or His and aliphatic protons of Leu and are labeled according to the assignment of the aliphatic proton. Vertical lines indicate spin systems of cross-peaks originating from the same aromatic proton and are labeled according to the assignment of the specific proton.

Figure 5. A docking model of IFNAR2/IFN α 2 based on inter-molecular NOEs

(A) Ensemble of best 10 structures from the highest ranking cluster in the calculation. IFNAR2 is shown in orange and IFN α 2 in green. The flexible N-terminal (R^2S1 - R^2C12) and C-terminal residues (R^2P204 - R^2S212) of IFNAR2 were removed for the presentation. (B) Stereo representation of an interface close-up of the docking model of IFNAR2/IFN α 2. Residues involved in the inter-molecular NOEs or DMC interactions and used in the docking are visible in stick representation. IFNAR-EC is colored in orange and IFN α 2 in green.

Figure 6. Summary of binding site residues in the current and previous docking models of IFNAR2/IFN α 2

(A) IFN α 2 residues and (B) IFNAR2 residues situated in the binding site of the current model are highlighted in yellow. Interface residues correspond to a minimal set determined by PISA (86), CMA (87) and MOLMOL (88) with a inter-molecular distance criterion ≤ 4 Å and are shown if present in at least 5 structures out of the 10 structures in the ensemble. Residues in the interface of the Quadt-Akabayov *et al.* model (26) are marked with magenta boxes while residues in the interface of the Chill *et al.* model (18) are marked with blue boxes. Asterisks mark the beginning and end of the IFN α 2 and IFNAR2 sequence present in the Chill *et al.* model (18). Secondary structure elements (helices or sheets) are shown in graphic representation marked with their name.

Figure 7. Open book representation of the IFNAR2/IFN α 2 complex and the observed NOE interactions

IFNAR2 (left) and IFN α 2 (right) are presented in a space-fill mode. Colored residues were found to be part of the binding interface in the current model according to the analysis in figure 6. Residues are colored as follows: light green, aliphatic; dark green, aromatic; red, negatively charged; blue, positively charged; cyan, asn and gln; indigo, his; orange, ser and thr. Residues marked with dots were found by mutagenesis studies to be important for binding. Residues giving rise to the observed inter-molecular NOEs used in the docking calculation are connected with black lines. Yellow contour lines include residues added to the binding interface in the present study.

Figure 8. The new and previous docking models of IFNAR2/IFNα2 – a change in the orientation of IFNAR2 relative to IFNα2

Representative structures of the current (IFNα2 in green and IFNAR2 in orange), the Quadt-Akabayov *et al.* (26) (dark grey) and the Chill *et al.* (18) (light grey) models are aligned with respect to IFNα2. The flexible N-terminal (^{R2}S1-^{R2}C12) and C-terminal residues (^{R2}P204-^{R2}S212) of IFNAR2 were removed from the new and the Quadt-Akabayov *et al.* (26) models for the presentation.

Figure 9. Effect of IFNAR1-EC binding to the binary IFNAR2/IFNα2 complex on IFNα2 binding site

Representative structures of the current and the Quadt-Akabayov *et al.* (26) models are aligned with respect to IFNα2. IFNα2 is shown in light green space fill representation while IFNAR2 from the current and the Quadt-Akabayov *et al.* (26) docking model are represented by an orange and a light grey ribbon diagram, respectively. The flexible N-terminal (R^2S1-R^2C12) and C-terminal residues ($R^2P204-R^2S212$) of IFNAR2 were removed from the new and the Quadt-Akabayov *et al.* (26) models for the presentation. Residues colored in cyan or yellow are IFNα2 binding site residues for IFNAR2 excluding those unaffected by IFNAR1 binding to IFNα2/IFNAR2 complex (101); residues which exhibited significant change in chemical shifts or could not be assigned due to large chemical shift changes or disappearance upon IFNAR1 binding are dotted. All the other colored residues could not be assigned due to overlap or lack of assignment in the binary complex (101). Residues situated in the binding interface of the previous model are shown in cyan (26). Residues added to the interface by the current model are shown in yellow.

Table 1A list of inter-molecular restraints used in the docking procedure^a

IFNAR2		
AIR	Active	M46, S47, K48, P49, E50, D51, L52, K53, V54, S74, H76, E77, V80, V82, C95, S96, H97, N98, W100, I103, D104
	Passive	S11, T13, K15, L42, T44, V55, R73, L83, E84, G85, S94, M105, L135, Q136, D138, L139, L141, L185, H187, S188, D189, Q191
	Flexible Segments	9–17; 40–57; 71–87; 92–107; 133–143; 183–193
IFNα2		
	Active	L26, F27, S28, C29, L30, K31, R33, H34, D35, F36, G37, F38, W140, A145, M148, R149, S152, L153
	Passive	R12, M16, A19, R22, I24, S25, Q40, E41, A118, K121, R125, L128, E132, K133, S136, P137, C138, N156, E159, S160
	Flexible Segments	10–43; 116–162
DMC		
	IFNα2	IFNAR2
	D35:OD*	K48:NZ
	R144:HG* or HD*	M46:HG* or HE*
	R149:HN*	E77:OE*
	S152:OG	H76:NE1 or NE2
NOE		
	IFNα2	IFNAR2
	D35:HN	K48:all non-labile side chain protons
	<i>F27:HZ or HE* or HD*</i>	<i>V80:HG*</i>
	<i>F27:HZ or HE* or HD*</i>	<i>L52:HD*</i>
	<i>F27:HZ or HE* or HD*</i>	<i>V82:HG*</i>
	<i>F27:HZ or HE* or HD*</i>	<i>T44:HG2*</i>
	<i>L15:HD*</i>	<i>W100: all aromatic protons</i>
	<i>L153:HD*</i>	<i>H76:HD2</i>
	<i>L15:HD*</i>	<i>H76:HE1</i>
	<i>R12:HG*</i>	<i>H76:HE1</i>
	<i>R12:HD*</i>	<i>H187:HE1</i>

^aInter-molecular NOEs used in the previous model are in regular font and the NOEs added in the current model are in italic font.

Table 2Docking and structural statistics for the 10 best IFNAR2/IFN α 2 model structures^a

	Ensemble	Representative structure
Docking statistics		
HADDOCK score	-135 $\pm$ 9	-133
E _{vdw} [kcal/mol]	-94 $\pm$ 10	-94
E _{elec} [kcal/mol]	-552 $\pm$ 46	-529
E _{inter} [kcal/mol]	-391 $\pm$ 45	-375
E _{AIR} [kcal/mol]	254 $\pm$ 16	248
BSA [$\text{\AA}^2$]	3060 $\pm$ 129	2949
RMSD from lowest energy structure [$\text{\AA}$]	0.69 $\pm$ 0.25	0.64
Cluster size	60	-
Number of AIR violations > 0.3 $\text{\AA}$	8 $\pm$ 1	8
Number of NOE or DMC violations > 0.3 $\text{\AA}$	0	0
Structural statistics		
RMSD backbone (heavy atom) [$\text{\AA}$]	0.76 $\pm$ 0.1 (1.08 $\pm$ 0.08)	-
RMSD all atoms at interface [$\text{\AA}$]	1.49 $\pm$ 0.1	-
RMSD backbone (heavy atom) from free IFNAR-EC [$\text{\AA}$]	0.99 $\pm$ 0.04 (1.42 $\pm$ 0.04)	0.95 (1.41)
RMSD backbone (heavy atom) from free IFN α 2 [$\text{\AA}$]	0.73 $\pm$ 0.08 (1.15 $\pm$ 0.06)	0.66 (1.09)
Deviations from idealized geometry		
RMS deviation for bond angles [$^\circ$]	0.6	0.6
RMS deviation for bond lengths [$\text{\AA}$]	0.004	0.004
Ramachandran analysis, residues in		
Most favored regions [%]	79.1	78.1
Additionally allowed regions [%]	19.4	20.3
Generously allowed regions [%]	0.6	0.9
Disallowed regions [%]	0.9	0.7

^aStructure validation parameters were calculated using PSVS – Protein Structure Validation Software suite (102).

Table 3A comparison between the structural statistics of the different models of the IFNAR2/IFN α 2 complex

	Quadt-Akabayov <i>et al.</i> model (26)	Chill <i>et al.</i> model (18)	Current model ^a
Number of IFNAR2 residues in the interface	15	24	36
Number of IFN α 2 residues in the interface	13	14	35
Buried surface area on IFNAR2	740 Å ²	800 Å ²	1556 Å ²
Buried surface area on IFN α 2	801 Å ²	650 Å ²	1611 Å ²
Number of inter-molecular hydrogen bonds	4	2	12
Number of inter-molecular salt bridges	8	6	11

^a Number of residues in interface was determined as in figure 7. Number of salt bridges and hydrogen bonds was determined as in table 3. Buried surface area was calculated using the program PISA (Protein interfaces, surfaces and assemblies service PISA at European Bioinformatics Institute, authored by E. Krissinel and K. Henrick (86)) for the representative structure of the ensemble.

Table 4

A list of inter-molecular salt bridges and hydrogen bonds formed in the docking model of IFNAR2/IFN α 2^a

	IFN α 2		IFNAR2	
	Residue	Atom	Residue	Atom
Hydrogen Bonds	T6	O	K48	NZ
	R12	NH2	D138	OD2
	R12	NH1	H187	O
	R13	NH2	S188	O
	R22	NH1	N98	OD1
	D35	OD1	K48	NZ
	E146	OE1	S47	OG
	E146	OE2	K48	NZ
	R149	NE	E77	OE2
	N156	OD1	Q136	NE2
	R162	NH1	D70	OD1
	R162	NH1	D70	OD1
Salt Bridges	R12	NH1	D138	OD2
	R12	NH2	D138	OD2
	R33	NH1	E50	OE1
	D35	OD1	K48	NZ
	E146	OE1	K48	NZ
	E146	OE2	K48	NZ
	R149	NE	E77	OE2
	R149	NH2	E77	OE2
	E159	OE1	H76	NE2
	R162	NH1	D70	OD1
	R162	NH2	D70	OD1

^aInter-molecular contacts were calculated using the program PISA (Protein interfaces, surfaces and assemblies service PISA at European Bioinformatics Institute, authored by E. Krissinel and K. Henrick (86)) and are reported if present in at least 5 structures out of the 10 structures in the ensemble.