Conformational Heterogeneity in Antibody-Protein Antigen Recognition

**IMPLICATIONS FOR HIGH AFFINITY PROTEIN COMPLEX FORMATION**

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**Background:** Antibodies are essential components of the immune system which recognize specific antigens with high affinity.

**Results:** Protein antigen binding sites on antibodies show conformational exchange on a millisecond to second timescale.

**Conclusion:** Conformational heterogeneity at high affinity protein-protein interaction sites may be common and facilitate efficient protein complex formation.

**Significance:** High affinity protein-protein interactions are critical for many biological processes.

Specific, high affinity protein-protein interactions lie at the heart of many essential biological processes, including the recognition of an apparently limitless range of foreign proteins by natural antibodies, which has been exploited to develop therapeutic antibodies. To mediate biological processes, high affinity protein complexes need to form on appropriate, relatively rapid timescales, which presents a challenge for the productive engagement of complexes with large and complex contact surfaces (~600–1800 Å²). We have obtained comprehensive backbone NMR assignments for two distinct, high affinity antibody fragments (single chain variable and antigen-binding (Fab) fragments), which recognize the structurally diverse cytokines interleukin-1β (IL-1β, β-sheet) and interleukin-6 (IL-6, α-helical). NMR studies have revealed that the hearts of the antigen binding sites in both free anti-IL-1β Fab and anti-IL-6 single chain variable exist in multiple conformations, which interconvert on a timescale comparable with the rates of antibody-antigen complex formation. In addition, we have identified a conserved antibody binding-induced change in the orientation of the two variable domains. The observed conformational heterogeneity and slow dynamics at protein antigen binding sites appears to be a conserved feature of many high affinity protein-protein interfaces structurally characterized by NMR, suggesting an essential role in protein complex formation. We propose that this behavior may reflect a soft capture, protein-protein docking mechanism, facilitating formation of high affinity protein complexes on a timescale consistent with biological processes.

Antibodies are both an integral component of the adaptive immune system and a highly important class of protein therapeutic. Their highly specific and modular nature, coupled with the potential to bind to a huge range of target molecules, makes them one of the most important types of therapeutic available today. Since monoclonal antibodies were first produced in the 1970s (1) there have been significant advances in the ability to engineer antibodies. For example, the successful humanization of mouse antibodies, together with the ability to now produce completely human monoclonal antibodies, has allowed the development of highly specific and potent therapeutics (2). Due to the modular nature of these proteins a number of antigen-binding derivatives have been developed as potential therapeutics, including the fragment antigen-binding (Fab) and single chain variable fragment (scFv). In addition, a variety of more elaborate antibody-based therapeutics is under evaluation, which provide further functionality required for specific applications (3).

To date, a number of Fab fragments have been licensed as therapeutics, with several other antibody fragments in various stages of clinical development. There are currently >30 United States Food and Drug Administration approved, antibody-derived therapeutics available, with at least nine of these generating revenues of >1 billion dollars per year (4). Antibody-based therapeutics now constitute a large proportion of global therapeutic sales, and with development times comparable with small molecule drugs and higher success rates, will remain a major part of the pharmaceutical industry.

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The high specificity and affinity of antigen recognition lie at the heart of both therapeutic antibody development and the adaptive immune response. This is primarily mediated by the hypervariable loops formed by the complementarity determining regions (CDRs), however, the conformational properties of the CDR loops, together with potential conformational changes induced by antigen binding, remain poorly characterized. The lack of information relating to the antigen binding site also includes very limited knowledge of potential conformational heterogeneity and dynamics on a slow or fast timescale (seconds to picoseconds).

Almost all of the high resolution structures currently available for antibodies have been determined by x-ray crystallography, which limits the ability to gain insights into functionally important conformational dynamics. In addition, there are a relatively limited number of cases where structures have been obtained for both the free antibody and the complex formed with its target protein (5). Consequently, no clear picture has emerged concerning the potential conformational changes induced by antigen binding, and the structural nature of the CDR loops in both the presence and absence of antigen. NMR spectroscopy-based structural biology has now advanced to the point at which detailed structural information can be obtained for proteins and complexes of at least 100 kDa in size (6). This enables the use of NMR spectroscopy to probe the conformational features and properties of both free and antigen-bound antibody fragments, such as scFvs and Fabs.

In this paper, we report the acquisition and analysis of high quality three-dimensional 15N/1H and 15N/13C/1H NMR spectra for both free and antigen-bound scFvs and Fabs. This has allowed the determination of comprehensive sequence-specific backbone resonance assignments for the antibody fragments, which have revealed conserved structural changes within distinct antibody fragments upon binding to structurally diverse protein antigens. Furthermore, the NMR data show that the core of the antigen binding sites, in particular the CDR3 loops, exist in multiple conformational states that interconvert on a relatively slow timescale (milliseconds to seconds). Interestingly, this behavior appears to be a conserved feature of many high affinity protein-protein interaction sites (7–13), suggesting a key role in the formation of tight protein complexes.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The humanized anti-IL-6 scFv and anti-IL-1β Fab antibody fragments, human IL-1β and human IL-6 were all produced as soluble proteins in *Escherichia coli*-based expression systems. Tandem pTTod-based expression vectors encoding the heavy and light chain of the antibody fragments were transformed into *E. coli*-competent cells for the production of 15N, 15N/2H, 15N/13C/2H, or unlabeled protein. Cells containing the anti-IL-1β Fab vector were grown in either Luria-Bertani (LB)-rich medium, or a modified Spizizen’s minimal medium (16, 17) and expression induced at 37 °C (14). Uniformly 15N labeled and unlabeled anti-IL-6 scFv were similarly expressed; however, 15N/2H- and 15N/13C/2H-labeled proteins were produced using a small volume, high cell density expression procedure (18) to obtain high yields of deuterated protein from 50-mL cultures.

IL-1β was expressed in Tuner (DE3) pLysS *E. coli* cells at 25 °C, as described previously (14). For the production of IL-6, the expression vector was transformed into Origami B DE3 pLysS *E. coli* cells, which were grown in either LB, modified Spizizen’s minimal medium, or high cell density minimal medium (18), containing 100 μg/ml carbenicillin, 12.5 μg/ml tetracycline, 15 μg/ml kanamycin, and 34 μg/ml chloramphenicol. Cultures were grown to an A600 of 0.3–0.6 at 37 °C, cooled to 17 °C, IL-6 expression induced by the addition of 100 μM IPTG, and cells harvested after approximately a 16-h induction.

The anti-IL-6 scFv, IL-1β, and IL-6 were purified as described previously (14, 15) using a combination of nickel affinity (Ni-nitrilotriacetic acid) and size exclusion chromatography (Superdex 75). The anti-IL-1β Fab was also purified as reported previously (14) using a combination of protein G affinity (protein G-Sepharose) and size exclusion chromatography (Superdex 75). During the purification of deuterated samples of the anti-IL-6 scFv, the protein was fully denatured and refolded after nickel affinity chromatography to ensure complete exchange of any buried backbone amide groups. The scFv was denatured by the addition of 5 M guanidine hydrochloride and refolded by dialysis into a 100 mM sodium chloride and 25 mM Tris buffer at pH 7.5. Purified samples of the deuterated anti-IL-1β Fab were similarly denatured (6 M guanidine hydrochloride) and refolded by dialysis (100 mM sodium chloride and 25 mM sodium phosphate buffer at pH 6.5) to obtain complete exchange of backbone amide groups.

**Surface Plasmon Resonance**—On and off rate constants for antigen binding to immobilized antibody fragments were determined by surface plasmon resonance experiments carried out essentially as described previously (15).

**NMR Spectroscopy**—NMR spectra were acquired from 350-μL samples of the free and antigen-bound antibody fragments (170–500 μM) and of free and antibody-bound 15N, and 15N/2H and IL-6 (200–400 μM). The IL-1β, anti-IL-1β Fab, and IL-1β Fab complex were in a 100 mM sodium chloride, 25 mM sodium phosphate, 10 μM EDTA, 100 μM AEBSF, and 0.02% (w/v) sodium azide buffer at pH 6.5 (90% H2O/10% D2O). Backbone resonance assignments were initially obtained for IL-6 in a 100 mM sodium chloride and 20 mM sodium phosphate buffer at pH 6.4 (95% H2O/5% D2O). The anti-IL-6 scFv, IL-6-scFv complex and comparable free IL-6 samples were in a similar buffer containing 100 mM sodium chloride, 25 mM sodium acetate, 10 μM EDTA, 200 μM PMSF, and 0.02% sodium azide buffer at pH 5.5 (90% H2O/10% D2O). NMR data were collected at 35, 40, and 45 °C for free IL-1β, 25, 35, and 40 °C for free IL-6, 40 °C for the free and antigen-bound anti-IL-6 scFv, and at 45 °C for the free and IL-1β-bound Fab, using either 600 MHz Bruker DRX or
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800 MHz Bruker Avance spectrometers equipped with triple resonance ($^{15}$N/$^1$H/$^1$H) cryoprobes. A series of two- and three-dimensional TROSY-based spectra (19) were acquired to obtain sequence specific backbone resonance assignments for the anti-IL-6 scFv, anti-IL-1β Fab, and antigen-bound complexes, including $^{15}$N/$^1$H TROSY (19), $^{15}$N/$^{13}$C/$^1$H HNCO, HNCA, HN(CO)CA (20), HN(CA)CAB (21) and HN(CO)CACB (22). Typical acquisition times for the two-dimensional experiments were 30 ms in $F_1$ ($^{15}$N) and 60 ms in $F_2$ ($^1$H), with a total experimental time of 30 to 90 min. Typical acquisition times for the triple-resonance three-dimensional experiments were 6–9 ms in indirect $^1$H, 9–12 ms in indirect $^{15}$N, and 60–90 ms in direct $^1$H, with an overall experimental time of 60 to 96 h. Long range backbone amide NOE data were also obtained from $^{15}$N/$^1$H NOESY-TROSY and NOESY-HSQC spectra (23), which were recorded with acquisition times of 12–15 ms in indirect $^1$H, 9–12 ms in indirect $^{15}$N, and 60–90 ms in direct $^1$H, with an NOE mixing time of 500 ms for the anti-IL-1β Fab, 450 ms for the anti-IL-6 scFv-IL6 complex, and 700 ms for the free scFv. Total acquisition times for the NOE experiments were 87–92 h. A series of double- and triple-resonance spectra were also recorded to obtain essentially complete sequence-specific backbone resonance assignments for IL-6 and IL-1β, as described previously (14, 15).

Partially aligned samples of the free and IL-1β-bound anti-IL-1β Fab were produced by the addition of Pf1 phage (Asla Biotech) at 3–4 mg/ml. Backbone amide residual dipolar coupling values were derived from measured differences in the $^{15}$N/$^1$H scalar couplings seen in spectra acquired from isotropic and partially aligned samples, with the separation between equivalent peaks in $^{15}$N/$^1$H HSQC and temperature-compensated TROSY spectra used to determine the couplings (24). Individual two-dimensional spectra were collected for 5–12 h, with acquisition times of 60 ms in direct $^1$H and 50 ms in indirect $^{15}$N.

All NMR data were processed using Topspin 2.1 (Bruker Biospin) and analyzed using the SPARKY package (Goddard and Kneller, SPARKY 3, University of California, San Francisco).

Chemical Shift-based Mapping of Interaction Sites—Backbone chemical shift ($^{15}$N, $^{13}$C, and $^1$H) comparisons between free and bound antibody fragments and antigens were made using the minimal shift method (7–9) and by direct comparison of assigned backbone signals for the free and bound proteins where available. For the anti-IL-1β Fab, peaks from the comprehensively assigned HNCO spectrum of the free protein were compared with the unassigned HNCO spectrum of the Fab-IL-1β complex. In the case of the anti-IL-6 scFv, this approach was extended to combine an assigned free versus bound comparison with minimal shift analysis for residues that were not assigned in both states, which provides the fullest overall picture of the backbone chemical shift changes induced by complex formation. Minimal shift analysis was used to map the affects of antibody binding on IL-1β and IL-6.

Modeling of the scFv and Fab Structures—Homology models of the anti-IL-1β Fab and anti-IL-6 scFv were produced as described previously, with the residual dipolar coupling refined model of the anti-IL-1β scFv (PDB accession code 2KH2) used as a template for the anti-IL-6 scFv (14). The homology model obtained for the anti-IL-1β Fab was further refined using HADDOCK (25), with a combination of backbone amide residual dipolar coupling data, chemical shift-derived backbone dihedral angles, and backbone H$_{\alpha}$-H$_{\beta}$ NOEs included as experimental restraints (14). Analysis of the scFv and Fab structural models produced, including the mapping of antigen binding-induced chemical shift changes, was carried out using the PyMOL molecular graphics package.

RESULTS AND DISCUSSION

NMR Spectroscopy of Antigen-binding Antibody Fragments—NMR samples of the anti-IL-6 scFv and anti-IL-1β Fab were found to be stable for many days at 40 and 45 °C, respectively, which allowed the acquisition of a range of high quality two-dimensional and three-dimensional NMR spectra, as illustrated in Figs. 1 and 2. The spectra obtained show excellent dispersion of backbone signals ($^{15}$N, $^{13}$C, and $^1$H) and good signal to noise ratios. The line widths observed for resonances in highly deuterated samples of the antibody fragments allowed the recording of an extensive set of triple resonance spectra for both the free and antigen-bound anti-IL-6 scFv and for the free anti-IL-1β Fab. The correlations detected between backbone signals in these spectra, together with backbone amide NOEs identified in three-dimensional $^{15}$N-edited NOESY experiments, enabled the determination of comprehensive sequence-specific backbone resonance assignments ($H_{\alpha}$, $N$, $C_\alpha$, $C_\beta$ and $C'$) for both the anti-IL-6 scFv (free and bound to IL-6) and the free anti-IL-1β Fab using well established procedures (7, 8, 14, 26, 27).

The majority of the detectable backbone amide signals ($^{15}$N and $^1$H) were assigned for the free anti-IL-6 scFv, with 192 of the 218 possible backbone amide groups identified (88% excluding the linker (20), His$_{\gamma}$ tag (6), prolines (9), and N-terminal residue). Backbone amide signals were not identified for Ser$_6$, Leu$_{77}$, Asp$_{50}$, Ser$_{77}$, Phe$_{96}$, Gly$_{110}$, His$_{145}$, Asp$_{162}$, Met$_{163}$, Tyr$_{224}$, Cys$_{225}$, Arg$_{227}$-Glu$_{236}$, Gly$_{241}$, and Thr$_{242}$ (Fig. 3). The extent of the backbone assignments obtained for the scFv bound to IL-6 was slightly higher, with 198 possible backbone amide groups identified (91%). Backbone amide resonances were not assigned for Leu$_{67}$, Asp$_{90}$, Asp$_{76}$, Ser$_{77}$, Ser$_{91}$, Tyr$_{94}$, Trp$_{96}$, Thr$_{97}$, Gly$_{138}$, Gly$_{145}$, Tyr$_{224}$, Cys$_{225}$, His$_{228}$-Thr$_{231}$, Asp$_{233}$, Tyr$_{234}$, and Thr$_{242}$. For both the free and IL-6-bound scFv nearly all of the backbone amide-associated spin systems observed in spectra were assigned to specific residues, with only a few very weak (3–4) or incomplete systems (5–6) remaining unidentified.

For the free anti-IL-1β Fab, $^{15}$N and $^1$H resonances from 338 of the 419 assignable backbone amide groups were assigned (81% excluding prolines (23) and the two N-terminal residues). Backbone amide signals were not assigned for Ile$_2$, Gly$_{5}$-Ser$_5$, Ser$_9$, Asp$_{28}$, Trp$_{35}$, Gly$_{41}$, Gly$_{55}$, Asn$_{59}$, Gly$_{66}$, His$_{90}$, Leu$_{94}$, Phe$_{96}$, Ser$_{156}$, Gly$_{157}$, Ser$_{168}$, Ser$_{202}$, Ser$_{203}$, Gly$_{212}$, Gly$_{213}$, Gly$_{215}$, Val$_{216}$, Gly$_{222}$, Asp$_{242}$, Asp$_{247}$, Leu$_{259}$, Val$_{262}$, Ala$_{263}$, Gly$_{268}$, Gly$_{270}$, Tyr$_{273}$, Phe$_{274}$, Asp$_{276}$, Thr$_{277}$, Gly$_{280}$, Tyr$_{308}$, Phe$_{320}$, Ser$_{333}$, Ser$_{347}$-Thr$_{355}$, Val$_{362}$-Phe$_{365}$, Thr$_{380}$, Gly$_{382}$, Ser$_{392}$, Ser$_{393}$, Ser$_{395}$, Lys$_{414}$-Lys$_{438}$, and His$_{440}$-Ala$_{443}$ (Fig. 3). More than 94% of the backbone amide-associated spin
systems detected for the Fab were assigned to specific residues, with the assignment of only approximately 20 backbone amide signals remaining unclear. We have previously reported essentially complete backbone resonance assignments for the equivalent scFv bound to IL-1/IL-9252 (14). Spectra acquired for the scFv and Fab bound to IL-1/IL-9252 show strikingly similar shifts for backbone signals (HN, N, and C') arising from the shared antigen binding variable domains (VH and VL), which clearly indicates an essentially identical structure for the two variable domains and the interface formed with IL-1/IL-9252. This is also reflected in the near perfect overlay of 15N/1H HSQC spectra acquired for IL-1/IL-9252-bound scFv and Fab (14).

Analysis of HNCO spectra collected for the free and antigen-bound anti-IL-1/IL-9252 Fab revealed the appearance of 17 new peaks upon binding to IL-1/IL-9252. Comparison with the fully assigned spectrum available for the equivalent scFv bound to IL-1/IL-9252 (14) clearly showed that these new peaks arose from the backbone amides of residues in the antigen binding CDR loops, in particular, Asn31, Phe91, Trp92, Ser93, Leu94, and Phe96 in the VL domain and Asp247, Gly268, Gly270, Gln313, Asn314, Lys315, Lys316, Leu317, Thr318, Trp319, and Phe320 in the VH domain (Figs. 3 and 4). Overall, by reference to the assigned spectra of the free anti-IL-1/IL-9252 Fab and equivalent scFv bound to IL-1/IL-9252, 355 of the 419 (85%) assignable backbone amide signals (15N and 1H) were identified for the IL-1/IL-9252-bound Fab.

The completeness of the backbone assignments obtained for the antigen binding variable domains of the anti-IL-6 scFv and anti-IL-1/IL-9252 Fab are shown in Fig. 3, which highlights many similarities. The overall assignment level for both proteins is high; however, the assignments are slightly more complete for the anti-IL-6 scFv than for the anti-IL-1/IL-9252 Fab, with better coverage for both the CDR loops and the framework residues. Interestingly, for both the IL-6- and IL-1/IL-9252-targeted antibodies the assignment of the CDRs is incomplete in the absence of bound...
antigen. This lack of assignments for the CDR loops reflects the absence of backbone amide signals from many residues present in these regions, in particular, for CDR3 of both the VL and VH domains, which indicates the presence of a number of discrete structural states interconverting on a relatively slow timescale (milliseconds to seconds) (28). The striking similarity in the behavior of the CDRs in both the anti-IL-1/β/H9252 Fab and anti-IL-6 scFv suggests that conformational heterogeneity is a common feature of antibody CDR loops, particularly toward the core of the antigen binding surface.

Comparison of the extent of the backbone assignments obtained for the variable domains (V_{\text{L}} and V_{\text{H}}) of the free and antigen-bound forms of the anti-IL-6 scFv (A) and anti-IL-1/β Fab (B). Residues for which backbone resonance assignments were made are highlighted in gray for both the free and antigen-bound antibody fragments. The positions of elements of regular secondary structure and CDR loops are also indicated, with α-helices shown as red bars, β-sheets as green arrows, and CDRs as green boxes. The overall assignment level for the free scFv is slightly higher than for the Fab, but for both antibodies backbone signals from residues in CDR3 of V_{\text{L}} and V_{\text{H}} remain largely unassigned due to the absence of detectable backbone amide resonances.

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Comparison of the extent of the backbone assignments obtained for the antigen-bound forms of the anti-IL-6 scFv and anti-IL-1/β Fab reveals a marked difference between the two antibody/antigen systems (Figs. 3 and 4). In both cases, a number of backbone amide signals were recovered upon antigen binding, which were assigned to CDR loop residues and are highlighted in green for both the free and antigen-bound antibody fragments. The majority of these signals appeared for the anti-IL-1/β Fab on binding to IL-1/β, but remained unobservable for the anti-IL-6 scFv bound to IL-6. The striking similarity in the behavior of the CDRs in both the anti-IL-1/β Fab and anti-IL-6 scFv suggests that conformational heterogeneity is a common feature of antibody CDR loops, particularly toward the core of the antigen binding surface.
bound to IL-6 a number of residues in $V_H$ CDR2 (Ser$^{179}$, Thr$^{181}$, Ser$^{183}$, Gly$^{184}$, Thr$^{187}$, Tyr$^{188}$, Arg$^{190}$, Ser$^{192}$, and Val$^{193}$) and either in, or adjacent to $V_H$ CDR3 (Leu$^{232}$, Phe$^{235}$, Tyr$^{237}$, Gln$^{240}$, and Gly$^{241}$), show significantly broader amide proton signals ($>30$ Hz) than observed for the majority of the protein (overall average of $26.3 \pm 4.2$ Hz), as shown in Fig. 5. This is consistent with some remaining slow conformational exchange behavior at the antigen binding site of the $V_H$ domain, which is presumably possible due to the relatively flexible nature of the IL-6 structure (15).

**Mapping of Antigen Binding Sites**—The effects of antigen binding on the backbone signals ($H_N$, $N$, and $C\alpha$) of the anti-IL-6 scFv and anti-IL-1$\beta$ Fab were determined by a combination of minimal and actual shift analyses of HNCO spectra obtained for the free and bound antibody fragments. The results from the chemical shift analyses were mapped on to a homology model of the anti-IL-6 scFv (Fig. 6) and a refined homology model of the anti-IL-1$\beta$ Fab (Fig. 7). The locations of residues affected by antigen binding further highlight the absence of NMR signals from a significant number of CDR residues, particularly in the CDR3s of both the $V_H$ and $V_L$ domains (Figs. 6 & 7). The binding-induced chemical shift changes also clearly show that the visible CDR signals are highly perturbed by antigen binding. An antigen binding site can be clearly identified on both the anti-IL-6 scFv and on the anti-IL-1$\beta$ Fab, which is further confirmed by NMR data obtained for the corresponding IL-1$\beta$ scFv (14).

Interestingly, for both antibody fragments further antigen-induced structural changes were revealed by chemical shift changes that lie beyond the CDR loops. These include changes in the framework residues supporting the CDR loops and in residues at the interface between the $V_L$ and $V_H$ domains. There are striking similarities in the regions affected for both antibody fragments despite binding to structurally diverse antigens, which strongly suggests that conserved conforma-

**FIGURE 5.** Linewidths of backbone amide proton NMR signals in a typical TROSY-HNCO spectrum of the anti-IL-6 scFv bound to IL-6. The positions of the variable domains ($V_L$ and $V_H$) and antigen binding CDR loops are indicated above the histogram. Significantly broader backbone amide signals were observed for a number of residues in $V_H$ CDR2 and either in, or adjacent to $V_H$ CDR3, which is consistent with slow conformational exchange in the antigen binding region of the $V_H$ domain.

**FIGURE 6.** Mapping of the IL-6 interaction site on the anti-IL-6 scFv. The combined $H_N$, $N$, and $C\alpha$ chemical shift changes for residues between free and IL-6-bound anti-IL-6 scFv were assigned a color depending on their magnitude, with shifts of $<0.002$ ppm colored white, $>0.03$ ppm colored red, and those in between colored on a linear gradient from white to red. Similarly, for residues with backbone signals that remained unassigned in the free or bound state antigen-induced changes were determined by the minimal shift approach, with shifts of $<0.002$ ppm colored white, $>0.03$ ppm colored blue, and those in between colored on a linear gradient from white to blue. Residues for which no chemical shift data could be obtained are colored yellow. The chemical shift changes observed are mapped onto both a ribbon representation of the scFv and a surface view of the antigen binding site. The most substantial changes are seen in the CDR loops and the interface between the two variable domains.

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Conformational changes beyond the CDR loops are important for effective antigen binding. In contrast to the variable domains, the constant domains present in the anti-IL-1/H9252 Fab show almost no signs of chemical shift perturbation except at the VL/CL interface. This most likely reflects movements of the VL domain relative to the rest of the protein rather than structural changes within the constant domain.

Mapping of Antibody Binding Sites—Nearly complete backbone and side chain assignments have been reported previously for IL-1/H9252 and IL-6 (29, 30), but under solution conditions different from those required for this study. It proved relatively straightforward to obtain essentially complete backbone resonance assignments for both cytokines under the new conditions, with backbone amide signals identified for all nonproline residues apart from Glu51, Lys54, and Glu69 in IL-6 (98%), and Ala1, Arg4, Asn53, and Glu64-Asn66 in IL-1/H9252 (96%). The backbone assignments obtained for IL-6 and IL-1β allowed further assessment of the nature of the interaction between the antibody fragments and their target antigens (14, 15), with minimal shift analysis providing information on the location of the antibody binding surfaces and of any structural changes induced in the bound proteins.

IL-1β shows a discreet antibody binding site, with substantial and highly localized chemical shift perturbations (Fig. 8A), consistent with a stable structure that is not significantly affected by antibody binding. In stark contrast, IL-6 shows significant perturbation of NMR signals throughout the protein as a result of scFv binding (Fig. 8B). This probably reflects the somewhat plastic and dynamic nature of the IL-6 structure, which has been described previously (15, 29), and may be related to the biological function of IL-6 and conformational changes associated with the formation of the IL-6/IL-6R/gp130 signaling complex (15, 31). This in turn may explain the continued lack of observable backbone amide signals for residues in both CDR3 loops of the anti-IL-6 scFv bound to IL-6, with the inherent flexibility of IL-6 allowing continued exchange between distinct structural states for the contacting CDR loops. This property of the IL-6-scFv complex is in contrast to the IL-1β-Fab, or equivalent IL-1β-scFv complex, in which the majority of the backbone amide CDR signals are seen (Fig. 4 and Ref. 14).

The exchange between multiple conformational states on a relatively slow timescale, particularly for the CDR3s, is a conserved feature of the two distinct antibodies that have been characterized. The lack of backbone amide signals for these regions implies that a number of discreet structural states are being sampled on a millisecond to second timescale. This behavior is conserved in two antibodies with very different antigen binding sites and has recently been seen in another heavy
chain antibody (32), which strongly suggests that conformational heterogeneity at the heart of the antigen binding site may be a critical feature to ensure effective binding of target proteins. This may simply reflect the need to accommodate flexible surfaces on protein antigens, but also has the potential to play a key role in facilitating productive binding to an extensive contact surface (typically 1700 ± 260 Å² (33)).

The slow dynamics of CDR loops at the heart of the antigen binding site and associated sampling of multiple conformations may allow antibodies to recognize and bind protein antigens when approaching at a less than optimal orientation. If this conformational heterogeneity in the CDRs allows the initiation of binding at some significant variance from the ideal orientation for interaction, it could greatly improve the chances of binding and therefore the on rate ($k_o$) for antibody-antigen complex formation (Fig. 9, anti-IL-1β $k_o = 1.4 \times 10^6$ M$^{-1}$s$^{-1}$, $K_D = 80$ pm and anti-IL-6 $k_o = 2.36 \times 10^6$ M$^{-1}$s$^{-1}$, $K_D = 41$ pm). This soft capture type mechanism would also allow initial intermolecular interactions at nonoptimal orientations, facilitating final docking of the proteins. The slow exchange between multiple conformations and relatively high affinity of interactions involving CDR3s appears well suited to the soft capture of protein antigens, perhaps assisted by movement of the variable domains relative to each other. This process could also serve to guide the target protein into the optimal orientation for interactions with residues in the apparently less dynamic CDR1 and CDR2 loops, which make important contributions to overall affinity and specificity (Fig. 10).

Conformational heterogeneity and associated slow motions appear to be a feature of many high affinity protein-protein interaction sites, involved in a diverse range of processes from receptor-mediated signaling to control of gene expression (7–13). This perhaps suggests that a soft capture type mechanism may be a key feature of many high affinity protein-protein interactions, facilitating a rate of complex formation consistent with that required by a range of biological processes.

Changes in the conformations and dynamics of the CDR3 loops induced by antigen binding appear to be accompanied by reorientations of the antibody variable domains relative to each other, with significant chemical changes seen for residues at the $V_{H}/V_{L}$ interface in both the anti-IL-1β Fab and anti IL-6 scFv (Figs. 6 and 7). A number of previous studies have reported antigen-induced changes in the orientation of the variable domains; however, no consistent picture has emerged (34–37). This may reflect the difficulty in detecting significant but small structural changes in proteins, which can now be reliably identified for relatively large proteins and complexes through the high sensitivity of backbone and side chain NMR chemical shifts to changes in conformation (6–9, 14, 15, 38).

CONCLUSIONS

The work reported here describes the results of an NMR-based study of the structural characteristics of the antigen binding regions of antibodies selected for their ability to recognize target proteins with very high specificity and affinity. This has revealed conformational heterogeneity and slow dynamics at
the heart of the antigen interaction sites, together with structural changes beyond the contact surfaces induced by antigen binding. The characterization of antibody fragments against two structurally distinct proteins, with IL-6 typical of a relatively flexible α-helical structure and IL-1β representative of a fairly rigid β-sheet topology, has allowed the identification of antibody features and properties associated with antigen recognition that are independent of the structure of the target protein.

A number of structural features and properties have been identified that are common to both the anti-IL-1β Fab and anti-IL-6 scFv. Perhaps the most interesting is the finding that the two distinct binding surfaces demonstrate similar conformational heterogeneity and slow dynamics for residues within the CDR3 regions of both variable domains prior to antigen binding. This sampling of a number of distinct conformations, leading to missing backbone NMR signals, appears to be an important feature of the heart of the antigen binding site, which is likely to play a key role in the recognition and/or binding of protein antigens. This inherent flexibility, sampling conformations on a millisecond to second timescale, may mediate an initial soft capture of the antigen over a range of suboptimal orientations of the two proteins, facilitating correct engagement of the antigen with other less flexible and potentially lower affinity CDR residues. This type of mechanism may help to guide initial antibody-antigen contacts down a productive binding pathway, resulting in an increased ability to bind at less than perfect orientations, which may significantly improve the rate of complex formation compared with rigid protein interaction surfaces.

Further antibody structural changes also take place following antigen binding, with an apparent change in the orientation of the two variable domains in both the anti-IL-1β Fab and anti-IL-6 scFv. Flexibility at the VH/VL interface may further facilitate the soft capture mechanism by allowing an additional level of adaptability. However, changes in VH/VL orientation could also be linked to processes beyond antigen recognition, such as the initiation of B cell receptor signaling, which remains poorly understood. The conformational heterogeneity and slow dynamics reported here appears to be shared by many high

**FIGURE 9. Binding of protein antigens to captured antibody fragments.** Typical surface plasmon resonance sensorgrams are shown for the binding of IL-6 and IL-1β to captured anti-IL-6 scFv (A) and anti-IL-1β Fab (B), respectively. The responses obtained indicate very high affinity antigen binding for both antibody fragments, with a $K_D$ of 41 pM determined for the anti-IL-6 scFv and 80 pM for the anti-IL-1β Fab.
affinity protein-protein interaction surfaces across a wide range of biological processes, which is consistent with a potentially important role for the soft capture, protein-protein docking mechanism in high affinity complex formation (7–13).

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