

Refined crystal structure of the interleukin-1 receptor antagonist Presence of a disulfide link and a *cis*-proline

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Interleukin-1 (IL-1) molecules are cytokines involved in the acute-phase response against infection and injury. Three naturally occurring IL-1 molecules are known, two agonists: IL-1 α and IL-1 β , and one antagonist, the IL-1 receptor antagonist (IL-1ra). Although IL-1 action protects the organism by enhancing the response to pathogens, its overproduction can lead to pathology and has been implicated in disease states that include septic shock, rheumatoid arthritis, graft versus host disease and certain leukemias. The crystal structure of IL-1ra has been solved at 0.21-nm resolution by molecular replacement using the IL-1 β structure as a search model. The crystals contain two independent IL-1ra molecules which are very similar. IL-1ra has the same fold as IL-1 α and IL-1 β . The fold consists of twelve β -strands which form a six-stranded β -barrel, closed on one side by three β -hairpin loops. Cys69 and Cys116 are linked via a disulfide bond and Pro53 has been built in the *cis*-conformation. Comparison of the IL-1ra structure with the IL-1 α and IL-1 β structures present in the Protein Data Bank shows that a putative receptor interaction region, involving the N-terminus up to the beginning of strand β 1 and the loops D and G, is very different in the three IL-1 molecules. Other putative interaction regions, as identified with mutagenesis studies, are structurally conserved and rigid, allowing precise and specific interactions with the IL-1 receptor.

Keywords. Cytokine; interleukin-1 receptor antagonist; acute-phase response; crystal structure; X-ray.

Cytokines are proteins that act as powerful regulatory factors between cells. They are produced by many mammalian cells and exert their influence via binding to surface receptors (Henderson and Blake, 1992). Interleukin-1 (IL-1) is a cytokine that is involved in the acute-phase response to infection and injury (see Henderson and Blake, 1992; Dinarello, 1991; and Baumann and Gauldy, 1994, for reviews). The production of IL-1 is extremely sensitive to endotoxins (bacterial lipopolysaccharides). Human blood monocytes start synthesizing IL-1 after stimulation by as little as 10–20 pg/ml of endotoxin. IL-1 produces a wide variety of biological and physiological responses. Intravenous infusion of low doses (1–10 ng/kg) of interleukin-1 β (IL-1 β) causes an increase in the number of circulating neutrophils and platelets (Tewari et al., 1990), while at higher doses it causes fever, sleepiness, hypotension and many other effects (Dinarello and Thompson, 1991). IL-1 is able to activate T-cells in the presence of suboptimal concentrations of antigens or mitogens.

At least four different cytokine molecules participate in eliciting the acute-phase response. These are the two IL-1 molecules which have been characterized: interleukin-1 α (IL-1 α) and IL-1 β (both 17.5 kDa), tumor necrosis factor α (17 kDa) and IL-6 (26 kDa). Two types of IL-1 receptor molecules have been

characterized. The type I receptor is a 80-kDa transmembrane protein with a proven signaling function (Stylianou et al., 1992). The type II receptor is a 68-kDa protein with only a short cytoplasmic tail. Evidence has been presented that the type II receptor inhibits IL-1 activity by acting as a decoy target (Colotta et al., 1993). Additional control of the IL-1 activity is provided by the IL-1 receptor antagonist (IL-1ra), a protein which binds to the IL-1 receptors, but which does not evoke any biological response (Hannum et al., 1990; Carter et al., 1990). The importance of the action of IL-1 is emphasized by the involvement of multiple cytokine molecules with overlapping functions while the need for tight control of this action is evidenced by the presence of multiple regulatory pathways.

Although IL-1 action protects the organism by enhancing the response to pathogens, its overproduction can lead to pathological symptoms. IL-1 is a key mediator of inflammation and has been implicated in disease states that include septic shock, rheumatoid arthritis, graft versus host disease and certain leukemias. Preliminary studies have indicated that inhibition of the action of IL-1, either by using the IL-1ra protein to block the IL-1 receptors, or by using soluble IL-1 receptors to bind excess IL-1, could have therapeutic benefits (Henderson and Blake, 1992; Dinarello and Thompson, 1991; Ohlsson et al., 1990). Recombinant IL-1ra is currently being tested in clinical trials for treating septic shock, rheumatoid arthritis and chronic myelogenous leukemias (Dinarello and Thompson, 1991; Bone, 1991). Studies in mice have shown that administration of soluble IL-1 receptor could arrest the progress of experimental arthritis (Schorlemmer

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Abbreviations. IL-1, interleukin-1; IL-1ra, interleukin-1 receptor antagonist; rms, root-mean-square.

Note. The coordinates published here have been deposited in the Brookhaven Protein Data Bank with reference code 1ILR.

et al., 1993) and that it had a profound inhibitory effect in the development of *in vivo* alloreactivity (Fanslow et al., 1990).

It is clear that compounds which are able to inhibit the action of IL-1 could have a significant potential as drugs. Knowledge of the three-dimensional structures of the two IL-1 molecules and of the IL-1ra molecule could assist the design of such inhibitors. The crystal structure of IL-1 α has been solved (Graves et al., 1990) and the structure of IL-1 β has been solved by at least five different groups by X-ray crystallography (Priestle et al., 1988; Finzel et al., 1989; van Oostrum et al., 1991; Veerapandian et al., 1992; Oldfield et al., 1993; Ohlendorf et al., Protein Data Bank entries 5I1B and 8I1B) and by one group with NMR (Clöre et al., 1991). In addition, the crystal structure of an IL-1 β mutant has been published (Camacho et al., 1993). In this paper, we present the crystal structure of the IL-1ra protein, refined at 0.21-nm resolution. The protein contains a disulfide link between Cys69 and Cys116 and Pro53 has been fitted in the *cis*-conformation. Two other groups have recently reported a similar crystal structure (Clancy et al., 1994; Vigers et al., 1994) but they did not report the presence of a disulfide link and a *cis*-proline.

EXPERIMENTAL PROCEDURES

Expression and purification. The IL-1ra gene was purchased from British Biotechnology Labs (BBL). For expression purposes, the gene was inserted into the TAC-*Bsp* vector (a modification of the TAC-RBS vector described by Zurawski et al., 1986). The construct was made by cutting the BBL IL-1ra vector with *Hind*III and *Bam*HI and isolating the *Hind*III-*Bam*HI fragment. This was then inserted into a *Bsp*MI/*Bam*HI-digested TAC-*Bsp* vector with the addition of two linker oligonucleotides to bridge the restriction site and to put the gene in frame.

Recombinant IL-1ra protein was expressed in *Escherichia coli* as follows. Recombinant *E. coli* was grown in a 6-l fermenter overnight at 37°C in Luria broth containing 50 µg/ml ampicillin. Bacterial cells were harvested, washed with Tris/EDTA (25 mM Tris/HCl pH 8.0 and 1 mM EDTA) and stored at -80°C until use. Frozen cells were resuspended in 100 ml 25 mM Tris/HCl pH 8.0, 10 mM EDTA and 1 µg/ml leupeptin (Calbiochem), and lysed by sonication. After centrifugation, ammonium sulfate to a final concentration of 1.5 M was added to the supernatant that was then incubated overnight at 4°C. The suspension was clarified by centrifugation and loaded on a Phenyl-Sepharose Fast Flow (Pharmacia) column (80 ml), pre-equilibrated with Tris/EDTA containing 1.5 M ammonium sulfate. The column was eluted with a 180-ml linear gradient of 1.5–0 M ammonium sulfate. Fractions containing IL-1ra (detected by SDS/PAGE analysis) were pooled, dialyzed against Tris/EDTA, and passed on a MonoQ column using the FPLC system (Pharmacia), with a linear gradient of 0–0.5 M NaCl in Tris/EDTA. Ammonium sulfate up to 1.5 M was added to the IL-1ra fraction and the solution was passed once again through the Phenyl-Sepharose column, using a step elution with Tris/EDTA containing no NaCl to concentrate and desalt. The protein was finally purified to homogeneity (by SDS/PAGE criteria) by gel filtration on a 150-ml Sephacryl-200 (Pharmacia) column. About 50 mg pure protein was obtained from a 6-l fermentation.

The recombinant protein has two differences with natural human IL-1ra: (a) it contains an extra Met at the N-terminus and (b) it is non-glycosylated. However, biological experiments have established that recombinant IL-1ra has identical behavior to the natural product (Eisenberg et al., 1990; Ann Akesson, unpublished results).

Crystallization. Recombinant IL-1ra was crystallized using the hanging-drop method under the following conditions. A pro-

tein solution was prepared by mixing a solution of 20 mg/ml IL-1ra, 30 mM NaCl and 10 mM sodium phosphate pH 7.3 with an equal volume of a solution of 5% (mass/vol.) poly(ethylene glycol) 8000, 100 mM Tris/HCl pH 8.0 and 100 mM ammonium acetate. Drops (5 µl) of the protein solution were set to equilibrate at 4°C against 1 ml of a solution of 30% (mass/vol.) poly(ethylene glycol) 8000. Lozenge-shaped crystals with dimensions of up to 0.2×0.6×0.8 mm³ appeared after one or two months. The crystals diffract to about 0.20-nm resolution. The space group is P₄₃2₁2 with *a* = *b* = 7.13 nm and *c* = 11.73 nm. The asymmetric unit contains two IL-1ra molecules. The specific volume *V*_m = 0.00229 nm³/Da and the solvent content calculated from this number (Matthews, 1968) is 46%. The space group is identical to the space group reported by Clancy et al. (1994) and Vigers et al. (1994) for their IL-1ra crystals and also the cell dimensions are quite similar.

Data collection. X-ray data were collected using a multiwire area detector (Siemens). The crystals were cooled to 4°C during data collection, the temperature at which they were grown. The crystal-detector distance was 160 mm and the exposure time was 120 s/frame of 0.25°. Nine scans at 2 θ = 30°, 2 θ = 25° and 2 θ = 10° were performed. Data were processed using the XGEN package (Howard et al., 1987). A total of 199878 reflections were measured, which yielded 16972 unique reflections, representing 92.7% of all possible reflections to 0.21 nm. The highest resolution shell (0.22–0.21 nm) is 60.1% complete and has an average *I*/ σ _i of 2.0.

In addition, we collected data on 13 crystals which had been soaked or crystallized in the presence of heavy atom reagents. However, these data sets were not used to solve the phase problem and will not be discussed here. The heavy atom positions obtained from a mercury cyanide derivative confirmed the results of the molecular replacement procedure.

Molecular replacement. IL-1ra has the same number of amino acids as IL-1 β and has 23% sequence identity. IL-1ra also binds to the same receptors as IL-1 β , although IL-1ra binding does not elicit a response. Also the NMR results by Stockman et al. (1992) indicated that both proteins have a similar fold. We attempted, therefore, to solve the IL-1ra structure with search models based on the structure of IL-1 β (Priestle et al., 1989). However, using the programs Merlot (Fitzgerald, 1988) and Xplor (Brünger, 1990), we did not find any clear solution, either with the complete IL-1 β model, or with truncated or backbone models of IL-1 β . Also, IL-1ra models obtained by homology building and models obtained by including published NMR restraints (Stockman et al., 1992) failed to produce a clear solution. This lack of results fits well with the experience of Clancy et al. (1994) who solved the IL-1ra structure with heavy atoms because their attempts to use molecular replacement were unsuccessful, and the experience of Vigers et al. (1994), who could only solve the molecular replacement problem for IL-1ra in a triclinic space group and not in the tetragonal space group used by us and by Clancy et al. (1994).

However, by using an automated procedure, as implemented in the program AMoRe (Navaza, 1994), using the IL-1 β structure as determined by Finzel et al. (1989) and by placing two molecules, we obtained a solution for data between 0.80–0.35-nm resolution with a correlation coefficient of 29.6%. The next best solution had a correlation of 23.4%. The molecular replacement procedure was repeated for the two enantiomorphic space groups P₄₁2₁2 and P₄₃2₁2, but a solution was only obtained in space group P₄₃2₁2, thus establishing the latter as the correct space group.

The position of the two search models is in agreement with solvent boundaries as observed in the preliminary electron density map based on two heavy atom derivatives (data not shown).

Table 1. Residues used in the superposition of IL-1ra with IL-1 α and IL-1 β .

IL-1ra	IL-1 α
13–28	18–33
29–37	35–43
42–52	46–56
54–71	64–81
78–89	86–97
95–125	102–132
128–135	133–140
140–148	142–150
IL-1ra	IL-1 β
8–27	3–22
28–52	24–48
54–83	56–85
95–138	96–139
140–152	140–152

and on the heavy atom positions we had found. When compared with the final model, the four mercury positions of a mercury(II) cyanide derivative are at 0.10–0.25-nm from cysteine S γ atoms. The first mercury position is at 0.12 nm of the S γ of Cys122I (I denotes the first independent IL-1ra molecule, II the second). The second mercury is at 0.25 nm of the S γ of Cys122II. The third mercury is at 0.14 nm of the S γ of Cys69I and at 0.10 nm of the S γ of Cys116I, which form a disulfide link in the native structure. Finally, the fourth heavy atom position is at 0.17 nm of the S γ of Cys69II and at 0.16 nm of the S γ of Cys116II. Two mercury ions appear to have inserted into disulfide links which is somewhat unusual because normally disulfide links need to be reduced first before they react with mercury. The different batch of protein used for the preparation of the heavy atoms might have lacked the disulfide link or, alternatively, the geometry or surroundings of the disulfide link might have promoted insertion of the mercury ions.

Refinement. All side chains of the two oriented and positioned IL-1 β molecules were changed into alanine molecules and the structure was refined using X-plor (Brünger, 1992). Energy minimization using data between 0.80–0.30 nm lowered the *R*-factor from 0.546 to 0.418. Subsequent molecular dynamics refinement at the same resolution, using a slow cooling protocol going from 3000 K to 300 K followed by refinement of individual, isotropic temperature factors, yielded an *R*-factor of 0.336. The electron density map based on this model allowed about 50% of the side chains to be placed. Subsequent molecular dynamics and individual, isotropic temperature factor refinement yielded an *R*-factor of 0.284 for data between 0.80–0.30 nm. Sixteen rounds of map inspection, rebuilding and refinement with gradually increasing resolution were performed. Water molecules were assigned by searching $F_o - F_c$ maps for peaks of at least 4 σ which are between 0.22–0.45 nm of protein atoms or other water molecules. During refinement, we discovered that the diffraction of our IL-1ra crystal was strongly anisotropic. Refinement of overall anisotropic B-factors yielded a value of 0.065 nm² for B11 and B22, and of –0.131 nm² for B33. The final model was obtained after removal of all water molecules with temperature factors higher than 0.70 nm² followed by two more rounds of map inspection, energy minimization and refinement of individual, isotropic temperature factors. The final model contains 139 water molecules (about 70/IL-1ra molecule). The final *R*-factor is 0.199 for all 16599 observed reflections between 0.80–0.21 nm and 0.186 for 14556 reflections greater

Table 2. Results of the superposition of IL-1ra with itself and with IL-1 α and with IL-1 β . Listed are the root-mean-square (rms) differences between equivalent C α atoms as defined in Table 1. I refers to the first independent IL-1ra molecule in the crystals, II to the second.

Superposition	Rms differences (number) for	
	all C α atoms	C α atoms differing <3.0 σ
	nm	
IL-1raI–IL-1raII	0.09 (144)	0.02 (101)
IL-1raI–IL-1 α	0.17 (114)	0.07 (78)
IL-1raII–IL-1 α	0.18 (114)	0.08 (79)
IL-1raI–IL-1 β	0.15 (132)	0.04 (72)
IL-1raII–IL-1 β	0.15 (132)	0.06 (94)

than 2 σ . Using data from infinity to 0.21-nm resolution, we obtain an *R*-factor of 0.275 for all 16962 reflections and an *R*-factor of 0.264 for 14914 reflections greater than 2 σ . These latter two *R*-factors are high because we did not apply any correction for the scattering of bulk solvent and the low resolution data were not included in the refinement.

Superpositions. The crystal structures of IL-1 α (Graves et al., 1990; Protein Data Bank code 2ILA) and IL-1 β (Finzel et al., 1989; Protein Data Bank code 1I1B) were superimposed onto the IL-1ra structure as follows. In a first step, the C α backbones of IL-1 α and IL-1 β were manually superimposed onto the backbone of IL-1ra. This manual superposition was used to define structurally equivalent regions. The superposition of these regions was subsequently optimized using the algorithm of Kabsch (1976). Residue pairs deviating more than three standard deviations were excluded from the superposition. The structurally equivalent regions were reassessed using the optimized superposition and the procedure was repeated until convergence was reached. The final parameters are listed in Tables 1 and 2.

RESULTS

Overall structure. The asymmetric unit of our crystals contains two independent IL-1ra molecules. The final model contains residues 8–152 of the first molecule and residues 9–152 of the second molecule. In addition, 139 water molecules have been added to the structure. The mean coordinate error as estimated from a Luzzati plot (Luzzati, 1952) is 0.02–0.03 nm. The structure has excellent geometry with root-mean-square (rms) deviations of 0.0008 nm for bond lengths and 1.5° for bond angles. All non-glycine residues have ϕ, ψ angles within or close to allowed regions (see Fig. 1). A disulfide link is present between Cys69 and Cys116 (see Fig. 2A). Pro53 has been modeled in the *cis*-conformation. It is part of a flexible loop and its electron density is not very well defined. However, attempts to refine Pro53 in the *trans*-conformation led to unallowed ϕ, ψ angles and $F_o - F_c$ difference maps showed peaks which clearly indicated that a *cis*-proline should be fitted. The fit of Pro53 in a $2F_o - F_c$ omit map is indicated in Fig. 2B.

The IL-1ra molecule has twelve β -strands, two very short 3_{10} helices and no α -helices (see Tables 3 and 4). Following Grütter et al. (1994), we numbered the β -strands 1–12 and named the connecting loops A–K (see Figs 3 and 6). The twelve β -strands form a six-stranded β -barrel (made by strands 1, 4, 5, 8, 9 and 12) which on one side is closed by three β -hairpin loops (made by strands 2, 3, 6, 7, 10 and 11). A schematic drawing of the IL-1ra molecule is presented in Fig. 3. A pseudo-three-fold axis

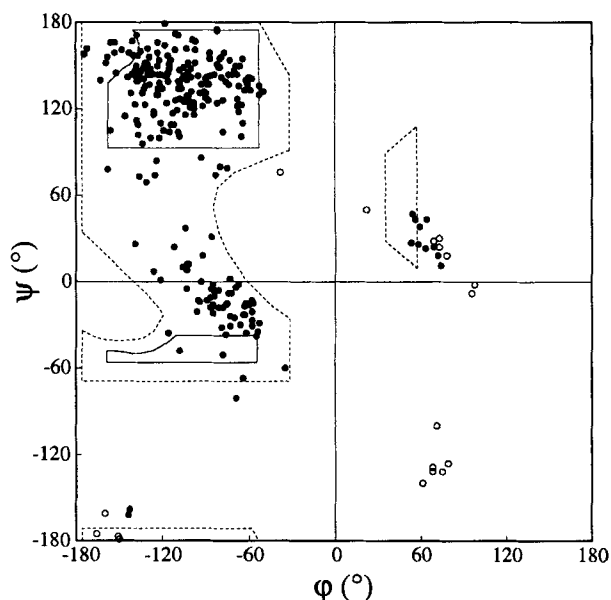


Fig. 1. ϕ/ψ plot of the two independent IL-1ra molecules. (○) Glycine residues; (●) non-glycine residues. All non-glycine residue are close to or within allowed regions. Pro53 in both molecules is in the *cis*-conformation. Vigers et al. (1994) report that Asn27 and Asp52 (the latter should be Glu52 according to the published sequences) are outliers in the ϕ/ψ plot. Asn27 in our model has ϕ/ψ angles of $54^\circ, 57^\circ$ (molecule I) and $59^\circ, 38^\circ$ (molecule II) which are in the region of a left-handed α -helix. The fit to the electron density map is reasonable but Asn27 is part of the flexible loop B which may have led to a different interpretation by Vigers et al. (1994). Our early models with Pro53 in the *trans*-conformation led to deviating ϕ, ψ angles for Glu52. The deviating ϕ, ψ angles of Asp(?)52 reported by Vigers et al. (1994) suggest that they have modeled Pro53 in the *trans*-conformation.

coincides with the barrel axis, relating three similar folding motifs consisting of a barrel strand, a β -hairpin loop followed by a second barrel strand. The two loops which connect the first three β -strands of the folding motif (loops A, B, E, F, I and J) are short (2–7 residues), while the third loop in each motif (loops C, G and K) is long (11–17 residues). The long loops C, G and K connect the hairpin loop with the second barrel strand (strands 4, 8 and 13) in the folding motif, and, as will be discussed below, these long loops vary with respect to IL-1 α and IL-1 β .

The topology of the IL-1ra molecule with its 12 antiparallel β -strands is identical to that of Kunitz-type soybean trypsin inhibitor (Sweet et al., 1974) and many other proteins including IL-1 α (Graves et al., 1990), IL-1 β (Priestle et al., 1988, 1989; Finzel et al., 1989; van Oostrum et al., 1991; Veerapandian et al., 1992; Oldfield et al., 1993; Ohlendorf et al., Protein Data Bank entries 5I1B and 8I1B; Clore et al., 1991), acidic and basic fibroblast growth factor (Eriksson et al., 1991, 1993; Zhang et al., 1991; Zhu et al., 1991), the ricin toxin B chain (Rutenber and Robertus, 1991), *Erythrina caffra* trypsin inhibitor (Onesti et al., 1991), hisactophilin (Habazetti et al., 1992), and K/ α amylase inhibitor (Zemke et al., 1991). This fold has been named Kunitz fold or interleukin-1 fold and has been described in great detail by Murzin et al. (1992) and by Swindels and Thornton (1993).

A superposition of the two independent IL-1ra molecules which are present in the crystals is shown in Fig. 4. The two molecules are virtually identical (0.02 nm rms difference for 101 equivalent Ca atoms). The only significant difference is a rigid body rotation of loop F between strands β_6 and β_7 which hinges around Lys71 and Thr76. Gly73, furthest away from the pivot

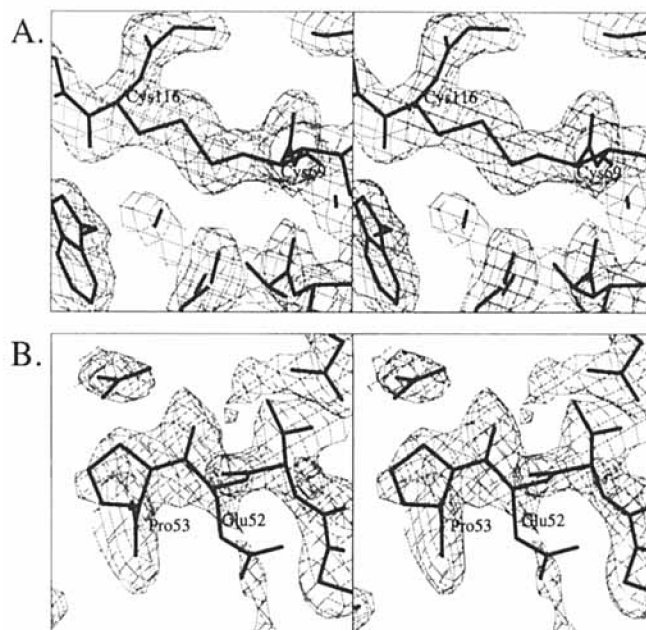


Fig. 2. Stereo diagrams of $2F_o - F_c$ omit-electron density maps of IL-1ra, contoured at 1.0σ . The maps were calculated after deleting the S γ atoms of Cys69 and Cys116 for A and Glu52 and Pro53 completely for B, followed by 120 cycles of positional refinement. (A) Omit-electron density of the disulfide link between Cys69 and Cys116 (molecule I). The strong and continuous electron density unambiguously establishes the presence of this disulfide link in the IL-1ra molecule I. The omit-electron density map looks the same for molecule II. (B) Omit-electron density of the *cis*-Pro53 (molecule I) which is part of the rather flexible loop D. Hardly any electron density is present for the side chain of Glu52, presumably because it moves freely around in the solvent. Attempts to refine Pro53 in the *trans*-conformation led to unacceptable ϕ, ψ angles and peaks in the difference maps which suggested the presence of the *cis*-conformation.

Table 3. β -strands in IL-1ra. Assignments were made with the program DSSP (Kabsch and Sander, 1983). Strands β_{10A} and β_{11A} form, together with a part of strand β_{11} , a very short irregular β -sheet. The conformation of this sheet is virtually identical in both molecules, although DSSP assigned strand β_{11A} only for molecule I.

Strand	Molecule I residues	Molecule II residues	Folding motif
β_1	11–17	11–17	I
β_2	22–26	22–26	
β_3	29–33	29–33	
β_4	46–51	46–51	
β_5	55–60	55–60	II
β_6	65–72	65–72	
β_7	75–81	75–81	
β_8	99–105	99–105	
β_9	108–113	108–113	III
β_{10}	120–124	120–122	
β_{11}	132–134	132–134	
β_{12}	146–150	146–150	
β_{10A}	129–130	129–130	
β_{11A}	142–143	–	

points, differs most (Ca distance 0.57 nm) between the two independent IL-1ra molecules.

Temperature factors. The average temperature factor of the two independent IL-1ra molecules is rather high (0.399 nm^2). A

Table 4. 3_{10} helices in IL-1ra. Assignments were made with the program DSSP (Kabsch and Sander, 1983). The main-chain conformation of residues 85–87 is virtually identical in both molecules. However, DSSP assigns a 3_{10} helical conformation to these residues in molecule I and a turn conformation in molecule II. We prefer to assign a turn conformation to this loop rather than to call it a single turn 3_{10} helix.

Helix	Molecule I residues	Molecule II residues
H1	37–42	37–42
H2a	85–87	—
H2	93–98	93–98

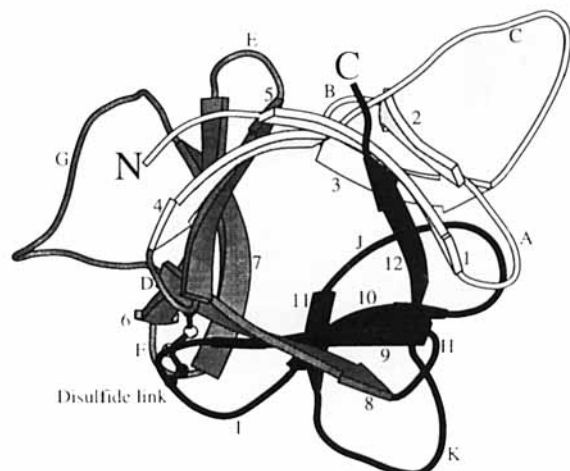


Fig. 3. Schematic drawing of the IL-1ra molecule produced by Molscript (Kraulis, 1991). β -Strands are indicated by arrows. The numbers indicate the strand numbers and the connecting loops are indicated by small capitals. The first folding unit (strands 1–4) is colored in light gray, the second unit (strands 5–8) in medium gray and the third unit (strands 9–12) in dark gray. The view is down the barrel axis.

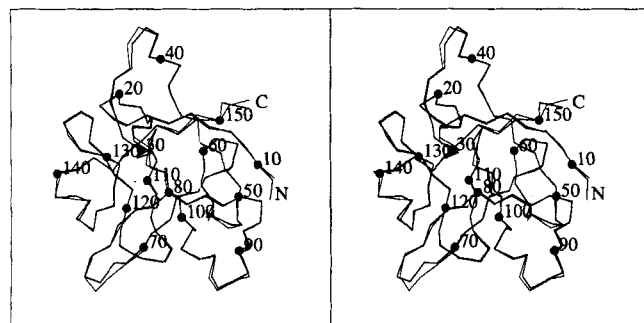


Fig. 4. $C\alpha$ traces of the two independent IL-1ra molecules after superposition. Molecule I is drawn in thick lines, molecule II in thin lines. Both molecules superimpose very well, the only exception is loop F between Lys71 and Thr76 which makes a hinge movement between the two molecules.

similar, relatively high, overall temperature factor has been reported for IL-1 β (Priestle et al., 1989). For IL-1ra, this high overall temperature factor seems to be caused by some extremely flexible loops. A plot of the distribution of temperature factors (Fig. 5) shows that the 12 β -strands which form the core of the IL-1ra molecule have temperature factors between 0.2–0.3 nm², while some of the interconnecting loops have temperature factors up to 1.0 nm². Continuous electron density is pre-

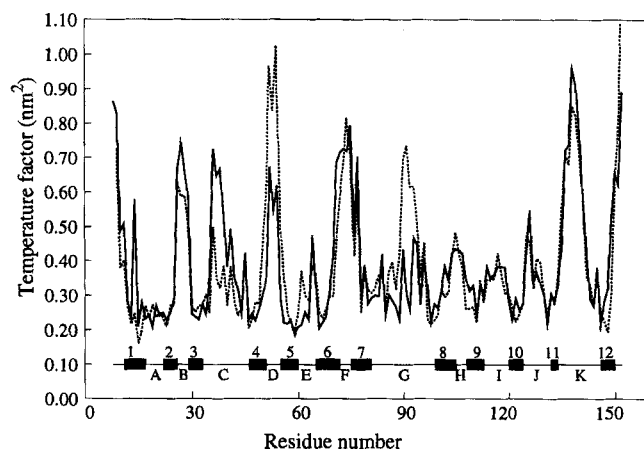


Fig. 5. Temperature factor distribution of the two independent IL-1ra molecules. The temperature factors of molecule I are drawn in continuous lines, of molecule II in broken lines. Bars indicate the 12 β -strands which make up the core of the IL-1ra molecule and capitals indicate the connecting loops. Most parts of the two molecules have very similar temperature factors, the exceptions are loops C, D and G.

sent in these regions for the main chain, but a number of side chains, especially those of Glu52, His54 and Glu139 have weak or absent electron density, suggesting that these side chains are freely moving in the solvent.

The presence of two independent molecules in our crystals allows us to use the structure as an example to study the influence of the crystal packing on the distribution of temperature factors. Most parts of the two independent molecules have very similar temperature factors, but loops C, D and G differ considerably. As will be discussed below, most of these differences can be explained by stabilization of flexible surface loops by crystal contacts. The two independent IL-1ra molecules have been arbitrarily named molecule I and molecule II and are thus indicated.

For loop C, a crystal contact ($d \leq 0.35$ nm) is present between Asn41I and Asn39II, both belonging to loop C. The loop C residues Gly37I and Pro38II have additional crystal contacts with Gly140II, which is part of the mobile loop K. Loop C residue Asn41II has an additional crystal contact with His61I, which is part of strand $\beta 5$ and has low mobility. The crystal contact of loop C of molecule II with the rigid His61I apparently has led to its much lower temperature factors. For loop D, we find crystal contacts of Pro53I with Asn28II and Met65II. No crystal contacts were found for loop D in molecule II, consistent with the much higher temperature factors of this loop in molecule II. For loop G, Asn91, Arg92 and Lys93 are involved in crystal contacts in both molecules. However, if we count the number of crystal contacts up to 0.45 nm, we find 125 such contacts in molecule I and only 41 in molecule II, which could explain the much lower temperature factors of loop K in molecule I.

Comparison with IL-1 α and IL-1 β . The structures of IL-1 α (only $C\alpha$ coordinates) and IL-1 β are available in the Protein Data Bank and allow for a comparison of the two IL-1 agonists (IL-1 α and IL-1 β) with the IL-1 antagonist (IL-1ra) structure solved by us. The IL-1 α and IL-1 β structures were superimposed onto the IL-1ra molecule I structure and the matching residues were optimized (see Tables 1 and 2). The sequence alignment, based on the three crystal structures is given in Fig. 6. Between the superimposed IL-1 α and IL-1ra, 63 $C\alpha$ atoms (41%) have positional differences of less than 0.10 nm and between the superimposed structures of IL-1 β and IL-1ra, 86 $C\alpha$ atoms (56%) differ less than 0.10 nm. These observations suggest that the IL-1ra

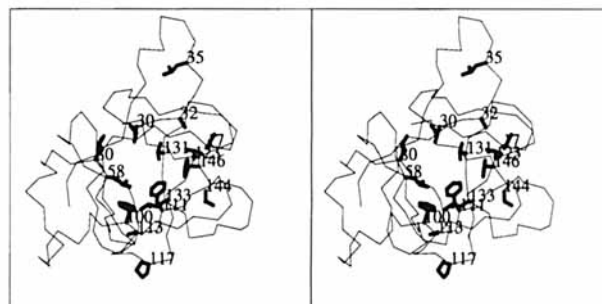
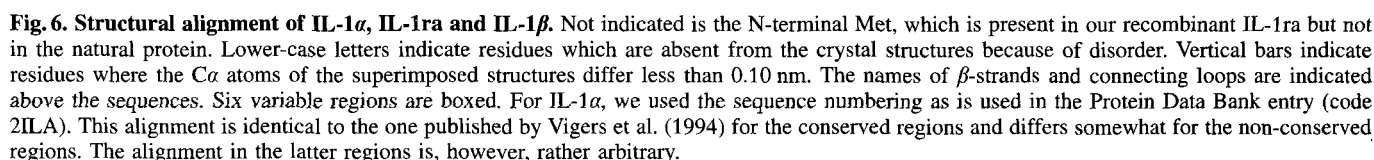


Fig. 8. C α backbone of IL-1ra with the side chains of 15 residues which are conserved between IL-1ra, IL-1 α and IL-1 β . These residues are: Asp17, Leu30, Ala32, Leu35, Leu58, Leu80, Phe100, Phe111, Ser113, Pro117, Thr123, Val131, Leu133, Thr144, and Phe146 (numbers refer to the IL-1ra sequence).

G (residues 97–99; strand BL according to Graves et al.) form an anti-parallel β -sheet-like structure with the N-terminal strand in the center. In the absence of full coordinates, it is not possible to determine whether these strands form a true β -sheet. Part of loop D (residues 59–61) is missing in the IL-1 α structure and appears to be disordered. The β -sheet-like structure is not present in the IL-1 β and IL-1ra structures. In IL-1 β , Asn48 of loop D is directly hydrogen bonded to Lys93 of loop G. No direct contacts between loop D and loop G are present in the IL-1ra structure. Loop D in IL-1ra is six residues shorter than in IL-1 α and IL-1 β . The N-terminus of IL-1ra, which is disordered in the crystal structure, could theoretically insert between loop D and loop G, like it does in IL-1 α .

In the structural alignment presented in Fig. 6, we find 15 residues, including 5 leucines, which are invariant in the human IL-1 α , IL-1 β and IL-1ra structures. These residues are shown in Fig. 8. Examination of an alignment of seven IL-1 sequences (Oldfield et al., 1993) reveals that, with two exceptions, these residues are completely conserved. As will be discussed below, these residues, with the exception of Thr144, seem to be important for the folding of the IL-1 molecule.

In IL-1 α , the N-terminal residues 6–10 (named strand N; Graves et al., 1990), part of loop D (residues 55–58), and loop

The first conserved residue, Asp17 (the IL-1ra sequence numbering is used throughout) is completely buried. It is situated at the end of strand β 1, just before a β -turn. Its O δ 1 atom is within hydrogen-bonding distance of the main-chain amide protons of Asn19, Gln20 and Lys21, in and just after the turn. Their three main-chain amide protons all point towards the Asp17 O δ 1, a situation reminiscent of the oxy-anion hole in serine proteinases. The O δ 2 of Asp17 is hydrogen-bonded to the conserved Thr123. Any mutation of Asp17 is likely to disrupt the structurally conserved loop A between strand β 1 and β 2.

The side chains of the conserved residues Leu30, Leu80 and Val131 are packed next to each other in the core of the IL-1 molecules. Ala32, which is not 100% conserved (it is Thr in murine IL-1 α), is situated next to Val131 and Thr123. The latter residue, as we have seen, is hydrogen-bonded to the conserved Asp17. The conserved residue Leu35 is not part of the core of the IL-1ra molecule, but is at the center of the relatively exposed loop C between strands β 3 and β 4 (see Fig. 8). In contrast, the conserved residues Leu58, Phe100, Phe111 and Phe146 are all clustered in the hydrophobic core. The side chain of the conserved Ser113 is buried and makes a hydrogen bond with the main-chain amide proton of Cys116, stabilizing the loop I between strands β 9 and β 10. This loop also contains the conserved Pro117, which is exposed and which may be important for the conformation of the structurally conserved loop I. The invariant Leu133 is at the surface, next to the conserved residues Phe100 and Ser113 mentioned above. Leu133 is not completely conserved, being Met in bovine IL-1 α .

Finally, Thr144 is the only conserved residue we found which is not buried and does not serve an obvious structural function. Interestingly, the adjacent residue Lys145 has been shown by site-directed mutagenesis to be crucial in determining agonist or antagonist activity. An aspartate at this position as in IL-1 α , IL-1 β and in the Lys145 $\rightarrow$ Asp mutant of IL-1ra leads to agonist activity, while a lysine at this position, as in IL-1ra and the Asp145 $\rightarrow$ Lys mutation of IL-1 β (Ju et al., 1991) leads to IL-1 antagonist activity. It is therefore likely that Thr144 is conserved because it is important for receptor interaction.

DISCUSSION

Two other groups have very recently reported the crystal structure of the IL-1ra, an initial analysis by Clancy et al. (1994) and a more detailed report by Vigers et al. (1994). However, neither group mentions the presence of a disulfide link nor of a *cis*-proline. Vigers et al. claim in their Experimental Procedures that no disulfide link is present in the IL-1ra. The disulfide link is in our case well defined (Fig. 2A) and at a resolution of 0.21 nm (our data) and 0.20 nm (Vigers et al., 1994) it is unlikely that this difference is due to uncertainties in the interpretation of the electron density maps. The presence/absence of the disulfide link must therefore have been caused by different handling of the protein samples. The IL-1ra has in both cases been produced in *E. coli*, which itself does not make disulfide links. Our disulfide link may have formed spontaneously due to exposure to air. IL-1ra is an extracellular protein which often contains disulfide links. It will be interesting to see whether natural IL-1ra, as it occurs in humans, contains a disulfide link.

The *cis*-Pro53 is part of the flexible loop D. The electron density (Fig. 2B) indicates some disorder which may be due to Pro53 being partially *cis* and partially *trans*. Stockman et al. (1992) reported NMR evidence for *cis/trans* isomerization for several proline residues. However, Pro53 in the *cis*-conformation fits the electron density much better than in the *trans*-conformation. In addition, the ϕ, ψ angles of Glu52 are in the disallowed

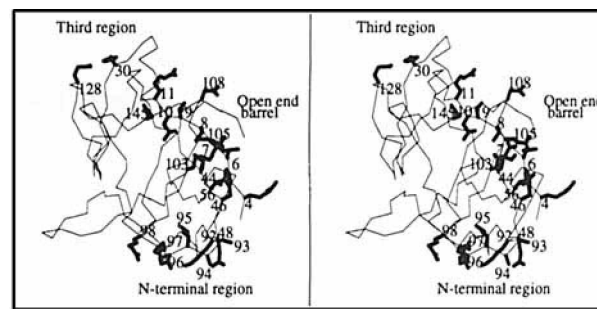


Fig. 9. Residues in IL-1 β which have been implicated by mutagenesis studies to be important for receptor interaction (see Table 5). The view is 90° rotated with respect to Figs 3, 4 and 8.

region with *trans*-Pro53, while they are in the allowed region with *cis*-Pro53 (Fig. 1).

In the absence of a structure of the IL-1 receptor or of a complex of the IL-1 receptor with one of its ligands, it is difficult to know exactly which residues are really important for receptor interaction. However, information on the crystal structures of the agonists IL-1 α and IL-1 β , and the antagonist IL-1ra can be combined with abundant data from mutagenesis experiments, mostly on IL-1 β (Table 5) and some data on IL-1 α (Yanofsky and Zurawski, 1990; Kawashima et al., 1992; Gayle et al., 1993; Labriola-Tompkins et al., 1993). Mutations in IL-1 β causing at least a fourfold change in receptor binding or biological activity are listed in Table 5 and their position in the molecule is indicated in Fig. 9. Mutations strongly affecting the receptor interaction cover a large part of the surface of the IL-1 β molecule and involve a region around the N-terminus, the open end of the β -barrel and loops C and J (indicated by 'third region' in Fig. 9).

The N-terminal region involves the N-terminus up to the beginning of strand β 1 and loops D (which includes Pro53) and G. Amino acid substitutions in this region strongly affect both receptor binding and biological activity (Table 5). Electrostatic calculations (Oldfield et al., 1993) revealed that this region corresponds to an area of positive charge which appears to be important for receptor interaction. Indeed, replacement in IL-1 β of Lys92 and Lys93 by anything except Arg leads to a drop in activity (see Table 5), while some (not all) mutations that enhance this positive charge (e.g. Ser5 $\rightarrow$ Arg and Glu51 $\rightarrow$ Arg; Labriola-Tompkins et al., 1991; Simon et al., 1993), also enhance receptor binding. Surprisingly, this region, where some mutations lead to less than 1% activity, is also the region with the largest structural differences between IL-1 α , IL-1 β and IL-1ra (see Fig. 7). In the crystals this region is flexible, its residues have high temperature factors and part of the N-termini in all three molecules and part of loop D in IL-1 α are disordered, suggesting that this region could adapt itself for optimal receptor interaction. It is likely that the disordered regions will become ordered upon receptor binding. Nevertheless, the large structural and sequence differences in this area suggest that the receptor interactions will be different for the three different IL-1 molecules.

The other regions are structurally almost identical in IL-1 α , IL-1 β and IL-1ra and encompass strands β 1, β 4, β 8, β 9, β 12, loop C and loop J. Two of the mutations listed in Table 5, Thr9 $\rightarrow$ Gly and Asp145 $\rightarrow$ Lys, cause a dramatic drop in biological activity while receptor affinity remains unchanged. Both residues are located near the open end of the barrel and point away from the barrel axis. Electrostatic calculations (Oldfield et al., 1993) indicate that the region around residue 145 carries a negative charge in IL-1 α and IL-1 β . In IL-1ra, this negative region

Table 5. Mutations in IL-1 β causing at least a fourfold change in receptor binding or biological activity. Listed are the affinities towards the IL-1 type-I receptors. The values given in this table are directly taken from the publications and can only be used in a qualitative sense since the authors of the different publications use different cell types and assay conditions. Multiple values are listed if different groups have tested the same mutations, or if the mutant protein has been tested with different cell lines. Mutation data were taken from the following references: (a) MacDonald et al., 1986; (b) Huang et al., 1987; (c) Wingfield et al., 1987; (d) Kamogashira et al., 1988a; (e) Kamogashira, et al., 1988b; (f) Gehrke et al., 1990; (g) Ju et al., 1991; (h) Labriola-Tompkins et al., 1991; (i) Simon et al., 1993; (j), Grütter et al., 1994. A dash indicates that the corresponding value was not mentioned in the publication.

Mutation	Reference	Binding	Activity	Comments
		%		
0 $\rightarrow$ Met	(c)	≈ 10	—	extra Met due to <i>E. coli</i> expression system
Ala1 $\rightarrow$ Thr	(b)	—	380	
Arg4 $\rightarrow$ Gln	(e, i)	19, 36	29, 57	reasonable activity with weak binding but figures for binding and activity are derived from different papers
$\rightarrow$ Gly	(e)	3.7	5.5	
$\rightarrow$ Asp	(e, h)	<0.01	0.11	
$\rightarrow$ Ala	(h)	<1	—	
$\rightarrow$ Lys	(h, i)	<1	47	
$\rightarrow$ Ser	(i, j)	40, 30, 60	7, 30, 20	
$\rightarrow$ Pro	(i)	9	12	open end barrel
$\rightarrow$ Val	(i)	23	4	
Leu6 $\rightarrow$ Ala	(h, i)	<1, 60	5	
Asn7 $\rightarrow$ Gly	(i)	25	16	open end barrel
Cys8 $\rightarrow$ Ser	(d, i)	—	2.7, 27	buried
Thr9 $\rightarrow$ Gly	(i)	100	0.4	buried; no activity with 100% binding affinity
$\rightarrow$ Trp	(i)	145	20	buried; loss of activity, but not of binding
$\rightarrow$ Gln	(i)	13	0.5	buried
$\rightarrow$ Glu	(i)	0.3	0.5	
Leu10 $\rightarrow$ Asn	(i)	<0.1	0.01	
$\rightarrow$ Thr	(i)	<0.1	0.2	
$\rightarrow$ Cys	(i)	<0.1	0.7	
$\rightarrow$ Ser	(i)	<0.1	0.1	
$\rightarrow$ Ala	(i)	<0.1	0.2	loss of activity with reasonable binding
Arg11 $\rightarrow$ Gly	(f)	75	1	
His30 $\rightarrow$ Asn	(a)	≈ 3	—	buried
$\rightarrow$ Arg	(a)	≈ 1	—	
Met44 $\rightarrow$ Ser	(h)	<1	—	
Phe46 $\rightarrow$ Asp	(h)	<1	—	
$\rightarrow$ Ala	(h)	<1	—	
Gln48 $\rightarrow$ Glu	(j)	30, 10, 100	—	
Ile56 $\rightarrow$ Ala	(h)	<1	—	increased activity and binding, showing the importance of positively charged residues in this region
Val58 $\rightarrow$ Ala	(h)	<1	—	
Lys92 $\rightarrow$ Glu	(h)	<1	—	
$\rightarrow$ Arg	(i)	472	306	
$\rightarrow$ Gln	(j)	70, 10, 100	—	
Lys93 $\rightarrow$ Leu	(h)	0.7	—	
$\rightarrow$ Ala	(h)	<1	—	loss of activity with reasonable binding
$\rightarrow$ Phe	(h)	<1	—	
$\rightarrow$ Ser	(h)	<1	—	
$\rightarrow$ Glu	(h)	<1	—	
$\rightarrow$ Met	(i)	23	9	
Lys94 $\rightarrow$ Trp	(i)	18	47	
$\rightarrow$ Gly	(i)	28	19	loss of activity with reasonable binding
$\rightarrow$ Asn	(j)	50, 20, 60	60, 30	
Met95 $\rightarrow$ Arg	(i)	14	7	loss of activity while retaining binding affinity
Glu96 $\rightarrow$ Gly	(i)	49	22	
Lys97 $\rightarrow$ Leu	(h)	10, 20, 40	—	loss of activity while retaining binding affinity
$\rightarrow$ Val	(i)	19	37	
Arg98 $\rightarrow$ Glu	(h)	10, 10, 20	—	loss of activity while retaining binding affinity
$\rightarrow$ Leu	(h)	10	—	
Lys103 $\rightarrow$ Ser	(h)	<1	—	loss of activity while retaining binding affinity
$\rightarrow$ Gln	(j)	90, 40	10, 10	
Glu105 $\rightarrow$ Ser	(h)	<1	—	loss of activity while retaining binding affinity
$\rightarrow$ Lys	(j)	20, 20, 60	30, 25	
Asn108 $\rightarrow$ Ala	(h)	10, 20, 20	—	loss of activity while retaining binding affinity
Glu128 $\rightarrow$ Lys	(j)	5, 2, 9	3, 5	
Asp145 $\rightarrow$ Lys	(g)	100, 27	9.4	loss of activity while retaining binding affinity

is interrupted due to the presence of Lys at position 145, instead of the Asp present in the agonists IL-1 α and IL-1 β and the authors argue that this positive charge may be important for antagonist action.

The crystallographic study presented in this paper clearly establishes that the folding of the IL-1ra molecule is very similar to the folding of the IL-1 α and IL-1 β molecules. One putative receptor interaction region, the N-terminal region, is quite variable and flexible, while other putative interaction regions are structurally conserved and more rigid, which would allow precise and specific interactions with the IL-1 receptor. Detailed structural information is now available for a natural receptor antagonist which can be used in interpretation of results from mutational studies and to aid the design of novel IL-1 agonist or antagonist molecules.

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