Pro-interleukin (IL)-1β Shares a Core Region of Stability as Compared with Mature IL-1β While Maintaining a Distinctly Different Configurational Landscape

**A COMPARATIVE HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY STUDY**

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Kendra L. Hailey, Sheng Li, Mette D. Andersen, Melinda Roy, Virgil L. Woods, Jr., and Patricia A. Jennings

From the Departments of Chemistry and Biochemistry and Medicine and Biological Graduate Program, University of California, La Jolla, California 92093

Interleukin-1β (IL-1β) is a master cytokine involved in initiating the innate immune response in vertebrates (Dinarello, C. A. (1994) *FASEB J.* 8, 1314–1325). It is first synthesized as an inactive 269-residue precursor (pro-interleukin-1β or pro-IL-1β). Pro-IL-1β requires processing by caspase-1 to generate the active, mature 153-residue cytokine. In this study, we combined hydrogen/deuterium exchange mass spectrometry, circular dichroism spectroscopy, and enzymatic digestion comparative studies to investigate the configurational landscape of pro-IL-1β and the role the N terminus plays in modulating the landscape. We find that the N terminus keeps pro-IL-1β in a protease-labile state while maintaining a core region of stability in the C-terminal region, the eventual mature protein. In mature IL-1β, this highly protected region maps back to the area protected earliest in the NMR studies characterizing an on-route kinetic refolding intermediate. This protected region also encompasses two important functional loops that participate in the IL-1β/receptor binding interface required for biological activity. We propose that the purpose of the N-terminal precursor region in pro-IL-1β is to suppress the function of the eventual mature region while keeping a structurally and functionally important core region primed for the final folding into the native, active state of the mature protein. The presence of the self-inhibiting precursor region provides yet another layer of regulation in the life cycle of this important cytokine.

Nearly all cell types respond to interleukin (IL)-1β, in a very sensitive manner, via binding to the interleukin-1 receptor type 1 (IL-1RI) (2). Although essential in the immune response, overproduction of IL-1β can lead to both acute (sepsis) as well as chronic (rheumatoid arthritis, atherosclerosis, obesity, and diabetes) disease states (3). Thus, the expression, activation, and secretion of this cytokine is tightly controlled (4). Although many cell types express IL-1β, it is predominately produced and secreted by monocytes and macrophages (1). The protein is synthesized as a biologically inactive 269-residue precursor molecule, pro-interleukin-1β (pro-IL-1β), and the 153-residue active mature IL-1β is generated from the C-terminal domain. Processing of the proprotein involves the recently discovered NALP-1 and NALP-3 inflammasomes, which are responsible for activating procaspase-1 (5). The inflammasome function is integral in wound repair as well as for combating infection (6–9).

In vivo, the 31-kDa pro-IL-1β precursor is processed to the active C-terminal 17-kDa form by the interleukin-1 converting enzyme, caspase-1 (10, 11). Caspase-1 is a cysteine protease that recognizes two cleavage sites in pro-IL-1β, the Asp27→Gly28 and Asp116→Ala117 peptide bonds (Fig. 1A). These cleavage sites are conserved across mammals (12–14). The activation pathway is believed to proceed with cleavage first at Asp27→Gly28 (site 1) followed by Asp116→Ala117 (site 2). These processing events lead to the generation of the mature, active IL-1β from the C-terminal domain of pro-IL-1β (15). After cleavage, the mature protein is exported via a cell-specific non-classical pathway (16). The events leading from caspase-1 activation to active IL-1β secretion are poorly understood and constitute an area of active research (16–20).

The native structure of IL-1β is classified as a β-trefoil. The global protein-fold contains three pseudo-symmetric βββloopβ motifs that coalesce to form a six-stranded barrel with three hairpins that form a six-stranded cap closing one end of the barrel (see Fig. 1B) (21). Mature IL-1β refolds relatively slowly (22), accessing multiple routes including a major route with a detectable intermediate population (23, 24). Recently, this slow folding has been attributed to repacking of a functionally important loop (the β-bulge) in the mature protein (see Fig. 1B, i) (25–27). Although much information is known about the structure, folding, and function of mature IL-1β, there is little information available on pro-IL-1β, despite the central importance of this molecule in mediating critical inflammatory processes (28–30). What is known is that the presence of the N-terminal 116 amino acids results in a highly protease-sensi-
Protein with no biological activity (31). Folding of mature IL-1β is believed to occur after cleavage of pro-IL-1β in vivo. Therefore, structural analysis of the precursor is essential for a better understanding of the role the precursor region plays in regulating folding events leading to the generation of the eventual mature protein.

The crystal structure of pro-IL-1β has not been determined, despite approximately 25 years of intensive efforts directed toward this goal, as a result of the dynamic nature of this molecule (32–34). Therefore, we used structure-sensitive methods to compare pro-IL-1β in reference to the mature protein. Optimal methods in combination with hydrogen/deuterium exchange mass spectrometric analysis (DXMS) and enzymatic digestion were used to investigate how the N-terminal precursor region modulates the properties of the C-terminal mature domain. DXMS is a well established technique for characterizing proteins refractory to standard crystallographic or NMR structure determination techniques (35–37). Taken together, our results indicate that the N terminus inhibits folding to the fully active trefoil structure in the C-terminal region, but maintains the protein in a conformation that is primed for efficient folding upon release after caspase-1 cleavage.

EXPERIMENTAL PROCEDURES

Protein Cloning, Expression, and Purification—Recombinant mature human IL-1β was prepared as previously described (38), with an additional purification step. Briefly, Escherichia coli BL21(DE3) cells (Novagen) containing the mature IL-1β expression vector were grown, induced with 1 mM isopropyl 1-thio-D-galactopyranoside, and harvested after 4 h at 37 °C. The cells were spun at 5,000 g for 30 min, and the supernatant was removed and dialyzed extensively in buffer A (25 mM ammonium acetate, 1 mM EDTA, pH 5.2). The dialyzed protein was injected onto HiTrap-SP cation exchange column (GE Healthcare) equilibrated with buffer A. IL-1β eluted in a single peak using a gradient of 15–50% buffer B (100 mM sodium acetate, 1 mM EDTA, pH 5.2) over 100 ml at 3 ml/min. The IL-1β fractions were pooled and extensively dialyzed into 20 mM sodium acetate, 1 mM EDTA, pH 5.2, and injected onto a Resource-S cation exchange column (GE Healthcare). Two isoforms of mature IL-1β, 1-Ala and 1-Pro (N terminus), eluted as single peaks with a gradient of 30–50% buffer B (100 mM sodium acetate, 1 mM EDTA, pH 5.2) over 200 ml at 1 ml/min. The 1-Ala fractions were pooled and the isoform identity was confirmed by matrix-assisted laser desorption ionization mass spectrometry.
Recombinant human pro-IL-1β wild type protein was prepared using the following procedure. The cDNA encoding the Glu6 isoform (IMAGE Consortium clone 3875593) was subcloned into a pET24-d(+) vector (Novagen) and transformed into E. coli BL21(DE3) cells (Novagen). Cells were grown in LB at 37 °C to an A(600) of 0.6, and protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The temperature was reduced to 30 °C, and the cells were harvested after 4 h. The harvested cultures were spun at 5,000 × g for 30 min, and the cell pellets were suspended in lysis buffer (25 mM Tris, 2 mM EDTA, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, pH 6.8). The cells were lysed by sonication at 4 °C, and then spun at 13,000 × g for 30 min. The soluble pro-IL-1β was made 25% saturated by ammonium sulfate, then precipitated by spinning at 13,000 × g for 30 min. The resulting pellet was suspended in buffer A (25 mM Tris, 2 mM EDTA, and 5 mM dithiothreitol, pH 6.8) and extensively dialyzed. The dia-lyzed protein was spun at 13,000 × g for 30 min, filtered by a 0.22-μm filter, and injected onto a HiTrap-Q anion exchange column (GE Healthcare). Pro-IL-1β eluted in a single peak using a linear gradient of 200–400 mM NaCl over 250 ml at a flow rate of 3.0 ml/min. The pro-IL-1β fractions were pooled and concentrated, then injected onto a 26/20 S-200 size exclusion column (GE Healthcare) equilibrated with buffer A (with 200 mM NaCl) at a flow rate of 2.5 ml/min. Purity of the elution was determined before starting the exchange time course experiments for pro-IL-1β.

Optimization of Fragmentation Conditions—The initial conditions for the sample composition and instrument parameters were determined before starting the exchange time course experiments. The initial set-up was previously described (39–41). A 5-μl stock of mature IL-1β was diluted with 15 μl of 1× MES and quenched with 30 μl of 0.5% formic acid, 16.6% glycerol, and 3.2 M guanidine HCl (quench buffer) at 0 °C. The samples were immediately flash-frozen in liquid nitrogen and stored at −80 °C. The samples were thawed at 0 °C and run at 100 μl/min on a pepsin-66 column to generate peptides. The peptides were separated using a C18 reversed-phase column (Vydac) running a gradient of 5–45%, 5% trifluoroacetic acid/acetonitrile, over 30 min before injecting onto the LCQ mass spectrometer (Thermo LCQ Classic, Thermo Finnigan). The peptides were identified using MS1 and MS2 data. The fragmentation conditions were determined by examining the quality of generated peptides and amount of peptide coverage over the full-length protein.

Deuterium On-exchange Experiments—The exchange time course experiments for pro-IL-1β and 1-Ala mature IL-1β were performed simultaneously at 25 °C with the following procedure. A full time course experiment was initiated by adding 100 μl of protein in 1× MES, pH 6.5, to 300 μl of the equivalent deuterated exchange buffer for a final 75% of D2O. Deuterated 1× MES was prepared using D2O and adjusted to a pH of 6.1 with DCl. The exchange was monitored over the course of 24 h, at intervals of 0.1, 1, 5, 15, 60, 120, 480, and 1440 min. Aliquots (20 μl) from the master reaction were removed and quenched in pre-chilled high pressure liquid chromatography vials containing quench buffer (30 μl). The vials were sealed and flash-frozen, then stored at −80 °C. The in-exchange control consisted of the protein added directly to the pre-chilled deuterated and quench buffers, then immediately followed by the normal sample preparation procedure. The back exchange control was determined by incubating the samples in 0.5% formic acid in D2O with varying concentrations of guanidine DCl (0, 2.0, and 4.0 M, Cambridge Isotopes) for 24 or 48 h. All samples were injected and run on the instrument with the same conditions listed in the fragmentation optimization section. Data for the time course exchanges were acquired in the MS1 mode.

Sequence Identification of Peptide Fragments—The most likely identity of the parent peptide ions was determined using the SEQUEST software program (Thermo Finnigan, Inc.) and MS1 and MS2 data. The quality of each peptide was monitored by individually examining each measured isotopic envelope spectrum for the entire time course exchange. The deuterium content was calculated for each time point by using specialized software as previously described (39, 40).

Enzymatic Digestion Assays—The chymotrypsin A and caspase-1 (Sigma) digests of pro- and mature IL-1β were performed in the following manner. Master reactions were made containing 200 μl of substrate at 15 μM in 1× MES buffer. The reactions were carried out at 30 °C. After adding the enzyme, time points were taken by quenching the reaction by adding each aliquot to 2× gel running buffer (200 mM Tris-HCl, 10% glycerol, 2% SDS, 0.5% bromphenol blue, and 10% β-mercaptoethanol) and flash freezing in liquid nitrogen. Reactions with chymotrypsin were quenched with the addition 1 mM phenylmethylsulfonyl fluoride to the gel running buffer showed no difference in the amount of substrate cleaved when compared with no phenylmethylsulfonyl fluoride added (data not shown). Substrate cleavage was monitored over time by change in band intensity on a Coomassie-stained, SDS-polyacrylamide gel. The enzymes were added based on the number of units necessary to digest the total amount of substrate in 15 min for chymotrypsin and 1 h for caspase-1.

Circular Dichroism—The spectra for near and far UV were recorded on an Aviv-2 Circular Dichroism Spectrometer at 25 °C in a 0.1-cm path length quartz cuvette. Each sample was 0.3 mg/ml final concentration in 1× MES, with or without 2 M guanidine HCl (also in 1× MES). The samples were equilibrated at least 24 h at room temperature overnight. The average time at each wavelength was 5 s, and the experiment was repeated in triplicate.

RESULTS

Generation of Mature IL-1β via Proteolysis—Human pro-IL-1β was cloned, expressed, and purified as described under
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“Experimental Procedures.” Pro-IL-1β is processed to the fully active mature protein by caspase-1 in vivo. In addition, a range of other proteases can cleave pro-IL-1β to generate mature proteins of varying activities (29, 30, 42–45). To investigate the properties of our expressed protein, we performed a series of proteolysis experiments on recombinant pro-IL-1β. Fig. 2A shows the time course for in vitro processing of the protein with caspase-1 as detected by SDS-PAGE analysis. The bacterially expressed full-length proprotein migrates as a single 31-kDa band as expected from the amino acid sequence. Upon addition of caspase-1, a band at 28-kDa accumulates, consistent with the expected molecular mass of the protein upon cleavage at the Asp27 → Asp28 site. Subsequent processing to the mature 17-kDa protein was observed with time, demonstrating that the recombinant pro-IL-1β holds the correct conformation to be processed by caspase-1 as observed in vivo. Comparative studies of chymotrypsin sensitivity of pro- and mature IL-1β are presented in Fig. 2, B and C, respectively. Shown by SDS-PAGE analysis, pro-IL-1β was efficiently cleaved by chymotrypsin to generate a 17-kDa product that is protected from further digestion as observed for the mature folded IL-1β. These results are consistent with facile generation of the mature folded protein from pro-IL-1β as there are many chymotrypsin recognition sites within the C-terminal 17-kDa domain.

CD Spectroscopy Demonstrates Less Secondary Structure in Pro-IL-1β Compared with Mature IL-1β—Although CD spectra of proteins containing the α-helix secondary structure exhibit characteristic signatures, CD spectra of β-sheet proteins are highly variable (46). There is only a weak intrinsic signal for the twist in the β-sheet and side chain signals such as those associated with disulfide bonds and/or exciton interactions that easily complicate spectra and make secondary structure interpretation difficult (47). Nonetheless, comparison of the CD spectra of folded/unfolded states of pro- and mature IL-1β can give information about secondary structural features relative to each other.

Representative spectra of pro- and mature IL-1β in the presence and absence of denaturant are shown in Fig. 3. There is a large difference between the spectra of the native precursor and mature proteins and between the spectra of folded and unfolded precursor protein in the far UV wavelength range. The difference between spectra of the pro- and mature proteins in the presence of denaturant is minimal, and consistent with denatured random coil. The unfolded spectra of pro- and mature IL-1β almost have the same overall shape and mean residue ellipticity. In the near UV range of the spectra (Fig. 3, inset), only the spectrum of native mature IL-1β is consistent with the presence of well ordered side chains. There are two maxima with positive mean residue ellipticity at ~270 and 300 nm. An earlier study attributes the majority of the near UV signal to Trp220 and Tyr68 in mature IL-1β (Trp236 and Tyr184 in pro-IL-1β) (48). The native pro-IL-1β spectrum lacks the same signal intensity. This indicates either a lack of ordered side chain packing, the presence of a nearby quenching moiety, or the cancellation of the aromatic side chain signals (46).

Mass Spectrometric Studies of the Pro-IL-1β Structure and Stability Relative to Mature IL-1β—H/D solvent exchange experiments require optimal sample conditions for pepsin digestion and minimal deuterium back exchange (39–41). These conditions were achieved using a 3.2 M guanidine HCl, 0.8% formic acid quencher buffer solution (final pH 2.5) for all samples (see “Experimental Procedures”). As mature and pro-IL-1β are stable at the low pH values necessary for optimal quenching of the H/D exchange, the addition of denaturant was necessary to generate good quality peptides by pepsin cleavage.

FIGURE 2. Generation of mature IL-1β via proteolysis. A, an SDS-PAGE gel showing the time course for processing of pro-IL-1β by caspase-1. A band at 28-kDa accumulates before the appearance of the 17-kDa band for the mature protein, indicating that site 1 is preferentially recognized and processed first. Here, numbers 1–9 indicate control (no enzyme), 0 s (directly into quench), 5, 15, 30, 60, 120, 240, and 1440 min, respectively. B, an SDS-PAGE gel showing the time course for processing of pro-IL-1β by catalytic amounts of chymotrypsin. As is evident in the analysis, pro-IL-1β is efficiently cleaved by chymotrypsin to generate a 17-kDa product that is protected from further digestion. C, control. Mature folded IL-1β is protected from further digestion. These results are consistent with the facile generation of the mature folded protein from pro-IL-1β. The numbers 1–9 for B and C correspond to the control (no enzyme), 0 s (directly into quench), 1, 5, 15, 30, 60, and 120 min, respectively.

FIGURE 3. Comparison of native pro- and mature IL-1β indicates the generation of additional ordered tertiary structure upon cleavage. The far and near UV circular dichroism spectra of native and unfolded pro- and mature IL-1β are shown. There is a very large distance between the spectra of the native pro- and mature proteins in the far UV range (solid and dashed black lines, respectively), but the spectra of the pro- and mature proteins in the presence of denaturant are minimal (solid and dashed gray lines, respectively). In the near UV range of the spectra (inset), only the spectrum of native mature IL-1β is consistent with the presence of well ordered side chains, while both pro-IL-1β spectra are similar in this region to unfolded mature IL-1β.
of the full-length proteins. Quenched samples were kept at −80 °C until analysis. The resulting pepsin fragmentation maps for a typical experiment for both pro- and mature IL-1β are shown in supplemental Fig. S1 (75), labeled in black and bold red sequence numbering, respectively. The conditions described generated 71 good quality peptides covering 91% of the pro-IL-1β sequence and 102 good quality peptides covering 100% of the mature IL-1β sequence in a representative data set.

The mass spectra of non-deuterated and fully deuterated peptides generated from mature IL-1β (Val72–Leu82) are given in supplemental Fig. S1, inset, to illustrate the deuterium incorporation into the peptide backbone over time after exposure to D₂O. We observed a range of exchange rates from fully exchanged before the first time point (10 s) to highly protected from exchange after the longest time point (1440 min or 24 h) for peptide fragments generated from both pro- and mature IL-1β. The average number of deuterons incorporated was calculated for each peptide at each time point (39–41). Log plots of the observed deuteron incorporation as a function of time for representative directly overlapping peptides are given in Fig. 4A. There are two categories of peptides, those sharing the identical sequence composition between pro- and mature IL-1β (e.g. mature IL-1β peptide 9–19 and pro-IL-1β peptide 125–135), and those that do not. The N-terminal of pro-IL-1β (residues 1–116) is, of course, absent in mature IL-1β. In addition, there is altered protease sensitivity between the two proteins resulting in different peptide coverage maps. Fig. 4B illustrates directly overlapping peptides over the exchange time course using the color block schematic representation of the percent of maximum deuterons incorporated. The coloring scale for the percent of maximum deuterons incorporated for the peptides over time is also indicated.

The overall relative protection profiles of amide protons against H/D exchange for the N-terminal region of pro-IL-1β (Fig. 5A), and the C-terminal region of pro- and mature IL-1β (Fig. 5B) are depicted in color blocks. The blocks representing the pro-IL-1β C-terminal sequence are located directly above the corresponding analogous sequence of the mature protein for ease of comparison. The N-terminal region of pro-IL-1β (residues 1–116) shows minimal protection and stability overall, with a very few scattered areas of moderate protection (Fig. 5A). Areas of moderate protection include the beginning of strand 1, a conserved stretch of aromatic and acidic residues (95–105), a stretch of residues (50–70) with predicted secondary structure, near cut site 2, and residues 77–80. Areas with no protection at all, i.e. exchanging faster than the first time point (10 s), include cut site 1 and the area surrounding Trp108 (pro-IL-1β numbering).

The C-terminal region of pro-IL-1β, residues 117–269 (which corresponds to 1–153 of the recombinant mature protein), shows substantially more overall protection compared with the N-terminal region. There are a number of areas of moderate to high protection, with pockets of little to no protection (Fig. 5B). The turn before and at the start of strand 2, the loop between the first 3–10 helix and start of strand 4, the region covering strands 5–7, strand 8, and the region covering strands 9 and 10 display high protection from exchange (note that these strands correspond to the secondary structure seen in structure of the mature protein). Interestingly, 3 of the 4 protected turns mark the barrel-to-cap transition and the other is between two strands of the barrel. Regions of moderate protection include the area near cut site 2, the region covering strands 2 and 3, the basic loop between strands 7 and 8, and the region preceding and including strand 11. Areas of little to no protection include strand 1, the loop between strands 3 and 4, the β-bulge (loop between strands 4 and 5), and the region covering strands 11 and 12 (the C terminus).

Mature IL-1β shows the most protection and stability overall (Fig. 5B, bottom set of color blocks). As compared to the C terminus of pro-IL-1β, the biggest difference lies in trefoil 1 (residues 1–45). Mature IL-1β shows more solvent-exchange protection and hence more secondary and tertiary structure here than the corresponding region in pro-IL-1β. Well protected areas in mature IL-1β are comprised of the region covering strands 1–3, the sequence preceding strand 4 and strand 4, the region covering strands 5–7, the region covering strands 9–11, and a small pocket preceding strand 12. These areas correspond well with the most protected residues seen by H/D exchange NMR, which corresponds in a residue-specific manner to the β-sheet secondary structure of the β-trefoil (49–51). Areas of moderate protection include the beginning of strand 1, the basic 90s loop, and strand 12. The least protected areas include the N and C termini, the loop between strands 11 and 12, the loop between strands 3 and 4, and the β-bulge loop.

DISCUSSION

Proteins over a wide array of fold and functional families are synthesized as larger precursor molecules (52). Many enzymes, including caspase-1, exist as larger inactive precursors known aszymogens before being activated (11). Precursor regions may also act as chaperones, facilitating folding of the eventual mature protein as in the case of the bovine pancreatic trypsin inhibitor (53). Some proteins would not fold without the presence of their precursor domain. The prodomain of the α-lytic protease is thought to reduce the conformational entropy of the active region thereby acting as a folding catalyst, allowing the mature protein to bypass the large kinetic folding barrier between it and its native state (54). Precursor domains also act as intracellular targeting sequences, as in the case of the outer-mitochondrial Fe-S cluster protein mitoNEET (55), or as extra-cellular targeting sequences, as with interleukin-1 receptor antagonist (IL-1Ra), the first identified naturally occurring antagonist (56).

The cytokine IL-1 family members contain the same fold, the β-trefoil. The antagonist for IL-1, IL-1Ra, also shares the same fold. This protein contains a short N-terminal precursor segment (24 residues) that acts as a recognition sequence directing it toward the endoplasmic reticulum for secretion via the classical endoplasmic reticulum/Golgi pathway (56). The agonist members of the IL-1 family, IL-1α, IL-1β, IL-18, and IL-33, are synthesized as much larger precursor molecules (57). All of these family members are secreted via a non-classical pathway, which is dependent on cell type for the respective protein. The functions of these precursor domains are still being investigated. The most well characterized to date is the N terminus of pro-IL-1α (processed by calpain to the mature form (58)), which contains a nuclear localization sequence and can act as a
FIGURE 4. Representative log plots showing deuteron incorporation over time for analogous peptides between pro- and mature IL-1β. A, the red circles correspond to the pro-IL-1β peptide, and the blue circles correspond to the exactly matching peptide in mature IL-1β. Both the pro and mature sequence numbering are indicated in each plot. The data represents an H/D exchange time course under the same conditions for each protein. Time points were taken at 10, 60, 300, 900, 3,600, 28,800, and 86,400 s. The color block representations of the percent of maximum deuterons incorporation over time for the directly overlapping peptides between pro- (top) and mature (bottom) are displayed in B. Each block represents an exchange time course for one peptide, analogous to the log plots in A. The percent of maximum deuterons incorporated for each for each time point in each block is colored according to the key located at the bottom right of the figure. The red numbers correspond to the mature and the black numbers to the precursor sequence, and the three trefoil subunits are indicated by brackets. The secondary structure of the mature protein calculated from the NMR structure (PDB code 6I1B) is represented by open arrows (β-strands) and the cylinder (3–10 helix) below the mature blocks.
transcription factor with or without the C-terminal region (59, 60). IL-1β and IL-18 are processed by caspase-1. The C-terminal mature forms of IL-1β, IL-18, and IL-33 all bind IL-1RI family receptors to activate their respective signaling cascades (57).

The N-terminal precursor domain for IL-1β contains no known localization sequence and has no assigned function. Only limited information about the structure and function of pro-IL-1β is available. A pull-down assay showed that when in the folded native state, mature IL-1β does not interact with the N-terminal sequence alone, suggesting that the conformational change after removal of the precursor sequence in one or both of the regions prevents further interaction (29). The expression, translation, processing, and secretion of mature IL-1β are tightly controlled events that require intricate networks of stimuli to occur. Recent work has shown how the inflammasome precisely orchestrates caspase-1 activation as a timely and unique response to a multitude of environmental and pathogenic assaults (7–9, 19). Along with the inflammasome, due to the potency of IL-1β as a key initiator of the innate immune response, the presence of a self-inhibiting precursor region adds yet another layer of regulation for this powerful cytokine.

**Prodomain Modulates Function of Mature Domain in Pro-IL-1β**

![Color block schematic of % of deuterium incorporation over time, representing relative amide solvent exchange protection in a full set of generated peptides.](image)

**FIGURE 5.** Color block schematic of % of deuterium incorporation over time, representing relative amide solvent exchange protection in a full set of generated peptides. Relative protection of peptides against solvent exchange in (A) the N-terminal region of pro-IL-1β, (B) side by side comparison of the C-terminal region of pro-IL-1β, which corresponds to mature IL-1β (top row) and mature IL-1β (bottom row). Each block represents a peptide or a group of overlapping peptides of the entire exchange time course, and each time point is colored by the percent of maximum of deuterons incorporated for that peptide. The level of protection is indicated by the coloring of that peptide over the entire time course, and the coloring scheme key is located at the far right of the figure. Due to the existence of many overlapping peptides, some peptides can only be partially represented. The protein sequence is indicated by the black line located above the blocks, and the sequence numbering in black corresponds to the pro-IL-1β sequence and in red for the mature protein sequence. Areas of high protection are generally <10–30% exchanged, areas of moderate exchange are >30–80% exchanged, and areas of little to no protection are >80–100% exchanged over the duration of the experiment. The areas with the most significant differences in protection between pro- and mature are bracketed by red arrows. Areas in both panels with no blocks indicate the absence of an available probe in that region, and the secondary structure of the mature protein is indicated in open arrows below the blocks in B.
resistant under the same brute force conditions (1:10 enzyme:substrate) (15, 28–31, 42). We performed experiments where we added chymotrypsin in catalytic amounts (1:1000, enzyme:substrate) and found that the enzyme preferentially acts on the N-terminal cut sites of pro-IL-1β, thus allowing the C-terminal region to fold into the protease-resistant β-trefoil of IL-1β prior to complete digestion (Fig. 2B). In this caspase-1 digest study, cut site 1 (Asp27↓Gly28) was accessed first and preferentially cleaved before cut site 2 (Asp116↓Ala117) (Fig. 2A). These findings suggest that even in the presence of the N terminus, the C terminus is still protected from degradation, although not to the same degree as the final folded, mature protein. Therefore, pro-IL-1β is a more protease labile state than the mature protein, but once separated from the prodomain after processing, the C-terminal region is primed to fold quickly and within a certain time frame into the final native conformation.

**Optical Studies Indicate Differences in Native Structure**—Circular dichroism spectroscopy was used to characterize a protein secondary structure. The signature of the native mature IL-1β spectrum displays the characteristic positive intensity between 230 and 240 nm, whereas the spectrum of native pro-IL-1β lacks this characteristic (Fig. 3). However, pro-IL-1β contains at least regular secondary structure as there is a large change in signal upon the addition of denaturant. The spectra of mature and pro-IL-1β overlay in the presence of added guanidine HCl (2 M), as both show the loss of the regular secondary structure upon exposure to denaturant (Fig. 3). The local environment around the aromatic side chains in native pro-IL-1β is different from that observed in the native mature protein, as indicated by the lack of a strong near UV signal from 250 to 320 nm (Fig. 3, inset). Although the possibility exists for the cancellation of the well defined peaks of Trp236 (120) and Tyr186 (68) by aromatics located throughout the precursor sequence, it is more likely that the local environment around these residues has changed, consistent with the lack of change in signal with and without the presence of denaturant. Indeed, the lack of intensity in this region is consistent with having more highly mobile side chains in pro-IL-1β versus the mature protein. More mobile side chains may be a result of the ability of the proprotein to sample locally unfolded states versus the more structured mature protein. This conformational heterogeneity can also account for the decreased resistance to protease degradation seen in the enzyme cleavage assays, and decreased protection seen in the H/D exchange analysis presented here. Taking the lack of stability in the overall exchange profile of the 116 N-terminal residues in pro-IL-1β into account, it appears that most of the secondary structure seen in the spectrum is found in the C-terminal region.

The **N terminus Region of Pro-IL-1β (Residues 1–116) Displays Only Limited Regions of H/D Exchange Protection**—The majority of the amide protons within the N-terminal region of pro-IL-1β are 70–100% exchanged for deuterons in the first minute of solvent exposure to D2O (Fig. 5A). The peptide covering the first caspase-1 cut site (Asp27↓Gly28) is fully exchanged within 10 s. The calculated average exchange half-life for the polypeptide at this pH and temperature is less than 1 s (61, 62). The peptides covering the second cut site (Asp116↓Ala117) still show some protection after 24 h. Therefore, we propose that the first cut site is recognized first, consistent with the degree of solvent exposure, as it is more exposed and easier to access. There is also no solvent exchange protection of the peptide covering Trp108, the only tryptophan residue in the N-terminal region of pro-IL-1β. This would facilitate preferential cleavage by chymotrypsin, because the enzyme recognizes aromatic residues in the P1 position of its substrate recognition sequence.

The N-terminal region does contain two pockets of moderate protection, one located at residues 95–105 and the other at residues 77–80. Residues 95–105 are highly conserved over mammalian species of IL-1β and also between IL-1β and IL-1α. This is particularly interesting because most of the sequence conservation between species lies within the C-terminal region (residues 117–269), and there are few regions in the N-terminal region (residues 1–116) of pro-IL-1β that are conserved over mammalian species (13, 63). Residues 95–105 consist of alternating acidic and hydrophobic residues, and are complementary to the basic 90s loop (residues 201–216) between strands 7 and 8 as identified from the structure of the mature protein (21). The other pocket of moderate protection is the peptide that covers residues 77–80. There is neither a predicted nor observed secondary structure in this region, but Val79 and Pro80 are highly conserved residues in higher mammals. Hence the lower rate of deuterium exchange may be the result of amino acid type rather than from the ordered structure. These residues do not suffer an anomalously high exchange rate (62). Residues 77–80 may be consistent with turn or secondary N to C terminus interactions, thus stabilizing them from exchange. A region of some limited protection (residues 45–75) encompasses two smaller regions of highly conserved residues (49–53 and 61–73), and also lies within an area with the predicted secondary structure. Interestingly, these moderately protected areas of predicted secondary structure also appear in the secondary structure prediction for pro-IL-1α (64).

The **C-terminal Region of Pro-IL-1β (Residues 117–269) Displays Decreased Amounts of Protection Relative to Mature IL-1β Overall, but Maintains a Core Region of Stability**—Fig. 5 shows the peptides generated from the N terminus of the proprotein (Fig. 5A) and the region of pro-IL-1β that corresponds to the mature protein and the peptides of the mature protein (Fig. 5B). Overall, the C-terminal region of pro-IL-1β, which corresponds to the mature protein, is less protected from exchange, which implies less stable secondary and tertiary structure overall for the IL-1β sequence within the intact precursor protein (see top row versus bottom row of blocks). For ease of discussion, all structural elements referred to are mature IL-1β, and we are not implying that these elements exist in pro-IL-1β here. The main differences between the top and bottom sets of blocks are within the residues corresponding to the first and third trefoil units of mature IL-1β (refer to Fig. 1B). The peptide covering the area representing the turn between the structure of strands 1 and 2 (125–135, 9–19, pro- and mature IL-1β numbering) is the most protected area in the trefoil 1 region (117–162, 1–45) of pro-IL-1β. In mature IL-1β, peptides starting in the middle of strand 1 up until the loop after strand 3 (8–28) display a high amount of solvent protection.
The areas in either the mature or pro-IL-1β that encompass the two important functional loops of the B-site, the β-bulge (residues 162–169 and 46–53 in pro-, mature IL-1β), and the basic 90s loop (residues 201–216 and 85–99, respectively) are protected the most. The main differences in protection are in the N and C termini, where the pro-IL-1β is significantly less protected than the corresponding region in the eventual mature protein.

**Geometric Frustration during Folding Is Linked to Function in Mature IL-1β**—IL-1β binds the IL-1RI receptor in two distinct sites, the A-site and B-site, indicated in Fig. 6 (65). The refolding of mature IL-1β is slow for globular proteins of its size, 17.4 kDa (on the order of minutes or longer depending on conditions to the native basin). Experimental and theoretical studies show that this slow folding largely results from geometric frustration (25, 26, 51). That is, the requirements for a particular functional substructure within a fold may not be as fully evolved for efficient folding as the rest of the structure, because only a limited number of contacts will lead to optimal activity. Some of the geometric frustration in refolding of the mature IL-1β is attributed to the β-bulge being packed into the correct conformation to interact with the receptor. Having the most geometrically frustrated but functionally important areas of the protein in close proximity to their final position in the precursor domain would not only ensure that it folds correctly after processing, but also quickly. Simplified model simulations indicate that there are multiple refolding routes accessible for mature IL-1β on its folding landscape (26, 66). The preferred route has the barrel form first, whereas an alternate route has the receptor binding B-site form before the barrel (27). The latter route has contacts involved in a “back-tracking” mode during folding, where contacts are initially made in and around the B-site, then unmade while other regions of the protein fold into place, before the B-site is finally able to take its final native structure. This back-tracking is attributed to geometrical complexity of packing the functionally important loop, the β-bulge (25). Having two or more competing routes frustrates the folding land-
the majority located in the trefoil 2 region (see Figs. 1B and 4). A direct comparison of exact peptides indicates a similar pattern of protection (Fig. 4B). The more comprehensive analysis of the data using peptides generated from the same regions of the sequence, but with non-equivalent residue composition, indicates large differences between pro- and mature IL-1β (Fig. 5B).

Because of the increased size of pro-IL-1β relative to the mature protein, one may infer that the proprotein would show increased solvent exchange protection solely based on increases in steric, or bulk non-polar interactions (71). However, we discovered and as indicated in Fig. 6B, that the directly overlapping peptides between pro- and mature IL-1β are well protected and show similar or reduced solvent exchange protection over time. As the exchange protection along the β-strands and in the surface 90s loop hydrophobic mini-core has been (well documented by NMR studies of the mature protein) attributed to secondary structure interactions (anti-parallel β strand hydrogen bonding), this is likely the cause for protection in pro-IL-1β as well (72). The pro-IL-1β native state heterogeneity appears likely due to the mobility of the N-terminal 116 residues as these residues have minimal stabilization to solvent exchange (Fig. 5A).

The full-length precursor protein neither binds IL-1RI nor is active (73). Presumably the N-terminal region of the proprotein interacts with the C-terminal to prevent its biological functioning. The charge complementarity between the N-terminal acidic/aromatic region (residues 95–105) and exposure of the C-terminal basic 90s loop (201–215 and 85–99, pro- and mature IL-1β) suggest this as a highly plausible interaction. Residues 95–105 are highly conserved and constitute one of the few regions of predicted secondary structure in the N terminus. In mature IL-1β, the 90s loop is one part of the B-site binding interface with the receptor (Fig. 6). Blocking this region would prevent the interaction between the 90s loop and receptor, thus preventing binding (Fig. 7). An additional interaction between the N- and C-terminal regions of pro-IL-1β includes the acidic

The Precursor Domain Suppresses the Folding and Function of the C Terminus—Peptide backbone 1H,15N resonances of mature IL-1β are well resolved in heteronuclear single quantum coherence spectra and studies on the effects of solvent conditions and/or mutations on the residue-specific stability of mature IL-1β to solvent exchange by two-dimensional heteronuclear NMR spectroscopy have been quite useful (49–51). However, the 1H,15N heteronuclear single quantum coherence spectrum of pro-IL-1β has limited chemical shift dispersion and this characterization, combined with a fairly large molecular weight, results in amide backbone resonances that are poorly resolved. Data from light scattering studies, size exclusion column chromatography, and one-dimensional diffusion NMR spectra preclude the possibility of aggregation under our experimental conditions. Poorly resolved amide proton resonances for pro-IL-1β are the result of a more heterogeneous conformational native state ensemble than is seen in the mature protein (67–70). Our results indicate that pro-IL-1β consists of a much more heterogeneous native state than the mature protein.

Both pro-IL-1β and the mature IL-1β have good peptide coverage and are highly amenable to DXMS analysis (supplemental Fig. S1A). The pattern of relative protection for peptides represented in the block diagram in Fig. 5B is consistent with residue-specific protection factors determined in previous hydro-

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schematic representation of the precursor domain preventing full barrel formation in the eventual mature domain is shown. Initially, the basic/hydrophobic 90s loop (red circles) and the β-bulge loop (blue circles) of the C-terminal region and the acidic/hydrophobic (residues 95–105, blue dashes) and basic/turn portions (residues 73–80, red dashes) of the N-terminal region interact via side chain/side chain charge complementarity. This interaction between the two domains creates a “pinching” effect, destabilizing the barrel (as in the mature IL-1β structure). The effect is transmitted to the opposite end of the protein, preventing the ends of the barrel from coming completely together. Subsequently, the proprotein is cleaved by caspase-1 or another protease capable of generating mature IL-1β. After the precursor region is removed, the functional loops are revealed and the C terminus is free to finish folding into the native state. The now fully folded mature IL-1β is stabilized further from exchange.

scapade of the mature protein. The addition of these proposed contacts between the N-terminal domain and the functional loop area could trap the C-terminal into a new well defined local minimum, mimicking an intermediate state of the mature protein. This intermediate-like state may prevent the formation of the functional loops while keeping them close to a structure that can easily access the native fold. Then, removing the N-terminal portion of the protein allows it to proceed quickly to its final native fold, bypassing the back-tracking route. The precursor protein not only suppresses the receptor binding function of itself, but promotes quick access to the native basin for the mature protein by abrogating the geometrical searching process (a “fast pass”).

The Precursor Domain Suppresses the Folding and Function of the C Terminus—Peptide backbone 1H,15N resonances of mature IL-1β are well resolved in heteronuclear single quantum coherence spectra and studies on the effects of solvent conditions and/or mutations on the residue-specific stability of mature IL-1β to solvent exchange by two-dimensional heteronuclear NMR spectroscopy have been quite useful (49–51). However, the 1H,15N heteronuclear single quantum coherence spectrum of pro-IL-1β has limited chemical shift dispersion and this characterization, combined with a fairly large molecular weight, results in amide backbone resonances that are poorly resolved. Data from light scattering studies, size exclusion column chromatography, and one-dimensional diffusion NMR spectra preclude the possibility of aggregation under our experimental conditions. Poorly resolved amide proton resonances for pro-IL-1β are the result of a more heterogeneous conformational native state ensemble than is seen in the mature protein (67–70). Our results indicate that pro-IL-1β consists of a much more heterogeneous native state than the mature protein.

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Both pro-IL-1β and the mature IL-1β have good peptide coverage and are highly amenable to DXMS analysis (supplemental Fig. S1A). The pattern of relative protection for peptides represented in the block diagram in Fig. 5B is consistent with residue-specific protection factors determined in previous hydro-gen/deuterium exchange NMR studies of mature IL-1β (51). The NMR and mass spectrometry results agree for studies of mature IL-1β. Therefore, any differences observed in the amino acid sequence corresponding to residues 117–269 are because of conformational/dynamic differences of this region within the proprotein.

Another indication of the difference in conformation of the mature region within the proprotein is the fact that the number of identical peptides generated during peptide cleavage for DXMS analysis is confined to a subset of peptides, despite overall good peptide coverage (supplemental Fig. S1A). The distribution of identical peptides varies by location within the sequence, with
\( \beta \) -bulge (162–169, 46–53) and a conserved basic/hydrophobic region (residues 73–80), further blocking the B-site.

Biological activity for the proprotein may also be precluded by the presence of the limited secondary/tertiary structure and an overall destabilized N-terminal region may inhibit the trefoil 1 region from fully folding. Only one small area in pro-IL-1\( \beta \) corresponding to the residues of trefoil 1 in the mature protein is protected over the exchange time course (Fig. 5B). These residues are most likely less stable to solvent exchange and in an altered conformation due to their proximity to the minimally protected, destabilized N terminus. The first trefoil region may act to buffer the effects seen in the remainder of the C-terminal region (eventual trefoils 2 and 3) due to the conformational heterogeneity of the N terminus. Only after the N terminus is removed can these residues fully fold into the native mature structure.

Taken together with previous studies our current results lead us to propose that the presence of the conserved N terminus of pro-IL-1\( \beta \) prevents the final folding to the native structure of this potent cytokine. This trait would block the function of the eventual mature protein, which is to bind IL-1RI (2). Overall destabilization allows the proprotein to be in a protease-labile state for quick processing while keeping the C-terminal region primed to fold quickly and correctly after cleavage. Only after the N-terminal region is fully removed can these residues be stabilized into their final native structure. If unneeded by the cell, pro-IL-1\( \beta \) can be quickly disposed of by proteases working alone or in concert without it erroneously escaping and eliciting destructive responses (4). Regulating IL-1\( \beta \) function in this layered, subtle fashion is truly an example of how nature accomplishes the many finely tuned complexities that constitute cell signaling.

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