Published in final edited form as:

Biochem Biophys Res Commun. 2007 August 31; 360(3): 604–608. doi:10.1016/j.bbrc.2007.06.099.

Molecular Cloning, Overexpression and Characterization of Human Interleukin 1 α

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Abstract

Interleukin-1 alpha (IL-1 α) regulates a wide range of important cellular processes. In this study for the first time we report the cloning, expression, biophysical and biological characterization of the human interleukin-1 α . Human IL-1 α has been expressed in *Escherichia coli* in high yields (~ 4 mg per liter of the bacterial culture). The protein was purified to homogeneity (~ 98% purity) using affinity chromatography and size exclusion chromatography. Results of the steady state fluorescence and 2D NMR experiments show that the recombinant IL-1 α is in a folded conformation. Far-UV circular dichroism (CD) data suggest that IL-1 α is an all β -sheet protein with a β -barrel architecture. Isothermal titration calorimetry (ITC) experiments show that the recombinant IL-1 α binds strongly ($K_d \sim 5.6 \times 10^{-7}$ M) to S100A13, a calcium binding protein that chaperones the *in vivo* release of IL-1 α into the extracellular compartment. Recombinant IL-1 α was observed to exhibit strong cytostatic effect on human umbilical vascular endothelial cells. The findings of the present study not only pave way for an in-depth structural investigation of the molecular mechanism(s) underlying the non-classical release of IL-1 α but also provide avenues for the rational design of potent inhibitors against IL-1 α mediated pathogenesis.

Keywords

Interleukin-1 alpha; beta-sheet protein; Non-classical release; cytostatic effects; S100A13; Isothermal calorimetry; Circular dichroism

Introduction

Interleukin-1 α (IL-1 α) is a ~ 19 kDa protein that plays a crucial role in the regulation of a number of key cellular processes. IL-1 α is a cytokine and a potent mediator of body's response to inflammation, microbial invasion, tissue injury and immunological response (1, 2). In addition, recent studies suggest that IL-1 α also has a role in wound healing, rheumatoid arthritis, Alzheimer's disease and tumor growth (3). IL-1 α exhibits its wide array of biological activities by binding to its specific cell surface protein kinase receptor. Interestingly, unlike many other proteins, IL-1 α lacks the N-terminal signal peptide and has

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been shown to be secreted through a non-classical route, which is independent of the conventional ER-Golgi secretory pathway (4). IL-1 α is secreted into the extracellular compartment as a binary complex with S100A13, calcium binding protein. The non-classical secretion of IL-1 α is proposed as ideal model to understand the ER-Golgi independent secretion of a large number of proteins that lack the N-terminal signal sequence (4).

The molecular mechanism underlying the wide spectrum of biological activities of IL-1 α is poorly understood. Most of the information that is currently available on the non-classical secretion and its mode of action are obtained from cell biology studies (5). Information on the structure-function relationship of IL-1 α is scarce because of its prohibitively high cost of production in mammalian cells. Several attempts to overexpress IL-1 α in bacterial hosts have been futile because the overexpressed protein was invariably trapped in inclusion bodies (6). To our knowledge, there is no prior report on the successful overexpression and purification of human IL-1 α in the biologically active form. In this context, in the present study, for the first time we report the successful cloning, overexpression, purification and biophysical characterization of biologically active recombinant human IL-1 α from *Escherichia coli*. In our opinion, the results of this study are expected to trigger a number structural studies aimed at understanding the mode of action of IL-1 α , which in-turn would pave way for the rational design of agonists and antagonists against IL-1 α induced pathogenesis.

Materials and Methods

General protocol and reagent

Taq DNA polymerase, thrombin, *Nde*I, and *Xho*I were purchased from Promega. *Escherichia coli* [BL21 (DE3) pLysS] and pET20b (+) were purchased from Novagen. Ni²⁺-sepharose was obtained from Amersham-Pharmacia Biotech. Labeled ¹⁵NH₄Cl, ¹³C-glucose, and D₂O were purchased from Cambridge Isotope Laboratories. Guanidium hydrochloride and imidazole were obtained from Sigma Chemical Co., St. Louis. All other chemicals used in this study were of high quality analytical grade.

Construction and expression of the human IL-1 α gene

cDNA encoding the human IL-1 α contains 483 bp was amplified by polymerase chain reaction (PCR), using primers containing *Nde*I and *Xho*I sites (Supplementary Fig. S1-A). The PCR product was digested with *Xho*I and *Nde*I, inserted into the vector pET20b(+). The authenticity of the clone construct was confirmed by nucleotide sequencing. *E. coli* cells transformed with pET20b(+) containing the human IL-1 α insert were grown in 1 L Luria broth (LB) medium which contained 100 μ g/mL ampicillin. Protein induction was achieved by the addition of IPTG (1 mM/L) when the OD₆₀₀ of the growing culture had reached about 0.6. The culture was incubated at 37 °C for additional 4 h and the cells were harvested and lysed by sonication. The expression of the IL-1 α was checked by SDS-PAGE.

Purification of recombinant IL-1 α

The first step of purification of IL-1 α was achieved on a nickel (Ni²⁺)-sepharose column (Amersham Biosciences, USA). Clear bacterial cell lysate was loaded on to a nickel (Ni²⁺)-sepharose column (Amersham Biosciences, USA), and the column was washed with 100 mL of 10 mM tris (pH 8.0) containing 100 mM NaCl and 20 mM imidazole. The proteins bound to the Ni²⁺-sepharose column were eluted using a stepwise gradient of imidazole. The elution of the proteins was monitored by absorbance at 280 nm. Protein fractions containing IL-1 α were pooled together and concentrated using an Amicon ultrafiltration set-up. The protein was further purified (at room temperature) by size-exclusion chromatography on a Superdex-75 column using AKTA-FPLC (Amersham Biosciences, USA). Pure IL-1 α

containing the His₆ affinity tag was incubated with 100 NIH units of thrombin for 12 hours (at 25 °C) to cleave the affinity tag. The mixture containing IL-1 α , His₆ affinity tag, and thrombin were loaded again onto a Ni²⁺-sepharose column. His₆ affinity tag and thrombin were eliminated by washing the column exhaustively (with 150 mL) with 10 mM tris (pH 8.0) containing 100 mM NaCl and 20 mM imidazole. The bound IL-1 α was eluted in 500 mM imidazole. The protein was further desalted and concentrated by ultrafiltration in 10 mM tris (pH 7.5) containing 100 mM NaCl. The homogeneity of the protein was assessed using SDS-PAGE. The authenticity of the sample was further verified by MALDI-TOF mass analysis. The concentration of t IL-1 α was estimated on the basis of the extinction coefficient value ($\Sigma_{280} = 21430 \text{ M}^{-1} \text{ cm}^{-1}$) calculated from the amino acid sequence of the protein.

Preparation of isotope-enriched IL-1 α

Uniform ¹⁵N labeling was achieved using M9 minimal medium containing ¹⁵NH₄Cl. To achieve maximal expression yields, the composition of the M9 medium was modified by the addition of a mixture of vitamins (7). The expression host strain *Escherichia coli* BL21 (DE3) pLysS is a vitamin B₁-deficient host, and hence, the medium was supplemented with thiamine (vitamin B₁).

Circular dichroism

All circular dichroism (CD) measurements were carried out at room temperature (298 K) on a Jasco J720 spectropolarimeter using a quartz cell of 0.02 cm pathlength. Each spectrum was an average of 10 scans. The concentration of the protein used for the CD measurements was 50 μ M. Necessary background corrections were made in all spectra.

Steady state fluorescence

Fluorescence experiments were performed on a Hitachi F2500 spectrofluorimeter. All fluorescence experiments were performed at 25 °C. The excitation wavelength was set at 280 nm, and bandwidths for excitation and emission were set at 2.5 nm and 10 nm, respectively. The concentration of the protein used for the fluorescence measurements was 10 μ M. Necessary background corrections were made in all spectra.

Equilibrium unfolding

Guanidinium hydrochloride-induced equilibrium unfolding of IL-1 α was performed at a protein concentration of 10 μ M and the unfolding of the protein was monitored by changes in the tryptophan fluorescence (at an emission wavelength of 338 nm). The excitation wavelength was set at 280 nm. The excitation and emission slit widths were set at 2.5 nm and 10 nm, respectively. Appropriate background corrections were made in all spectra.

NMR experiments

All NMR experiments were performed at 25 °C on a Bruker Avance 700 MHz NMR spectrometer equipped with a cryoprobe. ¹⁵N decoupling during acquisition was accomplished using the globally optimized altering-phase rectangular pulse sequence. 2D ¹H-¹⁵N HSQC spectra were acquired with 64 scans and 2048 complex data points in the ¹⁵N dimension. The concentration of the protein sample used for the 2D ¹H-¹⁵N HSQC experiment was 0.1 mM dissolved in 90% H₂O and 10% D₂O containing 10 mM tris-d₆ (pH 7.5) and 100 mM NaCl. All spectra were referenced to TSP-d₄ and were processed on a Windows workstation using XWIN-NMR and Sparky softwares.

Biological activity assay

Human Umbilical Vein Endothelial Cells (HUVEC) (Cambrex) at passage 5 were plated at the density of 5000 cells per well of a TC6 plate in the Endothelial Cell Growth Medium (Cambrex) supplemented with 10% fetal calf serum (Hyclone), in the presence or in the absence of 1 ng/ml recombinant IL-1 α . Cells were trypsinized at days 2 and 5 after plating, resuspended in 1 ml of the full medium and counted using a hemacytometer.

Isothermal titration calorimetry

Binding affinity of IL-1 α to S100A13 was assessed by measuring the heat change during the titration of IL-1 α and S100A13 using a VP-ITC titration microcalorimeter (MicroCal Inc., Northampton, MA). Protein and ligand (S100A13) solutions were degassed under vacuum and equilibrated at 25 °C prior to titration. The sample cell (1.4 mL) contained 0.03 mM IL-1 α dissolved in 10 mM tris (pH 7.5) containing 100 mM NaCl. The reference cell contained Milli Q water. Upon equilibration, a typical titration consisted of injecting 10 μ L aliquots of 1 mM ligand (S100A13) solutions into a 0.03 mM solution of IL-1 α after every 4 min to ensure that the titration peak returned to the baseline prior to the next injection. The resulting titration curves were corrected for the protein-free buffer [10 mM tris (pH 7.5) containing 100 mM NaCl] control and analyzed using the Origin ITC software supplied by MicroCal Inc. The binding constants were estimated from the obtained isotherms using the one-site binding model.

Results and Discussion

Overexpression and purification of recombinant interleukin-1 alpha (IL-1 α)

The human interleukin-1 α construct used in the present study is 162 amino acids long containing residues, 108 to 270 of the full-length human interleukin-1 α precursor (8) (Supplementary Fig. S1-B). SDS-PAGE of the bacterial cell lysate induced by IPTG showed an intense band at ~ 19 kDa corresponding to IL-1 α (Fig. 1A). SDS-PAGE of the bacterial lysate revealed that 25%–30% of the overexpressed IL-1 α was in the soluble fraction and the remainder was trapped in the insoluble form as inclusion bodies (Fig. 1A). The His₆-tag designed at the C-terminal end facilitated the purification of the recombinant IL-1 α (expressed in the soluble fraction) by affinity chromatography on a nickel affinity column. IL-1 α was bound to the nickel affinity column quite strongly and the unbound contaminating proteins were eliminated by washing the affinity column with 100 mL of the elution buffer [10 mM tris (pH 7.5) containing 100 mM sodium chloride and 20 mM imidazole]. Purification profile (on the nickel affinity column) obtained using a step-wise gradient of imidazole revealed that IL-1 α eluted in 150 mM, 250 mM, and 500 mM imidazole (Fig. 1B). All the three protein fractions showed an intense band on SDS PAGE corresponding to the molecular mass of IL-1 α (~ 19 kDa). The purity of IL-1 α in all the three fractions was about 70% (Fig. 1C). IL-1 α was further purified by size-exclusion chromatography using FPLC. IL-1 α eluted as a single peak with an elution time of 81 ± 1 minutes (Supplementary Fig. S2). SDS-PAGE of the re-purified IL-1 α sample yielded an intense single band with about 98% purity (Fig. 1D). MALDI-TOF mass spectrum analysis of the purified recombinant IL-1 α sample showed an expected molecular mass of 19,328 Da (Fig. 1E). The final yield of the pure IL-1 α was 4.0 ± 0.4 mg per liter of the bacterial culture.

Biophysical characterization of recombinant IL-1 α

We assessed the conformation of the recombinant IL-1 α using a variety of biophysical techniques including, Far UV circular dichroism (CD), fluorescence spectroscopy and multidimensional NMR spectroscopy. Far UV CD provides useful information on the secondary structure of proteins. Far-UV CD spectrum of IL-1 α revealed a prominent

positive CD band centered at around 228 nm and a negative ellipticity peak at about 205 nm (Supplementary Fig. 3A). These spectral features are reminiscent of an all β -barrel protein, with the constituent β -strands arranged antiparallely into a β -barrel structure (7, 9). The Far UV CD spectrum of IL-1 α in 6 M guanidium hydrochloride showed the complete loss of the 228 nm positive ellipticity band suggesting that the protein is denatured under these conditions (Supplementary Fig. S3A).

IL-1 α contains two tryptophan residues located at positions 115 and 141 in the primary sequence (1). Therefore, measurement of the intrinsic tryptophan fluorescence would serve as an excellent probe to monitor the tertiary structural changes that occur in the protein under different conditions. Fluorescence spectrum of IL-1 α in its native state showed an emission maximum around 338 nm suggesting that the tryptophan residues are buried in the interior of the well organized tertiary structure of the protein (Supplementary Fig. S3B, *inset*). Guanidinium hydrochloride-induced equilibrium unfolding of IL-1 α , monitored by changes in the intrinsic tryptophan fluorescence, showed that the protein completely unfolds in 4.0 M. The concentration of the denaturant at which 50% of the IL-1 α molecules exist in the denatured state(s) (C_m) is estimated to be about 2.2 ± 0.05 M. The free energy of unfolding of IL-1 α (ΔG_U) is estimated to be -4.3 ± 0.16 kcal.mol⁻¹. These results clearly indicate that the recombinant IL-1 α is in a stable folded conformation (Supplementary Fig S3B).

Structure and activity relationship monitored by NMR (SAR by NMR) is a versatile technique for screening protein-protein or protein-ligand or protein-drug interactions (9, 10). SAR by NMR technique mostly relies on the availability of ¹H-¹⁵N HSQC spectrum of the target protein. The ¹H-¹⁵N HSQC spectrum is a finger-print of the protein conformation and each crosspeak in the spectrum represents an amino acid residue in a protein. The dispersion of the crosspeaks in the ¹H-¹⁵N HSQC spectrum provides unambiguous and useful information on the conformational state of the protein. ¹H-¹⁵N HSQC spectrum obtained using ¹⁵N labeled IL-1 α is well-dispersed implying that the protein is structured (Fig. 2A). This is first time the ¹H-¹⁵N HSQC spectrum of IL-1 α is reported. The availability of the ¹H-¹⁵N HSQC spectrum of IL-1 α would help in mapping the interactions sites of various proteins/ligands/drugs on the protein (IL-1 α).

Biological activity of recombinant IL-1 alpha

It is important to verify if the recombinant IL-1 α is in its biologically active conformation. In order to test the biological activity of the recombinant IL-1 α , we studied its effect on the growth of human umbilical vascular endothelial cells (HUVEC). IL-1 α is known to exhibit a strong cytostatic effect on HUVEC (11). HUVEC were plated at low density in presence or absence of 1 ng/ml IL1 α in the medium. Cells were trypsinized and counted at days 2 and 5 after plating. By the day 5 of culture, IL-1 α induced a more than five fold decrease of cell growth (Fig. 2B). Conversely, IL-1 α did not cause cell detachment and death. These results clearly demonstrate that recombinant IL-1 α obtained in this is biologically active.

IL-1 α -S100A13 interaction

As mentioned earlier, IL-1 α is exported through a non-classical secretion pathway by forming a multiprotein release complex with S100A13 (12). Therefore, examination of the binding affinity of recombinant IL-1 α to S100A13 is an authentic test to ensure the “nativeness” of the tertiary fold of recombinant IL-1 α . Isothermal titration calorimetry (ITC) is a useful and popular technique to measure binding affinity of a protein to its ligand or protein partner. In addition, ITC is an important tool for studying both thermodynamic and kinetic properties of biological macromolecules by virtue of its general applicability and high level of precision (13). Therefore, we used ITC to measure the binding affinity of the

recombinant IL-1 α to its binding partner, S100A13. Isothermogram of the IL-1 α /S100A13 titration is sigmoidal (Fig. 3). The titration curve representing the binding of IL-1 α saturates at a protein to ligand ratio of 1:1. Also the binding of IL-1 α to S100A13 is accompanied by the evolution of heat (Fig. 3). The binding constant (K_d) is estimated to be 5.6×10^{-7} M. The results obtained unambiguously suggest that recombinant IL-1 α is indeed in its biologically active conformation capable of binding strongly to S100A13, which is essential for its non-classical release.

This is the first report of the cloning, overexpression, and purification of IL-1 α in a biologically active conformation. The findings of the present study are expected to trigger a number of structural studies aimed at understanding the molecular mechanism(s) underlying the regulation of the IL-1 α mediated biological activities. In addition, the demonstration of the feasibility of NMR experiments on the recombinant IL-1 α will not only provide impetus to embark on determination of the three-dimensional solution structure of IL-1 α using multidimensional NMR spectroscopy, but also is expected to generate new initiatives on the rational design of potent therapeutic principles against IL-1 α mediated pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institute for Health (NIH NCRR COBRE Grant 1 P20RR15569), the Department of Energy (DE-FG02-01ER15161) and the Arkansas Bioscience to TKSK. IP was supported by NIH grants HL32348, HL35627 and RR15555 (project 4).

Abbreviations

IL-1α	Interleukin-1 alpha
NMR	Nuclear magnetic resonance spectroscopy
CD	Circular dichroism
ITC	Isothermal titration calorimetry
SAR	Structure and activity relationship
HSQC	Hetero nuclear single quantum coherence
ER	Endoplasmic reticulum
HUVEC	Human umbilical vein endothelial cells
MALDI	Matrix assisted laser desorption/ionization
IPTG	Isopropyl β -D-1-thiogalactopyranoside

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Fig. 1.

Panel A- SDS-PAGE depicting the overexpression of IL-1α in *E. coli*. Lane M- represents the molecular weight marker; Lane 1- lysate of uninduced bacterial cells; Lane 2- lysate of IPTG-induced bacterial cells; Lane 3 – soluble fraction of the bacterial lysate; Lane 4 – insoluble fraction of the bacterial lysate. Panel B- Nickel (Ni²⁺)- affinity chromatography profile obtained in various concentrations of imidazole. Peak numbers 1, 2, 3, 4, 5, and 6 represent the fractions of proteins eluted in 20 mM, 50 mM, 100 mM, 150 mM, 250 mM, and 500 mM imidazole, respectively. The elution of the protein (at 25 °C) was monitored by absorbance at 280 nm. The eluent used was 10 mM tris (pH 8.0) containing 100 mM NaCl. Panel C- SDS-PAGE of the fractions collected at various concentrations of imidazole. Lane- M shows the molecular weight marker. Lanes- 1 to 6 depict protein bands contained in fractions collected at 20 mM, 50 mM, 100 mM, 150 mM, 250 mM, and 500 mM imidazole concentrations, respectively. Panel D- SDS-PAGE of recombinant IL-1α purified on FPLC (lane-1). Lane M- represents the molecular weight marker. Panel E- MALDI-TOF mass spectrum of the purified IL-1α sample.

Fig. 2.

Panel **A**- ^1H - ^{15}N HSQC spectrum of the IL-1 α obtained at pH 7.5 and 25 °C. The spectra were acquired in 10 mM d_6 -Tris-HCl (pH 7.5) containing 100 mM NaCl. The concentration of protein used was 0.1 mM. Panel **B**- Cytostatic effect of IL-1 α on HUVEC growth. Human Umbilical Vein Endothelial Cells (HUVEC) were plated at the density of 5000 cells per well of a TC6 plate in presence (filled) or absence (open) of 1 ng/ml recombinant IL-1 α . Cells were trypsinized at days 2 and 5 after plating and counted.

Fig. 3.

Isothermal titration calorimetry representing the interaction of S100A13 with IL-1 α . The upper panel represents the raw data and the bottom panel is best-fit of the raw data, after subtracting the heat of dilution. The solid line in the bottom panel represents the best-fit of the data. The isothermogram fits best to a one-site binding model. S100A13 binds to IL-1 α with a binding constant value of $\sim 5.6 \times 10^{-7}$ M. The concentration of the IL-1 α used was 30 μ M. Appropriate background corrections were made to account for the heats of dilution. Experiment was performed at 25 $^{\circ}$ C in 10 mM tris (pH 7.5) containing 100 mM NaCl.