Structural and Functional Similarity between *Yersinia pestis* Capsular Protein Caf1 and Human Interleukin-1β[†]

Vyacheslav M. Abramov,[‡] Anatoly M. Vasiliev,[‡] Raisa N. Vasilenko,[‡] Nataly L. Kulikova,[‡] Igor V. Kosarev,[‡] Valentin S. Khlebnikov,[‡] Alexander T. Ishchenko,[‡] Sheila MacIntyre,[§] Joel R. Gillespie,[∥] Ritu Khurana,[∥] Timo Korpela,[⊥] Anthony L. Fink,[∥] and Vladimir N. Uversky*,[‡,∥,#]

Institute of Immunological Engineering, 142380 Lyubuchany, Moscow Region, Russia, Microbiology Division, School of Animal and Microbial Sciences, University of Reading, P.O. Box 228, Reading RG6 6AJ, U.K., Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064, Finish-Russian Joint Biotechnology Laboratory, Department of Biochemistry, University of Turku, Bio-City SF-20520 Turku, Finland, and Institute for Biological Instrumentation, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia

Received November 22, 2000; Revised Manuscript Received February 27, 2001

ABSTRACT: A comparative study of the structural and functional properties of recombinant *Yersinia pestis* Caf1 and human IL-1β was performed. According to Fourier transform infrared spectroscopy (FTIR) and circular dichroism (CD) data, IL-1β and Caf1 are typical β-structural proteins. Neither protein interacts with the hydrophobic probe ANS (8-anilino-1-naphthalenesulfonate) under physiological conditions. Specific binding of Caf1 [$K_d = (5.4 \pm 0.1) \times 10^{-10}$ M] to interleukin-1 receptors (IL-1Rs) on the surface of finite mouse fibroblasts (line NIH 3T3) was observed. Caf1 is able to inhibit high-affinity binding of ^{125}I-labeled IL-1β to NIH 3T3 cells, and in the presence of Caf1, the binding of [^{125}I]IL-1β is characterized by a K_d of $(2.0 \pm 0.3) \times 10^{-9}$ M. Caf1 binding to IL-1R could reflect adhesive properties of the capsular subunits responsible for the contact of bacteria with the host immunocompetent cells. In its turn, this may represent a signal for the initiation of the expression and secretion of the proteins of *Y. pestis* Yop virulon. Thus, these results help to explain the importance of Caf1 in the interaction of *Y. pestis* with the host immune system.

Yersinia pestis is an agent of one of the most acute and especially dangerous diseases, namely, bubonic plague. The pathogenic properties of *Y. pestis* in a sensitive host are ensured through the regulation of coordinated expression of a set of virulence and pathogen factors with different functional properties. A key feature in the virulence of *Y. pestis* is its ability to resist the attack of host phagocytes (*1*, *2*). Moreover, *Y. pestis* virulence correlates not only with stability to phagocytic absorbance but also with its ability to survive and reproduce within phagocytes at the expense of inhibition of phagocyte antibacterial functions. Reproduction inside macrophages is a compulsory stage of plague pathogenesis (*1*). This property is connected with the function of the calcium-dependent plasmid (pCad)[1] or the low-calcium response plasmid, Lcr-plasmid (*3*, *4*), which are characteristic features of *Yersinia* species (*5*, *6*). Lcr-plasmid ensures production of a series of factors, of which Yop (*Yersina* outer protein) secretion proteins are important for the realization of the pathogenic properties of the agent (*7*). According to the model of the Yop virulon (*8*, *9*), Yop proteins are subdivided into two groups, effectors (YopE, YopH, YopM, YpkA, YopP, and YopT), which damage functions of phagocytic cells and cause their apoptosis, and translocators (YopB, YopD, and LcrV), which ensure transfer of effector proteins from the bacterial cell inside the phagocyte. One more Yop protein, YopN (LcrE), is involved in the regulation of biosynthesis and secretion of effector and translocator Yops. It has been suggested that in the absence of calcium (in vitro) or on contact of the pathogen with host cell receptors (in vivo) serve as the signal for initiation of expression and secretion of Yop proteins (*10*). Contact of pathogen with the host cell induces polarized translocation of Yop inside phagocytes only in the area of its interaction with the bacterium (*8*).

In pathogenic Gram-negative bacteria, special adhesive surface structures (*11*) [whose formation involves the chaperone−usher pathway (*12*)] are responsible for the contact with the host cell. Genes encoding the adhesin subunits and periplasmatic molecular chaperones of those adhesins (usher proteins, anchored adhesins on the bacteria surface), as well as the regulatory genes, belong to a specific

[†] This work was supported by grants from the International Science and Technology Center (United States and European Community) and the Russian Foundation for Basic Research.

* To whom correspondence should be addressed: Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064. Telephone: (831) 459-2915. Fax: (831) 459-2935. E-mail: uversky@hydrogen.ucsc.edu.

[‡] Institute of Immunological Engineering.

[§] University of Reading.

[∥] University of California.

[⊥] University of Turku.

[#] Russian Academy of Sciences.

[1] Abbreviations: FTIR, Fourier transform infrared spectroscopy; CD, circular dichroism; ANS, 8-anilino-1-naphthalenesulfonate; SAXS, small-angle X-ray scattering; Caf1, capsular antigen F1; IL, interleukin; IL-1R, interleukin-1 receptor; YOP, *Yersina* outer protein; pCad, calcium-dependent plasmid; Lcr-plasmid, low-calcium response plasmid.

10.1021/bi002678x CCC: $20.00
Published on Web 04/25/2001

operon class responsible for the synthesis and subsequent maintenance of the adhesion structures. It should be noted that the existence of such special surface adhesion structures in *Y. pestis* has not yet been confirmed.

The ability of *Y. pestis* bacteria to form a protective capsule was shown more than 100 years ago (*13*), and capsular antigen F1 (Caf1) was considered to be one of the factors of the plague microbe pathogenicity (*14*−*18*). Caf1 protein subunits are the main (but not only) capsular component (*19*). Caf1 consists of a single polypeptide chain of 149 amino acid residues (*20*). Genetic control of capsule formation involves the *f* operon (*20*), localized on the pFra plasmid. This operon consists of four genes: *caf1*, *caf1m*, *caf1a*, and *caf1r*, encoding Caf1 protein (*20*), Caf1M protein (molecular periplasmic chaperone of Caf1) (*21*), Caf1A protein (anchoring Caf1 on the surface of the bacteria) (*22*), and Caf1R protein (transcription regulator) (*23*), respectively. The fact that proteins encoded by the *f* operon have a high level of homology (more than 60%) with the proteins of the *pap* operon in uropathogenic *Escherichia coli* is of great interest (*11*, *24*, *25*).

Interleukin-1 (IL-1) is a key mediator in the immune response. This protein regulates proliferation and activation of T-lymphocytes and fibroblasts, leading to increased production of prostaglandins, collagenase (*26*−*28*), and other cytokines (including interleukin-2). IL-1 was detected in synovial fluid of patients with arthritis (*29*, *30*). The IL-1 family includes two different types of proteins: IL-1α and IL-1β which are ~25% homologous. Human IL-1β has 153 amino acid residues, with a molecular mass of 17 377 Da, and is formed from a precursor 31 kDa protein (*31*). IL-1β contains no disulfide bonds but has two free sulfhydryl groups (*32*).

Statistically significant homology between Caf1 of *Y. pestis* and human interleukins-1α and -1β and receptor antagonist (IL-1ra) has been established (*33*). On the other hand, Caf1 was shown to induce specific defense against plague (*34*−*38*), and specific anti-Caf1 antibodies exhibit preventive properties (*34*, *39*). Human IL-1β was shown to interact with *Y. pestis* Caf1A (*40*). All this suggests that Caf1 and IL-1β may have not only structural but also functional similarities, which appeared during the evolution of the host−parasite system.

We present here data on comparative studies of the structural and functional properties of Caf1 and IL-1β. The existence of considerable structural similarity between the two proteins is established. Effective binding of ^{125}I-labeled Caf1 to IL-1 receptors (IL-1Rs) on NIH 3T3 mouse fibroblasts {large number of IL-1Rs expressed on their surfaces [$(5.1 \pm 0.5) \times 10^4$] (*41*)} was found. Caf1 binding to IL-1R may explain the adhesive properties of the capsular subunits. Thus, this protein may be responsible for the contact of bacteria with immunocompetent host cells and, as a consequence, for the initiation of expression and secretion of *Y. pestis* Yop virulon proteins.

MATERIALS AND METHODS

Materials

Recombinant Y. pestis capsular protein Caf1 was isolated from *E. coli* HB101 cells transformed by plasmid pFS2 containing the *caf* operon. The protein was purified to homogeneity using TSK-Fractogel-HW-55 (F) column chromatography on a Pharmacia FPLC apparatus. After chromatography, SDS−PAGE showed only one protein with a molecular mass of 17 kDa. The specificity of recombinant Caf1 was proven by immunoblotting carried out using anti-Caf1 monoclonal antibodies. Since Caf1 does not contain tryptophan residues, this fact can also be used for the analysis of the sample purity. The absence of tryptophan residues in the analyzed Caf1 samples was supported by fluorescence spectroscopy, which shows the absence of any tryptophan contribution (see Figure 1).

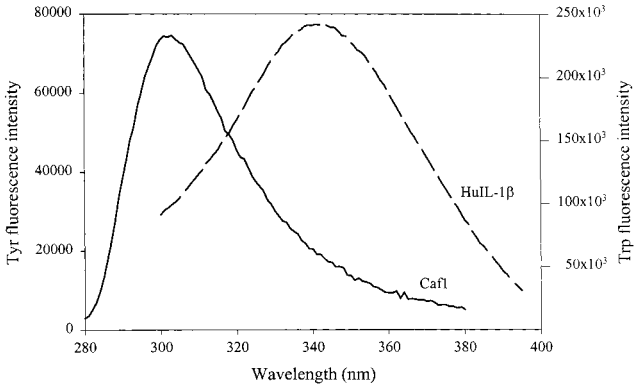

FIGURE 1: Intrinsic fluorescence of Caf1 (—) and human recombinant interleukin-1β (− − −). Measurements were carried out at pH 7.5 and 25 °C. The protein concentration was 0.1 mg/mL. Fluorescence was excited at 270 and 280 nm for Caf1 and huIL-1β, respectively.

Recombinant human IL-1β was produced by *E. coli* C600 cells transformed with the plasmid (pPR-TGATG-hLI-1$\tilde{b}$tsr), containing cDNA of IL-1β, and purified by gel filtration, ion exchange chromatography, and high-pressure liquid chromatography (*42*, *43*).

The number of amino acid residues was 153; the purity was more than 98% at the protein level, and its biological activity detected by murine thymocyte proliferative responses was of 1×10^8 units/mg of protein

Cell Cultures and Growth Conditions. Mouse fibroblast cell culture NIH 3T3 was used for studies on Caf1 and IL-1β reception. Cells were cultivated in 5% CO_2 at 37 °C in RPMI 1640 medium containing L-glutamine, 5−10% fetal-calf serum, and 50 μg/mL gentamycin.

Methods

SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the method of Laemmli (*44*). The gel was stained with Coomassie Brilliant Blue R250 or subjected to immunoblotting.

Circular dichroism measurements were carried out using an AVIV-60DS spectropolarimeter, equipped with a temperature-controlled cell holder. The cell path lengths were 1.0 and 10.0 mm for far- and near-UV CD measurements, respectively. The protein concentration was 0.6 mg/mL.

Intrinsic (tyrosine and tryptophan) and ANS fluorescence was measured with a Perkin-Elmer MPF-4 instrument with excitation at 270, 286, and 350 nm, respectively. The protein concentration was kept at 0.1 mg/mL. The ANS concentration was 25 μM.

FTIR spectra were collected on a Nicolet 800SX FTIR spectrophotometer equipped with an MCT detector. The IRE (72 mm × 10 mm × 6 mm, 45° germanium trapezoid) was held in a modified SPECAL out-of-compartment ATR apparatus. The hydrated thin films were prepared as described previously (*45*, *46*). Typically, 1024 interferograms were coadded at 4 cm^{-1} resolution. Data analysis was performed with GRAMS-32 (Galactic Industries). Secondary structure content was determined from curve fitting to spectra deconvoluted using second derivatives and Fourier self-deconvolution to identify component band position.

The molecular masses (M_r) of proteins were determined by equilibrium ultracentrifugation (*47*, *48*) or by gel filtration. Centrifugation measurements were performed on a MOM ultracentrifuge using interference optics. Sedimentation measurements were performed on a model E ultracentrifuge (Beckman) using Schleiren optics and UV absorption. Sedimentation coefficients were estimated at a protein concentration of 0.5−10 mg/mL and extrapolated to zero concentration.

Gel filtration measurements were carried out on a Superdex-75 column using a Pharmacia FPLC apparatus. To determine the molecular masses of proteins under native conditions, the gel filtration column was calibrated using the standard procedures (*49*−*52*). Proteins from a standard molecular mass (*M*) marker set were passed through the column, and the retention coefficient (K_d) for each individual protein was determined:

$$K_d = (V - V_o)/(V_t - V_o)$$

where V_o is the column void volume determined as the elution volume of blue dextran, V_t is the total solvent-accessible column volume determined as the elution volume of acetone, and V is the elution volume of the given protein. Least-squares analysis was used to fit the data to generate the calibration curve.

Hydrodynamic dimensions (Stokes radius, R_S) of proteins in different conformational states were also measured by size exclusion chromatography. In this case, a specific calibration curve, $1000/V$ versus R_s dependence, was used (*49*−*53*). A set of globular proteins with known R_S values was used for column calibration (*51*).

Small-angle X-ray scattering (SAXS) measurements were taken using the SAXS instrument on beamline 4-2 at Stanford Synchrotron Radiation Laboratory (*54*). X-ray energy was selected at 8980 eV (Cu edge) by a pair of Mo/B_4C multilayer monochromatic crystals (*55*). Scattering patterns were recorded by a linear position-sensitive proportional counter, which was filled with an 80% Xe/20% CO_2 gas mixture. Scattering patterns were normalized by incident X-ray fluctuations, which were measured with a short length ion chamber before the sample. The sample-to-detector distance was calibrated to be 230 cm, using a cholesterol myristate sample. To avoid radiation damage of the sample in equilibrium and manual mix measurements, the protein solution was continuously passed through a 1.3 mm path length observation flow cell with 25 μm mica windows. Background measurements were performed before and after each protein measurement and then averaged before being used for background subtraction. All SAXS measurements were performed at 23 ± 1 °C.

Radii of gyration (R_g) were calculated according to the Guinier approximation (*56*):

$$\ln I(Q) = \ln I(0) - R_g^2 Q^2/3$$

where Q is the scattering vector given by the equation $Q = (4\pi \sin \theta)/\lambda$ (where 2θ is the scattering angle and λ is the X-ray wavelength). $I(0)$, the forward scattering amplitude, is proportional to $n\rho_c^2V^2$, where n is the number of scatters (protein molecules) in solution, ρ_c is the electron density difference between the scatter and the solvent, and V is the volume of the scatter (*56*). Changes in $I(0)$ indicate changes in the association state of a protein. For example, if a protein sample were to go from a fully monomeric to a fully dimeric state, the number of scatters, n, would decrease by a factor of 2 and the volume of each scatter, V, would roughly increase by a factor of 2; the net effect would be a 2-fold increase in $I(0)$.

Determination of Caf1 Oligomeric Composition. ^{125}I-labeled Caf1 (concentration of 0.1 μg/mL) was added to Caf1 samples with concentrations of 1, 5, and 10 μg/mL. These samples were incubated for 48 h at 4 °C to reach equilibrium between the oligomers. Samples (50 μL) were fractionated on a Superose 12 HR 10/30 column, and the amount of radioactivity of 50 μL of each sample was measured.

Radiolabeling of protein Caf1 and IL-1β was carried out using the Iodogen technique described in ref *57*. Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl glycouril, Sigma) (1 mg) was dissolved in dichloromethane (25 mL), and 50 μL of this Iodogen solution was dispersed in the bottom of a polypropylene iodination vial and evaporated to dryness at room temperature under nitrogen. This removes the dichlormethane, produces a film of Iodogen, and ensures that the Iodogen does not form a suspension, which may give variable iodinations. These dried vials may be stored for up to 6 months at 20 °C.

Sodium phosphate buffer (0.05 M, 10 μL, pH 7.4) was added to the iodination vial, followed by $Na^{125}I$ (1 mCi, 50 μM, 10 μL) and 10 μg of Caf1 or rHuIL-1β in 0.05 M sodium phosphate buffer. The iodination was allowed to proceed for 10 min, ensuring that the reactants were in contact with the Iodogen film in the bottom of the vial, and terminated by adding 500 μL of 0.05 M protein-free sodium phosphate.

Labeled Caf1 and IL-1β were separated on a small Sephadex G-25 column (LKB). Fractions (100 μL) were collected, and fractions containing protein-bound radioactivity were pooled. The amount of radioactivity of ^{125}I-labeled Caf1 was 0.09 mKu/μg of protein, and that of ^{125}I-labeled IL-1β was 0.1 mKu/μg of protein.

Although the radiolabeled IL-1β lost ∼80% activity when assayed by murine thymocyte proliferative responses, it retaines binding activity to the rabbit anti-IL1β antibody. The radiolabeled Caf1 retained binding activity to the murine anti-CAf1 monoclonal antibody.

Assay of Binding of ^{125}I-Labeled Caf1 and ^{125}I-Labeled IL-1β to Cells. To study the reception of Caf1 and IL-1β by NIH 3T3 fibroblasts, cells at a density of 5×10^6 cells/mL were cultivated in 24-well plates (Costar) for 24 h until the cell monolayer was formed. Different doses of ^{125}I-labeled Caf1 and/or IL-1β were inserted into cell culture and incubated for 1 h at 4 °C. Then cells were washed three times

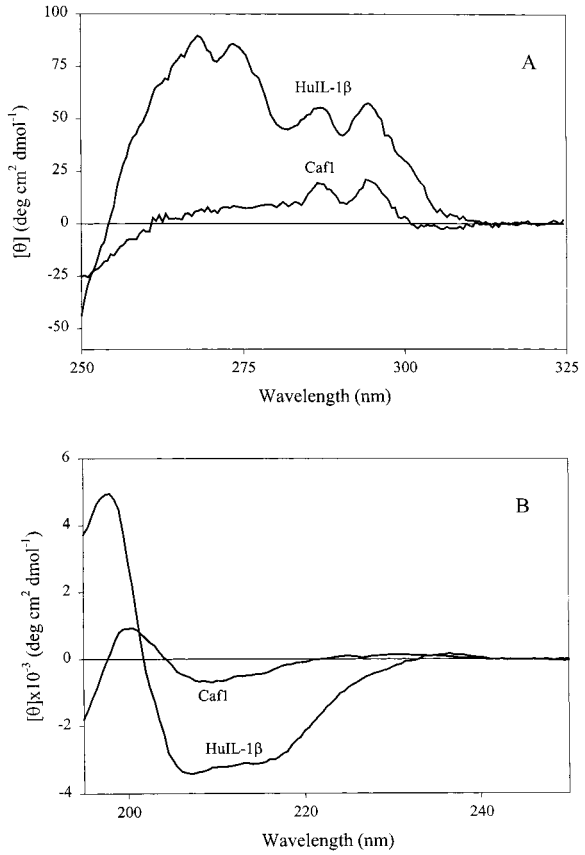

FIGURE 2: Near-UV (A) and far-UV (B) CD spectra of *Y. pestis* capsule protein Caf1 and human recombinant interleukin-1β. Measurements were carried out at pH 7.5 and 25 °C. The protein concentrations were 0.6 and 1.0 mg/mL whereas the cell path lengths 10 and 0.1 mm for near- and far-UV CD spectra measurements, respectively.

with culture medium (RPMI 1640, 1 mL per well). Then cells were treated with a tripsin−EDTA solution (ICN Biomedicals Inc.). In this case, 0.5 mL of a tripsin−EDTA solution per well was added and cells were incubated for 10 min. This process was repeated twice. The success of NIH 3T3 cell removal from the plastic surface was controlled microscopically. Cell suspension after the first and second trypsin treatment was joint, and the radioactivity level was measured by the γ-counter. For determination of the level of nonspecific binding of ^{125}I-labeled Caf1 and/or ^{125}I-labeled IL-1β, the 1000-fold surplus of unlabeled Caf1 and IL-1β was used. The results for specific binding were the average of triplicate cultures. These results were expressed as the mean number of counts per minute, in which the level of nonspecific binding was subtracted.

RESULTS

Structural Characterization of Caf1 and huIL-1β

Near- and Far-UV CD Spectra. Figure 2 represents CD spectra of Caf1 and huIL-1β. The near-UV CD spectrum of Caf1 has much lower intensity than that of huIL-1β (Figure 2A). This is due to the difference in the content of aromatic amino acids between the two proteins, as Caf1 does not have any Trp residues. The contribution of the single Trp120, and individual tyrosines (Tyr24, -68, -90, and -121), to the near-UV CD spectrum of huIL-1β has been examined by site-directed mutagenesis. It was assumed that the spectrum is largely a result of contributions from Trp120 and from Tyr68 (*58*). However, Figure 1 shows that not only huIL-1β but also Caf1 has well-pronounced positive peaks in the vicinity of 287 and 294 nm. As there is no Trp in Caf1, the presence of longer-wavelength peaks in the near-UV CD spectra suggests the existence of specific (and probably structurally close) clusters of aromatic residues in both proteins.

Far-UV CD spectra of Caf1 and huIL-1β are compared in Figure 2B. The spectrum of huIL-1β is typical of β-structural proteins, whereas the shape of the Caf1 spectrum is rather unusual (cf. ref *59*). It is known that in some cases far-UV CD spectra can be considerably distorted due to the contribution of aromatic side groups (*60*−*66*). Interestingly, such an effect is usually more pronounced for β-structural proteins, and the largest deviations from the "normal" spectrum (comparable to that of Caf1) were observed for human carbonic anhydrase B (*67*, *68*) and retinol-binding protein (*69*).

ANS Fluorescence. ANS is frequently used for characterization of the hydrophobicity of native proteins (*70*), and for the identification of non-native conformations in globular proteins (*71*). The reason is that the interaction of ANS with protein is accompanied by a considerable increase in the dye fluorescence intensity and by a pronounced blue shift of the fluorescence maximum. Surprisingly, we have established that spectrum of free ANS is very close to spectra of this dye in the presence of native huIL-1β or Caf1 (data not shown). This means that neither huIL-1β nor Caf1 binds ANS. In other words, these proteins do not have the appropriate sites for the interaction with a hydrophobic fluorescent probe under physiological conditions.

Secondary Structure Analysis by FTIR. The main advantage of FTIR in comparison with CD is that this method is much more sensitive to the β-structure. Figure 3 shows FTIR spectra measured for native huIL-1β (Figure 3A) and Caf1 (Figure 3B). Both huIL-1β and Caf1 are characterized by FTIR spectra typical of β-structural proteins. The curve-fit spectra for Caf1 and huIL-1β are compared in Figure 4, and the results of secondary structure analysis are summarized in Table 1. The data that are presented are consistent with the important conclusion that both proteins belong to the family of β-structural proteins (containing more than 40% β-sheet) and have very close (almost identical) secondary structure compositions.

Hydrodynamic Properties of Caf1 and huIL-1β. The molecular masses, M_r, of both proteins were determined at neutral and acidic pH by ultracentrifugation and gel filtration. Ultracentrifugation experiments showed that independent of the pH and protein concentration, Caf1 exists predominantly as oligomers. The degree of aggregation is diminished by a decrease in the protein concentration. For example, Caf1 with a high protein content (3−5 mg/mL) was characterized by an average molecular mass of ~7000 kDa, whereas gel electorophoretic analysis of Caf1 at 0.5 mg/mL is consistent with the conclusion that the protein sample represents a mixture of dimers, tetramers, octamers, etc. (not shown). At the same time, gel filtration investigations show that even very strong dilution (<0.001 mg/mL) does not lead to the dissociation of dimers into the monomers (see Table 2). This conclusion was confirmed by the results of SAXS analysis at 1.0 mg/mL. Thus, the dimer, with an approximate R_g of

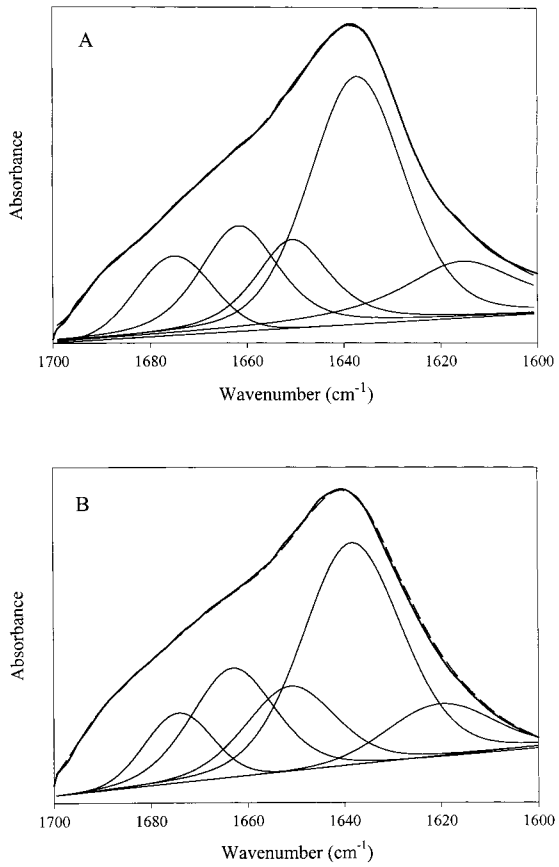

FIGURE 3: Secondary structure analysis of Caf1 (A) and huIL-1β (B) by FTIR. The figure depicts FTIR spectra of proteins (thick lines) and their curve-fit spectra (thin lines).

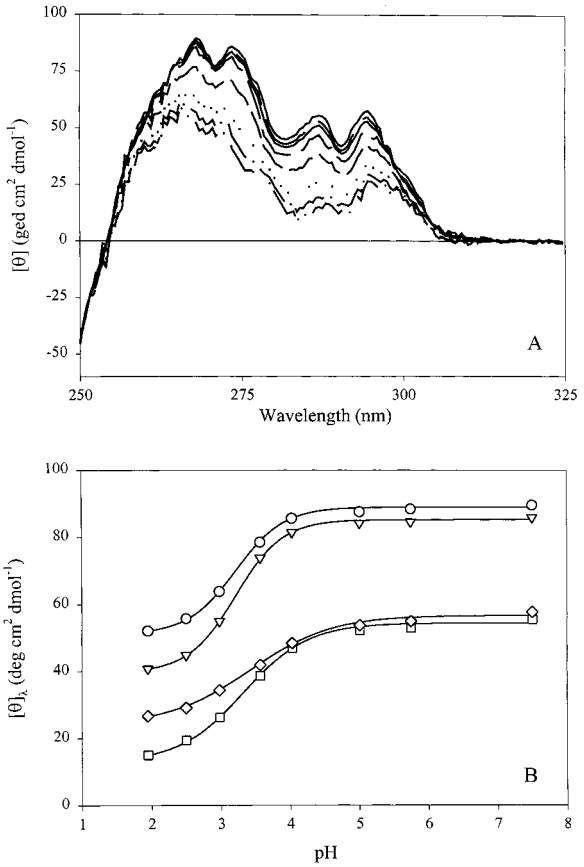

FIGURE 4: pH dependence of the near-UV CD spectrum of human recombinant interleukin-1β. (A) Spectra measured at pH 7.5, 5.75, 5.01, 4.03, 3.57, 2.98, 2.51, and 1.95 (in order of decreasing intensity). (B) Comparison of the effect of pH on different bands of the near-UV CD spectrum of human interleukin-1β: $[\theta]_{268}$ (○), $[\theta]_{273}$ (▽), $[\theta]_{287}$ (□), and $[\theta]_{294}$ (◇). All measurements were carried out at 25 °C. The protein concentration was 0.6 mg/mL, whereas the cell path length was 10 mm for these near-UV CD spectra measurements.

38.3 Å, should be considered as the smallest aggregated form of Caf1.

The ability of Caf1 protein to polymerize into oligimers with molecular masses of $>10^6$ kDa is one of the most important characteristics of the protein. The contribution of low-molecular mass (LMM) oligomers (from dimers to hexamers) to the total population of the aggregated Caf1 was unclear. This was especially true in the case of Caf1 functional studies, when solutions with low protein concentrations were used. Fractionation of ^{125}I-labeled Caf1 on a Superose 12 HR 10/30 column demonstrated that decreasing the protein concentration is accompanied by an increase in the population of LMM oligomers, reaching 22% at protein concentrations of 0.1 μg/mL (Table 3).

The gel filtration experiments with huIL-1β established that at neutral pH this protein migrates much faster than at moderately acidic pH (see Table 2). Analysis of the elution profiles shows that at pH 7.0 huIL-1β exists as a dimer;e decreasing the pH leads to the complete dissociation of the dimers, and between pH 4.0 and 2.0, huIL-1β is predominantly monomeric (see Table 2). This conclusion was confirmed by analytical ultracentrifugation (see Table 2) and SAXS experiments. In particular, decreasing the pH from 7.5 to 3.5 was accompanied by a 2-fold reduction in forward scattering amplitude [$I(0)$] and by a decrease in R_g from 39.7 to 27.3 Å.

Conformational Stability of Caf1 and huIL-1β

CD spectra of the Caf1 and huIL-1β were measured over a wide range of pH values. We have established that both near- and far-UV CD spectra of Caf1 remain unchanged over the course of the pH decrease from 7.5 to 2.0 (data not shown). This means that neither tertiary nor secondary structure of this protein is affected by the acidification of the solution.

Figure 4 shows that the tertiary structure of huIL-1β is invariant within the pH range of 8.0−4.0. A further decrease in pH is accompanied by the considerable reduction in near-UV CD spectrum intensity. However, even at pH 1.95 the aromatic CD spectrum of this protein still preserves infrastructure and considerable intensity (see Figure 4A). This means that the tertiary structure of huIL-1β is practically

Table 1: Secondary Structure Content of *Y. pestis* Capsular Protein Caf1 and Human Interleukin-1-β Determined by FTIR

	Y. pestis capsular protein Caf1		human interleukin-1-β	
structural assignment	wavenumber (cm^{-1})	%	wavenumber (cm^{-1})	%
turn	1688.7	3.7	1687.1	9.4
turn	1675.0	10.3	1674.3	8.0
α-helix/loop	1661.6	16.3	1663.2	18.2
disordered	1650.6	13.7	1651.5	14.5
β-sheet	1637.2	43.5	1638.8	40.8
β-sheet/side chains	1615.5	12.5	1620.9	9.1

Table 2: Hydrodynamic Properties and Apparent Molecular Masses Estimated for *Y. pestis* Capsular Protein Caf1 and Human Interleukin-1-β by Gel Filtration, Analytical Ultracentrifugation, and SAXS

protein and conditions	V_{el} (mL)	M_r^{SEC} (kDa)	R_s (Å)	M_r (kDa)	R_g (Å)
Caf1 at pH 7.5	14.21[a]	31.3[a]	25.4[a]	~7000	38.3
huIL-1β at pH 7.5	14.03	36.0	26.7	34.7	39.7
huIL-1β at pH 3.5	14.94	17.0	20.3	17.3	27.3
huIL-1β at pH 2.0	14.96	17.0	20.3	–	–

[a] Values estimated to be 0.001 mg/mL for the smallest aggregated form of Caf1.

Table 3: Evaluation of Caf1 Oligomer Contents Depending on Protein Concentration in Solution According to the Data from [^{125}I]Caf1 Gel Filtration on a Superose HR 10/30 Column

	oligomer content (%)			
[Caf1] (μg/mL)	Caf1 dimer	Caf1 tetramer	Caf1 hexamer	total oligomer contents with a mass of <100 kDa
10	0.5	2.2	3.7	16
5	0.6	2.3	3.7	16
1	0.77	2.5	4.1	18
0.1	>1.2	>2.7	>4.4	23

unaffected by the dimer dissociation (which is ended at pH 4.0). An extremely acidic environment (pH 2.0) induces some structural perturbation in the huIL-1β molecule, which still preserves a relatively rigid three-dimensional structure. Interestingly, Figure 4B shows that pH-induced changes occur simultaneously for different bands of the huIL-1β near-UV CD spectrum. This may indicate that the transition that is studied is a two-state one. As for the far-UV CD spectrum of huIL-1β, we have established that it remains practically unchanged for all the pH values that were measured (data not shown). All this allows us to conclude that huIL-1β preserves rigid native-like structure at acidic pH. Additional confirmations of this suggestion follow from the analysis of ANS fluorescence and studies of hydrodynamic properties of huIL-1β. Indeed, at pH 2.0 the spectrum of free ANS was still indistinguishable from that of the dye in the presence of huIL-1β. In other words, the decrease in pH values was not accompanied by the formation of ANS−protein complexes. Gel filtration experiments allowed us to conclude that the acidification of a solution was not accompanied by the unfolding of the huIL-1β molecule. Moreover, even at pH 2.0 this protein had the Stokes radius typical of the native globular protein with a molecular mass of ~17.0 kDa (see Table 2).

The data presented above are consistent with the suggestion that Caf1 and interleukin-1β belong to the type III proteins [in accordance with the classification of Fink et al. (*72*)], which show no significant structural changes at acidic pH.

Binding of huIL-1β and Caf1 to NIH 3T3 Mouse Fibroblasts

Fibroblasts play an important role in the regulation of immune response (*73*). Fibroblasts of the NIH 3T3 line are known to express a large number [$(5.1 \pm 0.5) \times 10^4$] of interleukin-1 receptors, IL-1Rs, on their surfaces (*41*). In other words, additional stimulation of receptor expression is not needed for these cells, and they represent a good model for the studies of receptor interaction.

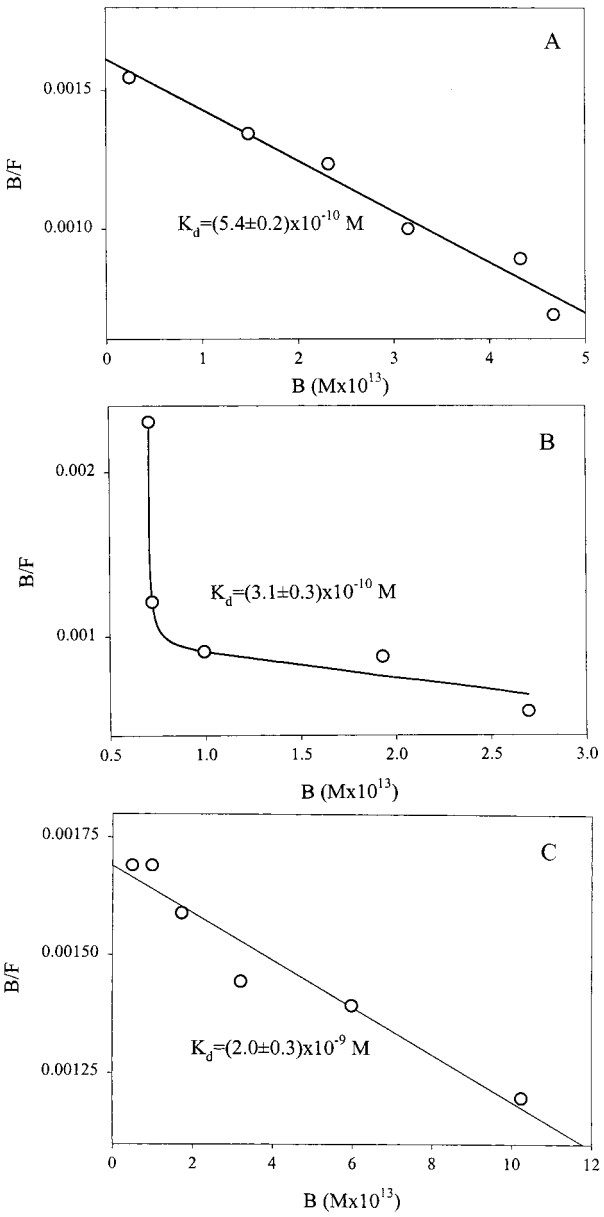

FIGURE 5: Scatchard analysis for the specific binding of [^{125}I]Caf1 (A) and [^{125}I]IL-1β with mouse fibroblast cell line NIH 3T3 (B). Caf1 effect on the specific binding of IL-1β with mouse fibroblast cell line NIH 3T3 (C).

Figure 5A shows that ^{125}I-labeled Caf1 binds specifically [$K_d = (5.41 \pm 0.1) \times 10^{-10}$ M] to mouse NIH 3T3 fibroblasts. Interaction of ^{125}I-labeled IL-1β with NIH 3T3 cells is characterized by negative cooperativeness with an apparent K_d of $(3.1 \pm 0.3) \times 10^{-10}$ M (Figure 5B). Addition of Caf1 to the incubation medium containing ^{125}I-labeled IL-1β drastically affects the interaction of IL-1β with the receptors. As a matter of fact, Figure 5C shows that the negative cooperativeness of the interaction disappears (Scatchard's plot represents a straight line). The essential drop in affinity also happened; there is a 15-fold decrease in the dissociation constant value [$K_d = (2.02 \pm 0.3) \times 10^{-9}$ M] induced by adding Caf1 to the reaction mixture.

DISCUSSION

The results presented in this paper show that there is an essential structural similarity between *Y. pestis* capsular protein Caf1 and huIL-1β. In fact, according to FTIR data, huIL-1β and Caf1 are typical β-structural proteins containing more than 40% β-sheet. Moreover, they have very close (almost identical) secondary structure compositions. The presence of long-wavelength peaks in the near-UV CD spectra ($\sim$300 nm) suggests the existence of specific (and probably structurally close) clusters of aromatic residues in both proteins. We have shown that the dimer with an approximate R_g of 38.3 Å should be considered as the smallest aggregated form of Caf1. On the other hand, we have established that at physiological pH huIL-1β exists as a dimer with an R_g of 39.7 Å. Both proteins possess similar conformational stability. Our data are consistent with the conclusion that Caf1 and interleukin-1β belong to the type III class of proteins [in accordance with the classification of Fink et al. (*72*)], which show no significant structural changes at acidic pH. This class includes proteins such as T4 lysozyme, chicken lysozyme, chymotrypsinogen, ubiquitin, concanavalin A, protein A, and β-lactoglobulin (*72*).

Finally, we have established that there is an essential functional similarity between *Y. pestis* Caf1 protein and huIL-1β. Particularly, specific binding of Caf1 to interleukin-1 receptors (IL-1R) on the surface of finite mouse fibroblasts was observed. Furthermore, Caf1 inhibits high-affinity binding of ^{125}I-labeled IL-1β to NIH 3T3 cells. Thus, our study represents an important stage in understanding the mechanisms of plague pathogenesis.

Analysis of binding of ^{125}I-labeled Caf1 or IL-1β to NIH 3T3 cells shows that IL-1Rs are involved in this interaction. Two types of IL-1Rs are known, IL-RI and IL-RII. Binding of IL-1α,β to the type I receptor (IL-RI) in the presence of so-called additional protein (IL-1R-AcP) initiates a cascade of reactions that results in a biological response of the target cell. The type II receptor (IL-1RII) is a functionally negative receptor or "trap" receptor, ensuring a tight binding of IL-1α,β, but it is unable to transmit the signal inside the cell (*75*, *76*). According to ref *77*, type I IL-1 receptors are expressed on fibroblasts and T-cells. At the same time, type II receptors are detected on B-cells and macrophages (*57*).

Cells synthesizing mainly IL-1RI have one class of binding sites for IL-1α (characterized by a K_d of $\sim 10^{-10}$ M) and two classes of binding sites for IL-1β. The first type of IL-1β binding sites has an affinity close to those of IL-1α sites. The second type, the dominating one, exhibits a lower IL-1β binding affinity ($K_d \sim 10^{-9}$ M) (*74*), and vice versa. Cells synthesizing IL-1RII had one class of binding sites for IL-1β ($K_d \sim 10^{-9}-10^{-10}$ M) and two classes of binding sites for IL-lα. One of these sites has high affinity ($K_d \sim 10^{-10}$ M), and the other shows lower IL-lα binding affinity ($K_d \sim 10^{-9}$ M) (*74*). These data taken together with our results on the binding of [^{125}I]Caf1 and [^{125}I]IL-1β to NIH 3T3 cells leave no doubt that these fibroblasts synthesized IL-1RI.

We have shown that Caf1 may be specifically bound to high-affinity sites on the surface of NIH 3T3 cells. On the other hand, [^{125}I]IL-1β loses its ability to interact with high-affinity binding sites of fibroblasts in the presence of Caf1 and binds only to the sites of average affinity. One of the possible explanations for this phenomenon could be the formation of a complex between IL-1β and low-molecular mass components of Caf1. Such a hypothesis did not contradict the recently published data on X-ray analysis of spatial structures of complexes of IL-1β (*78*) [and IL-1 receptor antagonist (IL-1RA) (*79*)] with the soluble extracellular part of IL-1RI. This issue will be the subject of our future studies.

In addition, the ability of Caf1 to interact with specific high-affinity IL-1 receptors on fibroblast surfaces suggests that this antigen may participate in very early stages of plague development. In fact, the ability of *Y. pestis* Caf1 protein to interact with IL-1 receptors indicates that the capsular subunits may regulate the contacts of bacteria with the host immunocompetent cells. As a result, Caf1 may be responsible for the initiation of the expression and secretion of *Y. pestis* Yop virulon proteins.

Interleukin-1 receptors are known either to be permanently present or to be primarily expressed on the surface of different types of immunocompetent cells as the result of their reaction with an antigenic stimulant. This may contribute to the increase in the level of *Y. pestis* adhesion to the host cells. Finally, the absence of interspecific differences between IL-1 receptors may probably explain the widening of the spectrum of hosts sensitive and receptive to *Y. pestis* species.

We suppose that dimeric or/and low-molecular mass oligomeric forms of Caf1, anchored with the usher protein CafA on the surface of *Y. pestis*, represent the most effective players in the realization of adhesion function of these bacteria. An interaction of free dimeric and/or LMM oligomeric forms of Caf1 with IL-1 receptors may represent the first stage of this reaction. Further contacts of LMM Caf1 with surface capsular molecules may trigger their copolymerization, providing contact between the bacteria and the host cell.

This model of the adhesive function of Caf1 subunits made it possible to explain the preventive properties of Caf1 specific antibodies. The specific antibodies, effectively bound to Caf1, may block or decrease the ability of *Y. pestis* bacteria to adhere to the host cell. As the result, *Y. pestis* will not apparently be able to realize the function of initiation of expression and secretion of Yop virulon proteins. The role of the Caf1 capsular antigen in the defense against infection by F1$^+$ strains of *Y. pestis* (*39*, *80*) and the exigency for Caf1 presence in the anti-plague vaccine are also becoming clear.

Interestingly, only one case of human plague infection by the F1$^-$ *Y. pestis* strain and a few cases of isolation of F1$^-$ strains from animals have been described (*80*). This testifies to the importance of Caf1 protein in the persistence of *Y. pestis*. However, information about the direct involvement of the Caf1 antigen in *Y. pestis* pathogenicity is rather ambiguous. Indeed, mice infected with F1$^-$ or F1$^+$ *Y. pestis* strains exhibited indistinguishable LD_{50} values, although the time of death of the animals with F1$^-$ strain challenge was prolonged twice as long on average. On the other hand, in experiments on guinea pigs, the F1$^-$ strain exhibited a 200-fold increase in LD_{50} as compared to F1$^+$ strain, though the available data also show that F1$^-$ strains preserve a level of virulence that is comparable with those of wild-type strains (*80*). It is possible that the adhesion function in F1$^-$ strains of *Y. pestis* is performed by V-antigen, or by a still unknown

protein of the outer membrane, which is confirmed by the fact that V-antigen specific antibodies possess preventive properties (*81*). Obviously, these antibodies may neutralize specific sites of extracellular V-antigen or V-antigen anchored on the outer membrane of bacteria (*82*). This may have functional importance for the attachment of *Y. pestis* to host cell and for the initiation of formation of a translocated channel of the Yop virulon system. As for the $F1^+$ strains, the functional interaction between Caf1 and V-antigen in this case may be postulated.

ACKNOWLEDGMENT

We thank Dr. I. S. Millett for his help with SAXS measurements. We are grateful to Prof. G. W. Anderson (Southern Research Institute, Birmingham, AL) for his valuable advice during the discussion of the results presented in this article.

REFERENCES

1. Cavanaugh, D. C., and Randall, R. (1959) *J. Immunol. 83*, 348−371.
2. Burrows, T. W. (1963) *Mikrobiol. Immun. Exp. Ther. 37*, 59−113.
3. Ferber, D. M., and Brubaker, R. R. (1981) *Infect. Immun. 31*, 831−841.
4. Portnoy, D. A., Moseley, S. L., and Falkow, S. (1981) *Infect. Immun. 31*, 775−782.
5. Portnoy, D. A., and Falkow, S. (1981) *J. Bacteriol. 148*, 877−883.
6. Portnoy, D. A., Wolf-Watz, H., Bolin, I., Beeder, A. B., and Falkow, S. (1984) *Infect. Immun. 43*, 108−114.
7. Bolin, I., Portnoy, D. A., and Wolf-Watz, H. (1985) *Immunology 48*, 234−240.
8. Cornelis, G. R., and Wolf-Watz, H. (1997) *Mol. Microbiol. 23*, 861−867.
9. Cornelis, G. (1998) *J. Bacteriol. 180*, 5495−5504.
10. Rosqvist, R., Magnusson, K.-E., and Wolf-Watz, H. (1994) *EMBO J. 13*, 964−972.
11. Hultgren, S. J., Abraham, S., Caparon, M., Falk, P., St. Geme, J. W., and Normark, S. (1993) *Cell 73*, 887−901.
12. Soto, G. E., and Hultgren, S. J. (1999) *J. Bacteriol. 181*, 1059−1071.
13. Kitazato, S. (1894) *Lancet 2*, 428−430.
14. Burrows, T. W., and Bacon, G. A. (1956) *Br. J. Exp. Pathol. 37*, 481−493.
15. Janssen, W. A., Lawton, W. D., Fukui, G. M., and Surgalla, M. J. (1963) *J. Infect. Dis. 113*, 139−143.
16. Williams, R. C., Gewurz, H., and Quie, P. G. (1972) *J. Infect. Dis. 126*, 235−241.
17. Hallett, A. F., Isaäcson, M., and Meyer, K. F. (1973) *Infect. Immun. 8*, 876−881.
18. Mayer, K. F., Hightower, J. A., and McCrumb, F. R. (1973) *J. Infect. Dis. Suppl. 129*, 41−45.
19. Englesberg, E. V., and Lewy, J. (1954) *J. Bacteriol. 67*, 438−449.
20. Galyov, E. E., Smirnov, O. Yu., Karlyshev, A. V., Volkovoy, K. I., Denesyuk, A. I., Nazimov, I. V., Rubtsov, K. S., Abramov, V. M., Dalvadyanz, S. M., and Zav'yalov, V. P. (1990) *FEBS Lett. 277*, 230−232.
21. Galyov, E. E., Karlyshev, A. V., Smirnov, O. Yu., Chernovskata, T. V., Dolgikh, D. A., Smirnov, O. Yu., Volkovoy, K. I., Abramov, V. M., and Zav'yalov, V. P. (1991) *FEBS Lett. 286*, 79−82.
22. Karlyshev, A. V., Galyov, E. E., Abramov, V. M., and Zav'yalov, V. P. (1992) *FEBS Lett. 305*, 37−40.
23. Karlyshev, A. V., Galyov, E. E., Smirnov, O. Yu., Guzaev, A. P., Abramov, V. M., and Zav'yalov, V. P. (1992) *FEBS Lett. 297*, 77−80.
24. Holmgren, A., Kuehn, M. J., Brändén, C. I., and Hultgren, S. J. (1992) *EMBO J. 11*, 1617−1622.
25. Zav'yalov, V. P., Zav'yalova, G. A., Denesyuk, A. I., and Korpela, T. (1995) *FEMS Immunol. Med. Microbiol. 11*, 19−24.
26. Schmidt, J. A., Mizel, S. B., Cohen, D., and Green, I. (1982) *J. Immunol. 128*, 2177−2182.
27. Krane, S. M., Dayer, J.-M., Simon, L. S., and Byrne, S. (1985) *Collagen Relat. Res. 5*, 99−117.
28. Dukovich, M., Severin, J. M., White, S. J., Yamazaki, S., and Mizel, S. B. (1986*) Clin. Immunol. Immunopathol. 38*, 381−389.
29. Wood, D. D., Ihrie, E. J., Dinarello, C. A., and Cohen, P. L. (1983) *Arthritis Rheum. 26*, 975−983.
30. Eastgate, J. A., Symons, J. A., Wood, N. C., Grinlinton, F. M., DiGiovine, F. S., and Duff, G. W. (1988) *Lancet 8613*, 706−709.
31. March, C. J., Mosley, B., Larsen, A., Cerretti, D B., Braedt, G., Price, V., Gillis, S., Henney, C. S., Kronheim, S. R., Grabstein, K., Conlon, P. J., Hopp, T. P., and Cosman, D. (1985) *Nature 315*, 641−647.
32. Wingfield, P., Payton, M., Tavernier, J., Barners, M., Shaw, A., Rose, K., Simona, M. G., Demczuk, S., Willamson, K. W., and Dayer, J. M. (1986) *Eur. J. Biochem. 160*, 491−497.
33. Zav'yalov, V. P., Denesyuk, A. I., Zav'yalova, G. A., and Korpela, T. (1995) *Immunol. Lett. 45*, 19−22.
34. Baker, E. E., Sommer, H., Foster, L. E., Meyer, E., and Meyer, K. F. (1952) *J. Immunol. 68*, 131−145.
35. Meyer, K. F., Hightower, J. A., and McCrumb, F. R. (1974) *J. Infect. Dis. 129*, S41−S45.
36. Simpson, W. J., Thomas, R. E., and Schawan, T. G. (1990) *Am. J. Trop. Med. Hyg. 43*, 389−396.
37. Oyston, P. C. F., Williamson, E. D., Leary, S. E. C., Eley, S. M., Griffin, K. F., and Titball, R. W. (1995) *Infect. Immun. 63*, 563−568.
38. Andrews, G. P., Heath, D. G., Anderson, G. W., Jr., Welkos, S. L., and Friedlander, A. M. (1996) *Infect. Immun. 64*, 2180−2187.
39. Anderson, G. W., Worsham, P. L., Bolt, C. R., Andrews, G. P., Welkos, S. L., Friedlander, A. M., and Burans, J. P. (1997) *Am. J. Trop. Med. Hyg. 56*, 471−473.
40. Zav'yalov, V. P., Chernovskaya, T. V., Navolotskaya, E. V., Karlyshev, A. V., MacIntyre, Sh., Vasiliev, A. M., and Abramov, V. M. (1995) *FEBS Lett. 371*, 65−68.
41. Dower, S. K., and Urdal, D. L. (1987) *Immunol. Today 8*, 46−50.
42. Mashko, S. V., Mochulsky, A. V., Kotenko, S. V., Lebedeva, M. I., Lapidus, A. I., Moshulskya, N. A., Isotova, L. S., Veiko, V. P., Vinetsky, Yu. P., Ketlinsky, S. A., and Debabov, V. G. (1991) *Gene 97*, 259−266.
43. Ketlinsky, S., Simbirtsev, A., Poltorak, A., Protasov, E., Solovjeva, L., Putchkova, G., Konusova, V., Pigareva, N., Kalinina, N., and Perumov, N. (1991) *Eur. Cytokine Network 2*, 17−26.
44. Laemmli, U. K. (1970) *Nature 227*, 680−685.
45. Oberg, K., and Fink, A. L. (1998) *Anal. Biochem. 256*, 92−106.
46. Oberg, K., Chrunyk, B. A., Wetzel, R., and Fink, A. L. (1994) *Biochemistry 33*, 2628−2634.
47. Van Holde, K. E., and Baldwin, R. L. (1958) *J. Phys. Chem. 62*, 734−743.
48. Yphantis, D. A. (1964) *Biochemistry 3*, 297−317.
49. Ackers, G. L. (1967) *J. Biol. Chem. 242*, 3237−3238.
50. Ackers, G. L. (1970) *Adv. Protein Chem. 24*, 343−446.
51. Uversky, V. N. (1993) *Biochemistry 32*, 13288−13298.
52. Uversky, V. N. (1994) *Int. J. Bio-Chromatogr. 1*, 103−114.
53. Corbett, R. J. T., and Roche, R. S. (1984) *Biochemistry 23*, 1888−1894.
54. Wakatsuki, S., Hodgson, K. O., Eliezer, D., Rice, M., Hubbard, S., Gillis, N., and Doniach, S. (1992) *Rev. Sci. Instrum. 63*, 1736−1740.
55. Tsuruta, H., Brennan, S., Rek, Z. U., Irving, T. C., Tompkins, W. H., and Hodgson, K. O. (1998) *J. Appl. Crystallogr. 31*, 672−682.
56. Glatter, O., and Kratky, O. (1982) *Small-angle X-ray scattering*, Academic Press, New York.

57. Dower, S. K., Qwarnstrom, E. E., Page, R. C., Blanton, R. A., Kupper, N. S., Raines, E., Ross, R., and Sims, J. E. (1990)-*Invest. Dermatol. 94*, 685.
58. Craig, S., Pain, R. H., Schmeissner, U., Virden, R., and Wingfield, P. T. (1989) *Int. J. Pept. Protein Res. 33*, 256−262.
59. Miller, J., Williamson, E. D., Lakey, J. H., Pearce, M. J., Jones, S. M., and Titball, R. W. (1998) *FEMS Immunol. Med. Microbiol. 21*, 213−221.
60. Bolotina, I. A., and Lugauskas, V. Yu. (1985) *Mol. Biol. (Moscow) 19*, 1409−1421.
61. Bolotina, I. A. (1987) *Mol. Biol. (Moscow) 21*, 1625−1635.
62. Manning, M. C., and Woody, R. W. (1989) *Biochemistry 28*, 8609−8613.
63. Perczel, A., Hollosi, M., Tusnady, G., and Fasman, G. D. (1991) *Protein Eng. 4*, 669−679.
64. Chaffotte, A. F., Guillou, Y., and Goldberg, M. E. (1992) *Biochemistry 31*, 9694−9602.
65. Chakrabartty, A., Kortemme, T., Padmanabhan, S., and Baldwin, R. L. (1993) *Biochemistry 32*, 5560−5565.
66. Uversky, V. N., and Ptitsyn, O. B. (1996) *J. Mol. Biol. 255*, 215−228.
67. Jagannadham, M. V., and Balasubramanian, D. (1985) *FEBS Lett. 188*, 326−330.
68. Rodionova, N. A., Semisotnov, G. V., Kutyshenko, V. P., Uversky, V. N., Bolotina, I. A., Bychkova, V. E., and Ptitsyn, O. B. (1989) *Mol. Biol. (Moscow) 23*, 683−692.
69. Bychkova, V. E., Berni, R., Rosi, J.-L., Kutyshenko, V. P., and Ptitsyn, O. B. (1992) *Biochemistry 31*, 7566−7571.
70. Stryer, L. (1965) *J. Mol. Biol. 13*, 482−485.
71. Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A. F., and Gilmanshin, R. I. (1991) *Biopolymers 31*, 119−128.
72. Fink, A. L., Calciano, L. J., Goto, Y., Kurotsu, T., and Palleros, D. R. (1994) *Biochemistry 33*, 12504−12511.
73. Phipps, R. P., Penney, D. P., Keng, P., Silvera, M., Harkins, S., and Derbak, S. (1990) *Immunol. Res. 9*, 275−286.
74. MacMahon, C. J., Slack, J. L., Mosley, B., Cosman, D., Lupton, S. D., Brunton, L. L., Grubin, C. E., Wignall, J. M., Jenkins, N. A., Brannan, C. I., Copeland, N. G., Huebner, K., Croce, C. M., Cannizzaro, L. A., Bejamin, D., Dower, S., Spriggs, M. K., and Sims, J. E. (1991) *EMBO J. 10*, 2821−2860.
75. Dinarello, C. A. (1996) *Blood 87*, 2095−2147.
76. Greenfeder, S. A., Nunes, P., Kwee, L., Labow, M., Chizzonite, R. A., and Ju, G. (1995) *J. Biol. Chem. 270*, 13757−l3765.
77. Dower, S. K., Call, S. M., Gillis, S., and Urdal, D. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1060−1064.
78. Vigers, G. P. A., Anderson, L. J., and Brandhuber, B. J. (1997) *Nature 386*, 190−194.
79. Schreuder, H., Tardif, C., Trump-Kallmeyer, S., Soffentini, A., Sarubbi, E., Akeson, A., Bowlin, T., Yanofsky, S., and Barrett, R. W. (1997) *Nature 386*, 194−200.
80. Friedlander, A. M., Welkos, S. L., Worsham, P. L., Andrews, G. P., Heath, D. G., Anderson, G. W., Pitt, M. L. M., Estep, J., and Davis, K. (1995) *Clin. Infect. Dis. 21* (Suppl. 2), S178−S181.
81. Motin, V. L., Nakajima, R., Smirnov, G. B., and Brubaker, R. R. (1994) *Infect. Immun. 62*, 4192−4201
82. Fiedls, A. K., and Starley, S. C. (1998) 7th International Congress on *Yersinia*, Nijmegen, The Netherlands, p 6.

BI002678X