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Preparation, characterization and application of interleukin-1β mutant proteins with surface-accessible cysteine residues

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Two mutants of interleukin-1β (K27C and K138C) were produced using site-specific mutagenesis in which lysine residues at positions 27 and 138 of the mature protein sequence were substituted by cysteine residues. The conformations of the mutant proteins were studied by ^{1}H-NMR spectroscopy and shown to be similar to the wild-type protein. The receptor-binding affinity and biological activity of K27C and K138C were also similar to wild-type protein. The substituted cysteines in both mutant proteins were shown to be solvent-accessible as judged by their reactivity towards sulfhydryl reagents. As the wild-type protein contains two cysteines, which are both solvent-inaccessible in the native state, the mutants offer the opportunity to introduce probes in a sequence-specific manner via reaction with sulfhydryl groups. Examples of this are described in which the K138C was disulfide-linked to phycobiliproteins. The highly fluorescent conjugates had similar receptor-binding affinities to that of the wild-type unconjugated protein and were found suitable for flow-cytometric analysis.

Interleukin-1 (IL-1) refers to at least two monocyte-derived proteins (IL-1α and β) which display a wide range of biological activities, including fever induction and augmentation of lymphocyte proliferation (reviews [1, 2]). IL-1α and β are both produced as 31-kDa precursors which are processed to give distinct bioactive 17-kDa proteins [3]. Although IL-1α and β have a relatively low sequence similarity (≈ 23%) they bind to the same cell receptor [4, 5] and appear to effect a similar range of biological activities [1, 2].

The availability of pure and well characterized recombinant-produced IL-1α and β [6–8] has enabled structural studies to be carried out using ^{1}H-NMR spectroscopy [9, 10] and X-ray crystallography [11]. The latter work has provided a 0.3-nm model of IL-1β and, in the near future, a refined structure will probably be described. Even with the structural information available, it is not possible to identify residues or regions of the IL-1 molecule important for receptor binding. Most of the information in this area has come so far from site-specific mutagenesis of IL-1β. Large decreases in the receptor-binding affinity of IL-1β are observed upon substitution of the single histidine residue located at position 30 of the mature sequence [12]. The altered binding affinities were shown by ^{1}H-NMR spectroscopy to be due to small local perturbations rather than gross conformational changes [12]. Other changes in receptor-binding affinity have been noted for various amino acid substitutions, extensions and deletions to the N-terminal region of IL-1β [13–15].

In this study we describe two new mutant proteins of IL-1β. In these mutant proteins we have substituted lysine residues for cysteine residues. These mutant proteins allow the introduction of both low- and high-molecular-mass probes to specific regions of the IL-1β molecule. This approach has given additional information on the regions of IL-1β important for receptor binding. A practical application for one of the mutant proteins is also described in which the protein is coupled to phycoerythrin forming a highly fluorescent conjugate useful for quantitative flow-microfluorometric analysis of cells expressing IL-1 receptors.

MATERIALS AND METHODS

Preparation of IL-1β wild-type and mutant proteins

Site-specific mutagenesis was used to introduce point mutations in the IL-1β molecule as described previously [9, 12]. The mutant proteins, K27C and K138C, were constructed such that the lysines at positions 27 and 138, respectively, were replaced by cysteine residues. Fermentation and protein-purification procedures were essentially as described previously for the recombinant-derived wild-type protein [6]. The method used for purification of the mutant proteins differed from that used for the wild-type protein as follows. The buffer used for dialysis prior to and during the CM-Sepharose chromatography stage was 50 mM sodium acetate, pH 4.0 containing 1 mM dithiothreitol (a pH 5.7 buffer was used for the wild-type protein); the buffer used for the Ultrogel AcA54 gel-filtration stage was 100 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol and 1 mM EDTA. Immediately before using a protein in the labelling and conjugation reactions

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Abbreviations. IL-1, interleukin-1; K27C and K138C, interleukin-1β mutant proteins with cysteine substituted for lysine at positions 27 and 138, respectively; IL-1/LAF assay, lymphocyte-activating-factor assay; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); HOHAHA spectroscopy, homonuclear two-dimensional Hartmann-Hahn spectroscopy.

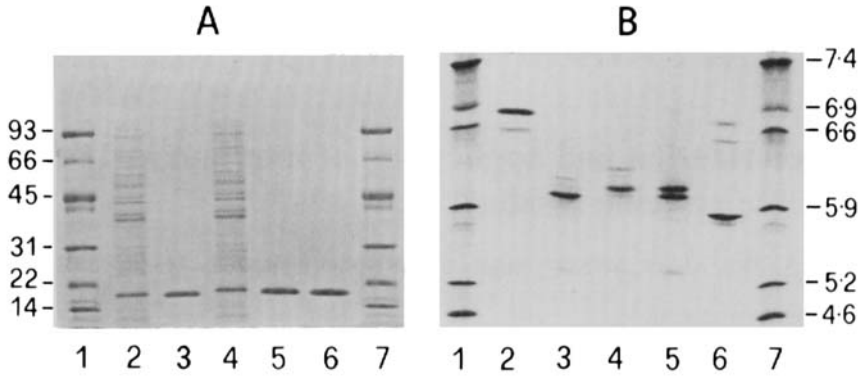

Fig. 1. *SDS/PAGE and IEF of interleukin-1β mutant proteins.* (A) Results of SDS/PAGE using a 10–15% (mass/vol.) gradient polyacrylamide gel stained with Coomassie blue. Lanes 1 and 7, standard proteins: from top to bottom phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme. Molecular mass (kDa) given in the left-hand margin. Lane 2, cell extract containing mutant protein IL-1β K27C. Lane 3, purified mutant protein IL-1β K27C. Lane 4, cell extract containing K138C. Lane 5, purified K138C. Lane 6, purified wild-type human IL-1β. (B) Results of IEF on the polyacrylamide gel. Lanes 1 and 7, standard proteins: from top to bottom myoglobin basic band, myoglobin acidic band, human carbonic anhydrase B, bovine carbonic anhydrase, β-lactoglobulin A and soybean trypsin inhibitor. pI values given in right-hand margin. Lane 2, human wild-type IL-1β. Lane 3, pI 5.9 species of mutant protein IL-1β K138C isolated by chromatofocusing. Lane 4, pI 6.0 species of K138C isolated by chromatofocusing. Lane 5, K138C after Ultrogel AcA54 chromatography. Lane 6, mutant protein IL-1β K27C after Ultrogel AcA54 chromatography

described below, dithiothreitol and EDTA were removed by gel filtration using a 5 cm × 1.5 cm PD-10 Sephadex G-25M column [Pharmacia-pdf-(Schweiz) AG] equilibrated in 100 mM Tris/HCl pH 7.9.

Protein concentration determinations

Protein concentrations of wild-type and mutant proteins were determined from ultraviolet absorption spectra. A value of $A^{1\%}_{1\,cm} = 6.30$ at 280 nm was used [6].

Chromatofocusing of K138C

Protein, 1 mg in 1 ml 25 mM Tris/HCl, pH 7.5 was applied to an FPLC Mono P HR5/20 column (Pharmacia) equilibrated with 25 mM Bistris/acetic acid, pH 6.7. The column was eluted with 40 ml of a Polybuffer 96/74 mixture (1:15) diluted 1:10 with water and adjusted to pH 5.5 with acetic acid (Polybuffers from Pharmacia). Polybuffers were removed by hydrophobic chromatography using a Phenyl-Superose HR10/10 column (Pharmacia), using the sample application and elution procedure recommended by the manufacturer.

Alkylation of the IL-1β mutant proteins

Proteins (1–2 mg/ml) in 100 mM Tris/HCl, pH 7.9, were incubated with either 20 mM iodoacetamide or with 10 mM 5-iodoacetamidofluorescein (Molecular Probes Inc., Eugene, Oregon, USA) for 15 min at 20 °C in the dark. Excess reagent was removed by gel filtration using Sephadex G-25.

Conjugation of K138C with phycobiliprotein

A mixture of K138C (3.5 mg/ml) and B- or R-phycoerythrin pyridylsulfide derivatives (1.0 mg/ml) were incubated in 100 mM Tris/HCl, pH 7.9 containing 5 μM $CuSO_4$ for 18 h at 20 °C (the phycobiliprotein derivatives were from Molecular Probes Inc.). Conjugated K138C was separated from non-conjugated protein by gel filtration on a FPLC Superose 12 HR 10/30 column. The column buffer was 100 mM Tris/HCl, pH 7.9, containing 1 mM sodium azide. The conjugate was stored at 0–4 °C.

Analytical separation methods

Native PAGE and SDS/PAGE were carried out on PhastGel 10–15% (mass/vol.) polyacrylamide gradient gels (Pharmacia). Electrophoresis and staining with Coomassie blue were performed with the Pharmacia PhastSystem. Isoelectric focusing on thin-layer polyacrylamide gels (LKB Ampholine PAG plates pH 3.5–9.5) and Coomassie-blue staining were according to the manufacturer's instructions. pI values were determined using an isoelectric focusing calibration kit (Pharmacia).

Chemical analysis

Amino acid analysis and N-terminal sequence analysis were performed as previously described [6]. Sulfhydryl titrations with 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) and 4,4′-dithiopyridine were performed in 0.1 M Tris/HCl, pH 7.9, containing 2 mM EDTA. The assay volume was 1.5 ml and contained 40–50 μM test protein and 0.33 mM of the respective reagent. In some assays the proteins were unfolded by the addition of 1% (mass/vol.) SDS. Titrations with Nbs_2 and 4,4′-dithiopyridine were monitored at 412 nm ($\varepsilon = 1.36$ mM cm^{-1}) [16] and 324 nm ($\varepsilon = 1.98$ mM cm^{-1}) [17], respectively.

Tryptic digestion and peptide mapping of labelled IL-1β mutant proteins

Tryptic digestion of modified proteins (0.5–1.0 mg/ml) was carried out in 50 mM NH_4HCO_3, pH 8.0. One addition of 1% (by mass) trypsin was followed 3 h later by a similar addition. The total digestion period was 6 h at 37 °C. The digested sample was acidified by the addition of 0.1% (mass/vol.) CF_3COOH (solvent A) and the peptides were separated by HPLC using an Aquapore RP-300 (C-8) 150 mm × 4.6 mm column. The peptides were eluted at room temperature with a

Table 1. *Amino acid analyses of IL-1β mutant proteins*
The compositions were determined from 24-h acid hydrolysates and are, in each case, the average of three determinations. The cysteine contents were estimated as *S*-carboxymethylcysteine (CmCys) derived from protein alkylated with iodoacetamide. WT refers to wild-type IL-1β. The values in parentheses are those predicted from the DNA sequences; nd, not determined

Residue	Composition		
	WT	K27C	K138C
	mol/mol		
CmCys	2.2 (2)	3.1 (3)	3.3 (3)
Asx	16.2 (17)	16.6 (17)	16.3 (17)
Thr	5.7 (6)	5.4 (6)	5.7 (6)
Ser	11.1 (14)	11.3 (14)	12.1 (14)
Glx	22.8 (23)	22.1 (23)	23.3 (23)
Pro	8.2 (8)	8.2 (8)	7.4 (8)
Gly	8.2 (8)	8.1 (8)	8.1 (8)
Ala	5.2 (5)	5.4 (5)	5.2 (5)
Val	11.2 (11)	10.8 (11)	10.7 (11)
Met	5.5 (6)	4.9 (6)	4.6 (6)
Ile	5.1 (5)	4.8 (5)	5.1 (5)
Leu	15.5 (15)	15.1 (15)	15.1 (15)
Tyr	3.9 (4)	3.9 (4)	4.2 (4)
Phe	9.3 (9)	8.5 (9)	8.9 (9)
His	1.4 (1)	1.0 (1)	1.0 (1)
Lys	14.9 (15)	13.8 (14)	13.7 (14)
Arg	3.1 (3)	3.1 (3)	3.0 (3)
Trp	0.93 (1)	nd (1)	nd (1)

mixture of solvents A and B (0.1% CF_3COOH in acetonitrile) using a linear 0–80% gradient of B over 80 min at a flow rate of 1 ml/min. A_{214} was recorded.

^{1}H-NMR spectroscopy

Samples for ^{1}H-NMR spectroscopy contained 1–2 mM protein in 99.996% D_2O (Cambridge Isotope Laboratories, Woburn, MA, USA) containing 100 mM sodium phosphate, pH 7.1, 2.5 mM EDTA and 0.5 mM dithiothreitol. All NMR experiments were recorded at 27 °C on a Bruker AM600 spectrometer. Homonuclear Hartman-Hahn experiments [18] were carried out using a WALTZ17$_y$ mixing sequence [19]. All spectra were recorded with sweep widths of 8042 Hz. The digital resolution was 3.93 Hz/point in the t_2 dimension and 7.86 Hz/point in the t_1 dimension. This was achieved by zero-filling once in both the t_1 and t_2 dimensions. Eight scans were collected for each of 1024 t_1 increments with a relaxation delay of 2 s between successive transients.

Flow microfluorometry

Quantitation of binding of K138C conjugated to R- or B-phycoerythrin to EL4-6.1 thymoma cells was carried out by flow microfluorometry. EL4-6.1 cells (5×10^5/100 µl) were incubated for 3 h at 4 °C with various concentrations of phycoerythrin-conjugated K138C in the presence or absence of a 100-fold excess of unlabelled wild-type IL-1β. Cells were then washed and analyzed on a FACS II flow cytometer equipped with a 5 W Argon laser (Becton-Dickinson FACS Systems, Sunnyvale, CA). R-phycoerythrin was excited at 488 nm whereas B-phycoerythrin was excited at 515 nm. Emission in both cases was measured using a 575 ± 6-nm band pass filter.

Fluorescence histograms (corresponding to 10^4 viable cells gated according to forward light scatter) were accumulated using linear amplification.

IL-1-receptor-binding assay

Competition-binding analysis using EL4-6.1 cells, radiolabelled IL-1α and various concentrations of unlabelled test proteins was carried out as described previously [4, 12].

Bioassay

The lymphocyte-activating-factor (IL-1/LAF) assay, which measures the stimulation of murine thymocyte proliferation in the presence of phytohemagglutinin, was performed as described by Mizel [20]. One unit of IL-1 activity is arbitrarily defined as the concentration of protein which induces 50% of the maximal response under the assay conditions.

RESULTS

Rationale for choice of mutants

As the two cysteine residues of wild-type IL-1β are essentially solvent-inaccessible [6] the introduction of a solvent-accessible cysteine residue which has no effect on the overall conformation of the protein, would allow specific labelling using sulfhydryl reagents. When this study was initiated we had no information on the surface exposure of individual residues. We therefore chose to substitute lysine residues as they are usually located on the surface of proteins [21].

There is a high degree of sequence similarity among the human, rat, rabbit and mouse IL-1β proteins. Furthermore these proteins all bind with similar affinity to the EL4-6.1 cell line (unpublished data). The lysyl residues are strongly conserved among IL-1β from the above different species except for Lys-138 which is only found in the human protein. This residue was therefore unlikely to be directly involved in receptor binding and was chosen for substitution.

The recent crystal structure of human IL-1β indicates that Lys-138 is in a so-called 'loop region' [22] connecting two antiparallel β-strands and which is located at the protein surface [11].

For the second substitution Lys-27 was chosen. In a previous study we had shown that substitution of His-30 for various other residues resulted in decreases in receptor binding [12]. The introduction of a potentially reactive cysteine residue to a region of the protein possibly involved in receptor binding was thought to be of interest.

Production and characterization of mutant proteins

Both IL-1β mutant proteins, K27C and K138C, were produced in *Escherichia coli* to the same level as the wild-type protein, namely about 8% of the total cell protein (Fig. 1 A). Also, similar to the wild-type protein, both proteins accumulated in the soluble phase of the bacterial cytoplasm. Purification of the mutant proteins was achieved using the same method that we previously described for the wild-type protein with the modification that a lower pH buffer was required for the cation-exchange chromatography stage (Materials and Methods). This was necessary, as both mutants, as expected, had isoelectric points lower than that of the wild-type protein

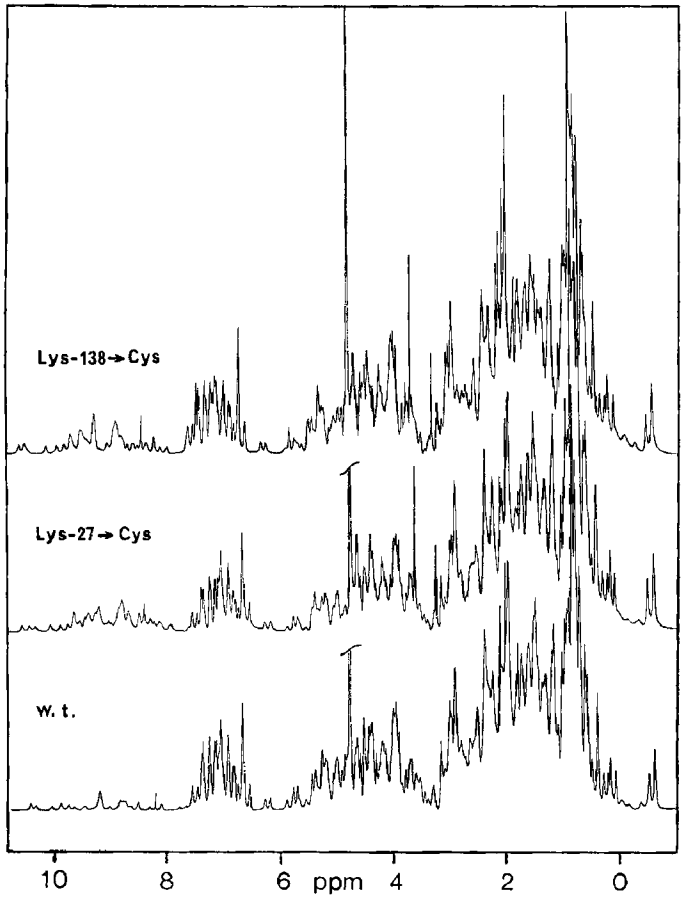

Fig. 2. *600 MHz ^{1}H-NMR spectra of wild-type IL-1β and IL-1β mutant proteins.* Spectra were recorded at 27°C in sodium phosphate, pH 7.5. Lys-138→Cys, K138C; Lys-27→Cys, K27C; w.t., wild-type

(see below). In addition, a reductant was included in most buffers to prevent potential intermolecular disulfide-bond formation. For each mutant, about 5 mg purified protein was recovered from 1 g wet cells.

SDS/PAGE of the mutant proteins (Fig. 1 A) indicates that they were pure and, whereas K27C migrated with the same M_r ($\approx$ 17000) as the wild-type protein, K138C migrated with a slightly higher M_r (17700). Analysis of samples not pretreated with the reductant dithiothreitol also gave the same results as shown in Fig. 1 A indicating the absence of intermolecular disulphide bonds in the mutant proteins. Under native conditions both proteins were monomeric, behaving identically to the wild-type protein during size-exclusion chromatography on Ultrogel AcA54.

Isoelectric focusing of the mutant proteins (Fig. 1 B) indicated that K27C was a single-charge species with an estimated pI of 5.7. K138C on the other hand exhibited charge heterogeneity, two main species of pI 6.0 and 5.9 being observed. The pI values of all IL-1β species were lower than that of the wild-type protein (pI 6.8); this was expected because in each mutant protein a basic residue (lysine) has been substituted by a weakly acidic residue (cysteine).

Amino acid analysis of IL-1β mutant proteins carboxymethylated with iodoacetamide indicated an amino acid composition similar to the wild-type protein except for the predicted one residue more of *S*-carboxymethylcysteine (derived from cysteine) and one residue less of lysine (Table 1). N-terminal amino acid sequence analysis of K27C indicated the sequence: Ala-Pro-Val-Arg-Ser-Leu. This sequence was as expected from the DNA sequence of the wild-type protein [3] with no N-terminal methionine being present. The two species of K138C were separated by chromatofocusing (Materials and Methods) and each sequenced. The pI = 6.0 species had the same N-terminal sequence as K27C (see above); however, the pI = 5.9 species had the sequence Met-Ala-Pro-Val-Arg-Leu. The difference in pI between the two K138C species thus appears to be due to the presence of unprocessed N-terminal Met. Charge heterogeneity in the wild-type protein for the same reason has been previously reported [13, 23, 24]. Most of the studies described below with K138C were carried out on protein not fractionated by chromatofocusing.

Sulfhydryl modification

Sulfhydryl modification of K138C, under native conditions at pH 7.9 using Nbs_2 (reagent: protein = 10 mol/mol), one sulfhydryl residue/mol protein was titrated within 1 min. There was no sulfhydryl modification under the same conditions with the wild-type protein. Similarly, labelling of the mutant with iodoacetamide at pH 7.9 (reagent: protein = 100 mol/mol) for 10 min at room temperature followed by amino acid analysis of acid hydrolysates indicated about 1 mol *S*-carboxymethylcysteine/mol protein. To confirm that only Cys-138 was being modified, protein was labelled under native conditions with [1-^{14}C]iodoacetamide, digested with trypsin and the resultant peptides separated by HPLC (see Materials and Methods). No radioactivity was associated with the peptides containing only Cys-8 and Cys-71 which are normally present in the wild-type protein. These findings indicate that Cys-138 is solvent-accessible and can be specifically labelled under conditions where Cys-8 and Cys-71 are not modified.

In contrast to K138C, K27C reacted slowly with Nbs_2; after 60 min, under the same conditions as those indicated above, about 0.20 mol sulfhydryl group/mol protein was titrated. On the other hand when the neutral sulfhydryl reagent 4,4′-dithiopyridine was used, 0.80 mol sulfhydryl group/mol protein was titrated within 10 min. Treatment with iodoacetamide for 60 min also resulted in modification of about 0.80 mol sulfhydryl group/mol protein. Tryptic mapping of [1-^{14}C]iodoacetamide-labelled protein indicated that 80% of the radioactivity was associated with a peptide containing Cys-27 and 20% with a peptide containing Cys-8. The cysteine at position 27 can thus be labelled with neutral sulfhydryl reagents, but only slowly with anionic sulfhydryl reagents such as Nbs_2. This is probably due to electrostatic repulsion between the glutamate carboxylate which is positioned near the cysteine side chain on the same side of the β-sheet. Furthermore, the specificity of labelling is not as high as with K138C.

Conformation and activity

As ^{1}H-NMR spectroscopy is extremely sensitive for protein tertiary structure [25], ^{1}H-NMR spectroscopy was performed in order to examine possible conformation differences between the wild-type and mutant proteins. Comparison of the one-dimensional ^{1}H-NMR spectra of the wild-type and the two mutant proteins (Fig. 2) indicates that no major structural rearrangements were caused by the amino acid substitutions. Minor changes, however, certainly did occur, as can be appreciated from small shifts of cross peaks in the

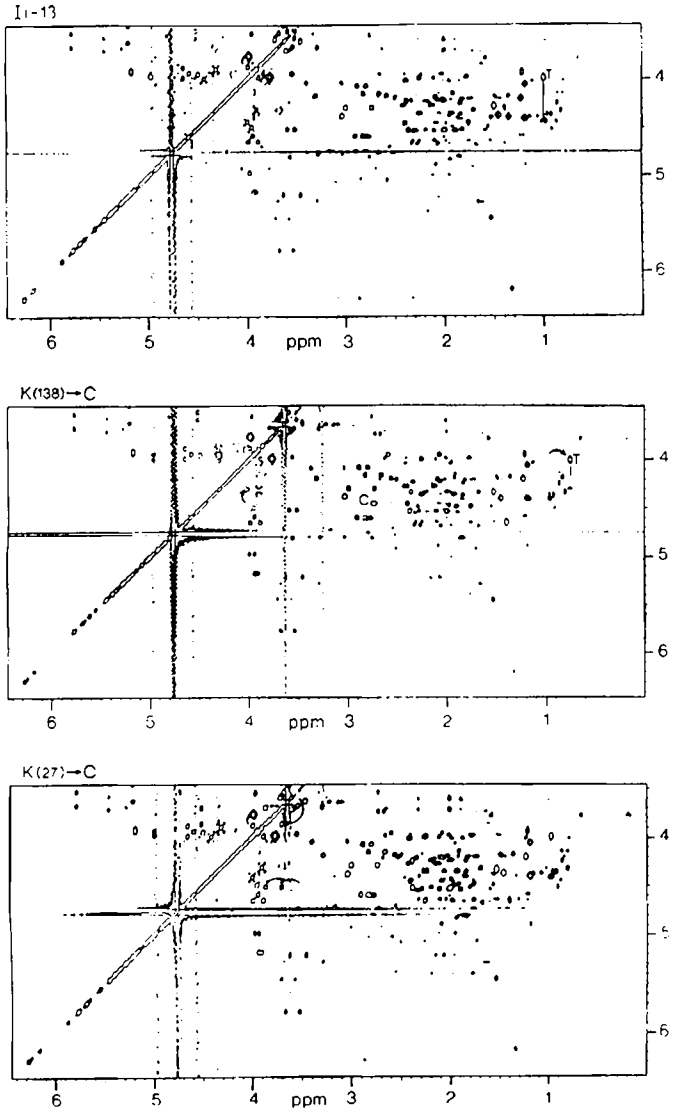

Fig. 3. *Portion of the $C^{\alpha}H$ (F1 axis) – aliphatic (F2 axis) region of the 45-ms HOHAHA spectrum of wild-type and IL-1β mutant proteins in D_2O at 600 MHz.* Some of the shifted resonances are indicated by arrows. In the spectrum of K138C, the new cysteine α/β cross-peak is labelled (C) as well as shifted threonine spin system (T). IL-1β, native interleukin-1β; K(138)→C, mutant IL-1β substituted with cysteine at position 138; K(27)→C, mutant IL-1β substituted with cysteine at position 27

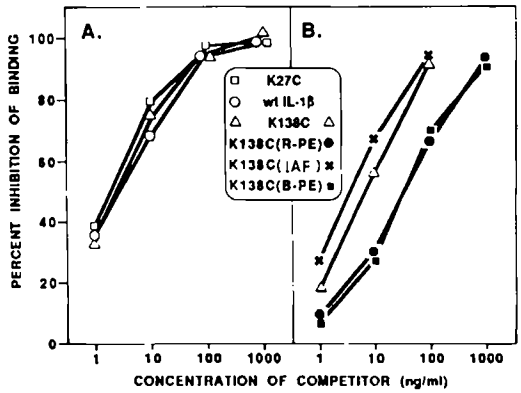

Fig. 4. *Competition-binding analysis of IL-1β mutant proteins and fluorescent conjugates.* EL4-6.1 cells (5×10^5) were incubated with ^{125}I-labelled IL-1α (1 ng/ml) in the presence or absence of various concentrations of non-radioactive IL-1β, IL-1β mutant proteins and conjugates. After 3 h at 4 °C, bound and free radioactivity were separated on a oil gradient. Data are presented as percentage inhibition of ^{125}I-IL-1α binding compared to control. (A) Demethionylated IL-1β mutant proteins, K27C and K138C, are compared to the corresponding wild-type preparation. The corresponding methionylated molecules had 5 – 10-fold-reduced activity (not shown). (B) Unconjugated IL-1β mutant protein K138C is compared to its R-phycoerythrin (R-PE), B-phycoerythrin (B-PE), and 5-IAF conjugates. wt, wild-type

Table 2. *Flow-microfluorometric analysis of binding of 5-iodoacetamide- and phycoerythrin-conjugated IL-1β mutant protein K138C to EL4-6.1 thymona cells*

EL4-6.1 cells (5×10^5) were incubated for 3 h at 4 °C with the indicated concentration of phycoerythrin-conjugated K138C. Samples were washed and analyzed by flow microfluorometry (cf. Fig. 5). IAF, 5-iodocetamidofluorescein

IL-1β conjugate	Concentration	Mean fluorescence
	ng/ml	
B-Phycoerythrin	1000	84.8
	100	63.6
	10	28.4
	1	15.3
	0	13.5
	100[a]	17.7
R-Phycoerythrin	100	67.1
	0	25.3
	100[a]	25.3
IAF	100	40.5
	0	36.8
	100[a]	37.3

[a] Incubations carried out in the presence of 100-fold excess of cold IL-1β wild-type protein

two-dimensional HOHAHA spectra (Fig. 3). Without attempting to carry out a detailed comparison, which is only possible if all spectra have been completely assigned, we believe that the shifted threonine spin system in K138C belongs to the neighbouring amino acid at position 137. As both amino acids are positioned in a loop connecting two β-strands [11], there should be enough flexibility in this region of the polypeptide chain to allow the new amino acid to position itself in the most favourable conformation. For K27C, we were unable to identify the new cysteine spin system by just inspecting the ^{1}H-NMR spectrum. This will have to await a more detailed study based on sequential resonance assignments.

The effect of the substitutions on IL-1-receptor binding was also tested. As observed previously for wild-type IL-1β [13], N-terminal-methionylated K138C was 5 – 10-fold less active than demethionylated protein (data not shown). Demethionylated forms of K27C and K138C bound to EL4-6.1 cells with similar affinity, as compared to an equivalent preparation of wild-type IL-1β (Fig. 4A).

Using a biological assay (IL-1/LAF) which measures stimulation of murine thymocyte proliferation, unfractionated K138C had a specific activity of about 5×10^6 units/mg protein (see Materials and Methods for definition of unit). This was about threefold lower than the control wild-type protein. When the cysteine side chain was modified with

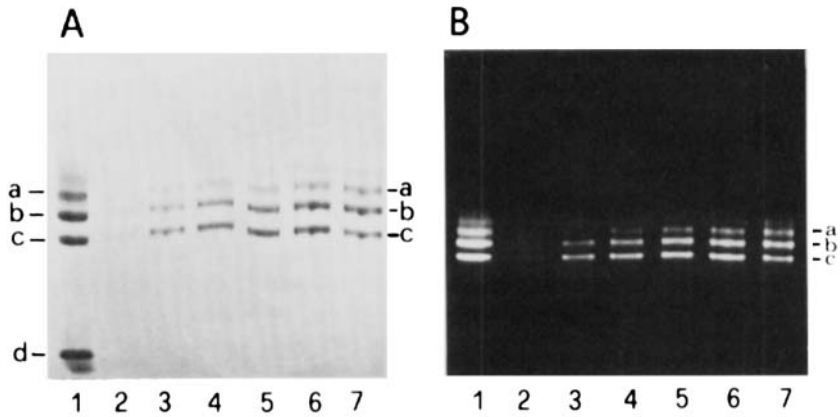

Fig. 5. *Native PAGE of phycoerythrin conjugates of the interleukin-1β mutant protein K138C.* (A) Results of native PAGE on a 10–15% (mass/vol.) gradient polyacrylamide gel stained with Coomassie blue. The pH was 8.8 and direction of migration was towards the anode (bottom of gel). Lane 1, conjugate and excess IL-1β mutant protein K138C prior to fractionation on a FPLC-Superose 12 column. (a) Phycoerythrin conjugated to two IL-1β molecules; (b) phycoerythrin conjugated to one IL-1β molecule; (c) unconjugated phycoerythrin; (d) unconjugated IL-1β. Lanes 2–7, Superose 12 column fractions in order of decreasing elution volume across the phycoerythrin-containing peak. It can be seen that there is little separation of conjugated from unconjugated phycoerythrin. (B) Native PAGE shown in (A) visualized by irradiation of the gel with ultraviolet light

iodoacetamide (Materials and Methods) the specific activity was similar to that of wild-type protein. These results indicate that the reduced activity of the unmodified mutant is probably due to the presence of a reactive sulfhydryl group which can take part in side reactions such as disulfide exchange with cellular proteins. These reactions would reduce the effective concentration of the biologically active IL-1β mutant protein. K27C had a IL-1/LAF specific activity similar to that of the wild-type protein, consistent with the receptor-binding analysis.

Fluorescent labelling and flow microfluorometry

Having established for K138C that Cys-138 is freely solvent-accessible and that the substitution has no effect on receptor binding, we used the protein to introduce both low-molecular-mass (e.g. fluorescein) and high-molecular-mass (e.g. phycobiliprotein) fluorescent probes.

K138C labelled with 5-iodoacetamidofluorescein (1 mol/mol) bound to the IL-1 receptor on EL4-6.1 cells with an affinity similar to that of the wild-type protein (Fig. 4B). The modified protein also had an IL-1/LAF specific activity similar to the wild-type protein (data not shown). Flow-cytometric analysis of the labelled-cells (Table 2) indicated weak fluorescence emission just above the background level. As there are about 10^4 IL-1 receptors/cell on the EL4-cell line used [4], then 10^4 fluorescein molecules/cell is just above the theoretical limit of detection using the FACS II system (estimated to be $\approx 5 \times 10^3$ molecules/cell).

To increase the detection limit of IL-1β bound to receptor, we prepared conjugates between highly fluorescent phycobiliprotein [26] and K138C. The method of conjugation was based on intermolecular disulfide-bond formation between Cys-138 of the IL-1β mutant protein and pyridyldisulfide-modified (2.1 mol/mol protein) phycoerythrin. After separation of excess (unconjugated) mutant IL-1β by size-exclusion chromatography, native polyacrylamide gel electrophoresis (Fig. 5) indicated the presence of three major bands corresponding to unconjugated phycobiliprotein (45% of the total protein) and phycobiliprotein conjugated with one or two molecules of IL-1β, which represented 35% or 20%, respectively, of the total protein. The molecular mass of the various conjugates (258–275 kDa) and unconjugated phycobiliprotein (240 kDa) were too similar to effect clean separations by size-exclusion chromatography.

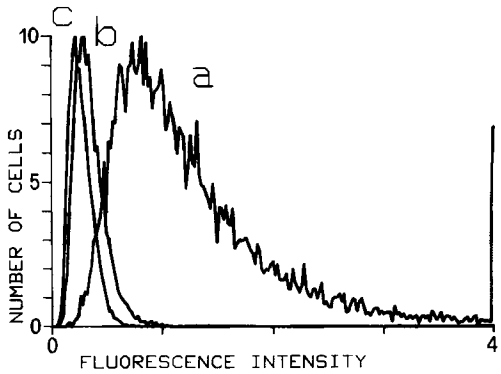

Fig. 6. *Flow-microfluorometric analysis of IL-1 receptors on EL4-6.1 thymoma cells.* EL4-6.1 cells were incubated for 3 h at 4°C with 100 ng/ml of B-phycoerythrin-conjugated IL-1β mutant protein K138C in the absence (a) or presence (b) of a 100-fold excess of cold wild-type IL-1β. Unlabelled cells are included for comparison (c). All samples were passed on a FACS II flow cytometer, gated to exclude non-viable cells. Diagrams each represent 10^4 cells accumulated using linear fluorescence amplification. The values given on both axes are in arbitrary linear units

The phycoerythrin-conjugated IL-1β mutant protein had a receptor-binding affinity only slightly lower than unconjugated wild-type protein (Fig. 4B). This result indicated that the region of IL-1β important for receptor binding was not sterically hindered by the high-M_r phycobiliprotein coupled to Cys-138. However, the conjugate had about a 50-fold-reduced biological activity on IL-1/LAF.

The fact that receptor binding of the conjugate was similar to that of the unconjugated protein, but with biological activity significantly reduced, may indicate that internalization of IL-1β is required for signal transduction (see [27] for recent work supporting this view). Due to either conjugated IL-1β

not being internalized or to the IL-1/LAF assay conditions (i.e. long incubation periods at 37°C in complex medium), free IL-1β is generated which is the active species.

In contrast to the fluorescein conjugate, binding of R- or B-phycoerythrin-conjugated K138C to EL4-6.1 cells was readily detected by flow microfluorometry (Fig. 6, Table 2). This binding was dose-dependent and could be completely inhibited by unlabelled wild-type IL-1β. Inspection of the fluorescence diagram (Fig. 6) indicated relatively heterogeneous expression of IL-1 receptors at the single-cell level. Attempts to isolate stable subclones of EL4-6.1 expressing high levels of IL-1 receptors by cell sorting are currently in progress.

DISCUSSION

The single histidine residue (His-30) of IL-1β is located on the surface of the molecule [11]. This residue is probably hydrogen bonded as it has an abnormally high pK_a and its substitution by site-directed mutagenesis results in reduced receptor-binding affinity [12]. Moreover, this reduced binding affinity is not a result of gross conformational change [12]. Here we have shown that substitution of a residue (Lys-27) close to His-30 does not affect receptor binding. His-30 and Lys-27 are both located in the same β-strand (number three) of the all-β-strand IL-1β molecule [11].

Changes in receptor binding have also been noted when various substitutions or deletions were made in the N-terminal region of IL-1β [13–15]. This region (residues 3–12) is a β-strand (number one) which forms part of a six-stranded β-barrel structure and is located close to the beginning of β-strand three [11]. The two portions of the protein where modifications alter receptor binding are thus located on the same face of the tetrahedrally shaped IL-1β molecule.

In this study additional information on the receptor binding region(s) of IL-1β has been obtained by conjugation of phycoerythrin to the mutant IL-1β protein, K138C. Attachment of this high-M_r (240000) multiprotein complex to Cys-138 of the mutant protein, which is located in the loop region connecting β-strands 11 and 12 [11], had little effect on receptor-binding affinity. This loop region and its immediate surroundings must therefore be oriented away from the region(s) involved in receptor binding. The above-mentioned mutagenesis studies are consistent with this, as β-strand three and the loop region are located at different corners of the tetrahedral structure.

In K138C the reactive cysteine offers the opportunity of introducing both low- and high-molecular-mass compounds to a defined region of the protein not important for receptor binding. We have described conjugates of mutant IL-1β and phycoerythrins suitable for flow-cytometric analysis. Such conjugates can already be used to analyse and sort cells (or transfectants) expressing the IL-1 receptor at high levels. Furthermore, increased sensitivity of fluorescent labelling could lead to detection of IL-1 receptors on normal cells and hence analysis of receptor heterogeneity at the single-cell level.

Another application for K138C is in the preparation of affinity matrices suitable for purification of detergent-solubilized IL-1 receptor from EL4-6.1 cells. The mutant protein can be immobilized via Cys-138 to various supports, such as thiol-activated Sepharose. The immobilized protein is, thus, oriented with the region involved in receptor binding facing away from the matrix. Both K27C and K138C will also be useful for various spectroscopic studies, such as fluorescence energy transfer, as they enable the introduction of spectroscopic probes in a sequence-specific manner.

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