Identification of a Reactive Lysyl Residue (Lys^{103}) of Recombinant Human Interleukin-1β. Mechanism of Its Reactivity and Implication of Its Functional Role in Receptor Binding

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ABSTRACT: Recombinant human interleukin-1β (h-IL-1β) was chemically modified with 4-(*N*,*N*-dimethylamino)-4′-isothiocyanatoazobenzene-2′-sulfonic acid (S-DABITC), a water-soluble color reagent specific for lysine labeling. Modified h-IL-1β was digested by lysyl endopeptidase. Peptides containing labeled lysines were detected at the visible wavelength (436 nm) and isolated by HPLC. The modification sites were eventually determined by sequence analysis. The results revealed that Lys^{103}, Lys^{92}, Lys^{93}, and Lys^{94} of h-IL-1β reacted selectively with S-DABITC. A 1-h incubation with 1 mM S-DABITC at room temperature resulted in a quantitative modification of Lys^{103}, 22% of Lys^{92}, 27% of Lys^{93}, and 18% of Lys^{94}, respectively. This modification was accompanied by a 20-fold decrease of the protein's ability to bind to the receptor. Furthermore, a mutant of h-IL-1β (M9, Glu^{105} substituted by Lys) exhibits markedly impaired receptor binding, and the S-DABITC reactivity of its Lys^{103} was found to be reduced by 90%. These findings suggest that Lys^{103} of h-IL-1β might play an important role in the h-IL-1β/receptor interaction.

Human interleukin-1β (h-IL-1β)[1] is an immunomodulator which participates in the regulation of numerous immunological and inflammatory processes (Dinarello, 1984; Durum et al., 1985; Krakauer, 1986). The physiological functions of h-IL-1β are triggered by its binding to specific cell surface receptors (Kilian et al., 1986; Dower & Urdal, 1987; Horuk et al., 1987; Dower et al., 1990). Although the X-ray crystal structure of recombinant human IL-1β has been known (Priestle et al., 1988, 1989), the three-dimensional structure of the IL-1β/receptor complex is still not available. So far, the mechanism of the interaction of h-IL-1β with its receptor has been largely deduced from the study of h-IL-1β mutants produced by site-directed mutagenesis (Gronenborn et al., 1986; Horuk et al., 1987; Huang et al., 1987; Kamogashira et al., 1988; Joblings et al., 1988; Gehrke et al., 1990). Specifically, it was demonstrated that deletion of the N-terminal amino acid residues caused loss of biological activity (Mosley et al., 1987; Lillquist et al., 1988). Chemical modification has also been employed to locate the amino acid residues that are essential for the biological activity of h-IL-1β (Mizel et al., 1981; Dinarello et al., 1982; Krakauer, 1984). Yem et al. (1989) employed biotinylation and identified specific modification of Lys^{93}, Lys^{94}, and Lys^{103}. The relative reactivity among these three lysines, however, was not quantitatively measured by these authors, and it was concluded that these lysyl residues were not crucial for the activity of h-IL-1β.

We describe in this report chemical modification of lysyl residues of recombinant human IL-1β and two mutants of IL-1β, obtained by site-directed mutagenesis, using a color reagent, 4-(*N*,*N*-dimethylamino)-4′-isothiocyanatoazobenzene-2′-sulfonic acid (S-DABITC) (Chang, 1989a). Attempts were made to quantitate the relative S-DABITC reactivity of lysines in h-IL-1β and to correlate the extent of modification with impaired biological activity.

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Experimental Procedures

Materials. Recombinant h-IL-1β and its mutants, M2 (Glu^{25} to Lys^{25}) and M9 (Glu^{105} to Lys^{105}), were purified by a method already described (van Oostrum et al., submitted for publication). 4-(*N*,*N*-Dimethylamino)-4′-isothiocyanatoazobenzene-2′-sulfonic acid (S-DABITC) was synthesized in our laboratory (Chang, 1989a). Trinitrobenzenesulfonic acid, sulfonated *N*-hydroxysuccinimide-modified biotin (sulfo-NHS-biotin) and NHS-LC-biotin were purchased from Pierce. 4-(*N*,*N*-Dimethylamino)azobenzene-4′-sulfonic acid (methyl orange) was obtained from Fluka. Lysyl endopeptidase (from *Achromobacter lyticus*) is a product of Wako Pure Chemical Industries Inc.

S-DABITC Modification of h-IL-1β and Its Mutants. h-IL-1β (2 mg) was dissolved in 800 μL of 50 mM sodium bicarbonate (pH 8.3) and then was added to 800 μL of a S-DABITC solution (2 mM in the same sodium bicarbonate buffer). Derivatization was carried out at 22 °C. At different time points (20 min and 1, 3, and 6 h), an aliquot (360 μL) of the sample was withdrawn and immediately passed through a disposable PD-10 column (Pharmacia) equilibrated with 50 mM ammonium bicarbonate. Modified h-IL-1β was visible during desalting as an orange-red color band and was recovered in a total volume of 1.2 mL. A control sample was prepared by treating 500 μg of h-IL-1β with the same sodium bicarbonate buffer for 1 h and was similarly desalted through a PD-10 column. These derivatized h-IL-1β samples were directly used for biological assay or directly mixed with lysyl endopeptidase for enzyme digestion. The extent of S-DABITC labeling (moles of S-DABITC per mole of h-IL-1β) was estimated on the basis of the molar extinction coefficient of the S-DABITC derivative of 29 000 at 465 nm. The protein

[1] Abbreviations: h-IL-1β, human recombinant interleukin-1β; S-DABITC, 4-(*N*,*N*-dimethylamino)-4′-isothiocyanatoazobenzene-2′-sulfonic acid; PBS, phosphate-buffered saline.

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Table I: Sequences of S-DABITC-Labeled Peptides of h-IL-1β[a]

peptides[b]	amino acid sequences[c]	sequence position
a	(*)-Lys	93–94
b	Arg-Phe-Val-Phe-Asn-(*)	98–103
c	(*)-Met-Glu-Lys	94–97
d	Asn-Tyr-Pro-(*)-Lys	89–93
e	(*)-Ile-Glu-Ile-Asn-Asn-Lys	103–109
f	Ser-Gly-Pro-Tyr-(*)-Leu-Lys	21–27
g	Ser-Leu-Val-Met-Ser-Gly-Pro-Tyr-(*)-Leu-Lys	17–27
h	(*)-Ile-(*)-Ile-Asn-Asn-Lys	103–109
i	Arg-Phe-Val-Phe-Asn-(*)-Ile-Glu-Ile-Asn-Asn-Lys	98–109
j	Arg-Phe-Val-Phe-Asn-(*)-Ile-(*)-Ile-Asn-Asn-Lys	98–109

[a] The sequences were determined using an Applied Biosystems Inc. 477A sequencer. [b] The nomenclature of peptides is indicated under the chromatograms of Figures 1 and 2. Peptides f and g were derived from mutant M2 only. Peptides h and j were unique products of mutant M9. Peptides e, f, and h were produced by nonspecific cleavage of lysyl endopeptidase. [c] All peptides were more than 90% pure except for peptide g. An asterisk indicates S-DABITC-labeled lysine.

concentration was determined by amino acid analysis (Knecht & Chang, 1986). In the experiments of competitive labeling, S-DABITC (2 mM) was premixed with an equal concentration of a second lysine-specific reagent prior to the mixing with the h-IL-1β solution.

Structural Characterization of S-DABITC-Labeled h-IL-1β. S-DABITC-derivatized h-IL-1β (100 μg in 0.3 mL of ammonium bicarbonate solution) was digested with 10 μg of lysyl endopeptidase and incubated at 22 °C overnight. The digested sample was directly injected for HPLC analysis using the conditions described in the legend of Figure 1. The color peptides were collected, and their structures were analyzed by both quantitative N-terminal analysis (Chang, 1988) and amino acid sequencing (Hunkapiller et al., 1983) in order to identify the labeled lysines.

Biological Assays of S-DABITC-Modified h-IL-1β. The biological activities of modified h-IL-1β were evaluated by their ability to bind to the receptor or to induce cell adhesiveness of endothelial cells. The receptor binding assay, a modification of that described by Loewenthal et al. (1986), was performed in flexible microtiter plates (Joss et al., 1991) using EL-4.61 cells as a receptor source (provided by H. R. MacDonald, Ludwig Institute for Cancer Research, Lausanne, Switzerland) and ^{125}I-IL-1β as a radioligand (ANAWA, CH-8601 Wangen, Switzerland). Briefly, a suspension of 400 000 cells/well in a total volume of 40 μL was incubated in the presence of a final concentration of 0.5 nM ^{125}I-IL-1β with or without unlabeled IL-1β (for the standard curve) or S-DABITC-modified IL-1β at various concentrations. Incubation on ice was terminated after 3 h. Separation of bound and free ^{125}I-IL-1β was achieved by addition of 150 μL of phosphate-buffered saline to the cell mixture and centrifugation of the microtiter plates at 1000*g* for 10 min. The supernatants were aspirated, and the cell-bound specific radioactivity was counted.

The induction of endothelial cell adhesiveness for leukocytes by h-IL-1β and its derivatives was determined essentially as described by Bevilacqua et al. (1985). Monolayers of human umbilical vein endothelial cells in microtiter plates were incubated at 37 °C for 4 h in the presence of the indicated concentrations of h-IL-1β or its derivatives, and 1×10^5 HL-60 cells labeled with ^{51}Cr in 100 μL of PBS containing 1 g/L D-glucose were added. After 20 min of incubation at 37 °C, unbound cells were removed by washing twice with PBS, and the number of bound cells was estimated from the bound radioactivity.

Results

S-DABITC Labeling of h-IL-1β and Its Mutants. The extent of S-DABITC incorporation into h-IL-1β increased with reaction time: 20 min (1.47 mol of S-DABITC/mol of h-IL-1β), 1 h (2.51), 3 h (3.9), and 6 h (5.18). These derivatized samples were digested by lysyl endopeptidase followed by HPLC analysis. Color peptide mapping (Figure 1) displayed about five major labeled peptides (fractions a, c, d, e, and i of Figure 1), suggesting that there were about five lysines which were preferentially labeled by S-DABITC. Sequence analysis revealed that peptides a, c, and d each contain one S-DABITC modification site at Lys^{93}, Lys^{94}, and Lys^{92}, respectively (Table I). Peptides e and i, however, were derived from the same modification site (Lys^{103})—a consequence of nonspecific cleavage by lysyl endopeptidase. These results show that Lys^{103} is the most reactive lysine of h-IL-1β. A 1-h incubation with S-DABITC resulted in a nearly quantitative labeling of Lys^{103} and approximately 20%–25% modification of Lys^{92}, Lys^{93}, and Lys^{94}. The extent of modification at these four lysines was reduced by 50%–60% when S-DABITC labeling was carried out in the presence of an equal concentration of either trinitrobenzenesulfonic acid, NHS-LC-biotin, or sulfo-NHS-biotin (Table II). These data demonstrate comparable specificity and reactivity among S-DABITC and various lysine labeling reagents. The extent of S-DABITC labeling of h-IL-1β remains unchanged when reactions are performed in the presence of an excess amount of methyl orange [4-(*N*,*N*-dimethylamino)azobenzene-4′-sulfonic acid] (Table II). Methyl orange is structurally similar to the azo-dye moiety of S-DABITC. This experiment confirmed that the azo-dye structure of the reagent played no significant role in influencing the specificity and reactivity of the S-DABITC labeling of h-IL-1β.

Table II: S-DABITC Labeling of h-IL-1β in the Presence of Trinitrobenzenesulfonic Acid (TNBS), Sulfo-NHS-biotin, NHS-LC-biotin, and Methyl Orange[a]

labeling conditions	% Lys^{93}	% Lys^{94}	% Lys^{92}	% Lys^{103}
control (1 mM S-DABITC)	100	100	100	100
1 mm S-DABITC + 1 mM TNBS	30	83	44	37
1 mM S-DABITC + 1 mM sulfo-NHS-biotin	10	53	37	32
1 mM S-DABITC + 1 mM NHS-LC-biotin	18	66	61	45
1 mM S-DABITC + 1 mM methyl orange	102		102	104
1 mM S-DABITC + 5 mM methyl orange	81	83	89	90

[a] The extent of the S-DABITC labeling of the control sample was taken as 100% for each lysine modified. Modification was carried out at 22 °C for 1 h.

The S-DABITC labeling pattern of mutant M2 (Glu^{25} substituted by Lys) differed only slightly from that of wild-type h-IL-1β (Figure 2). Lys^{103} of mutant M2 remained chemically the most reactive lysine. In addition, the newly introduced Lys^{25} also reacted with S-DABITC. The reactivity of

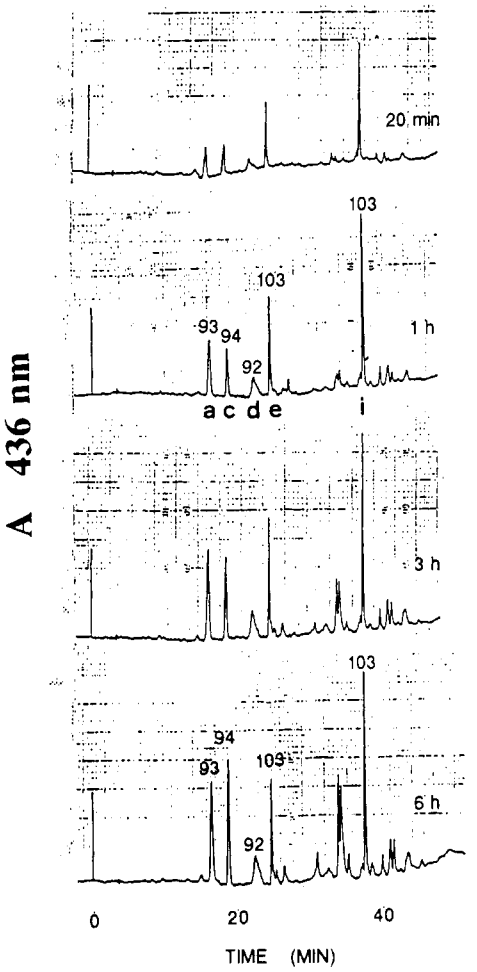

FIGURE 1: Peptide mapping of S-DABITC-derivatized h-IL-1β. The derivatized h-IL-1β was digested by lysyl endopeptidase. Peptides were monitored at the visible wavelength, and therefore only those which contained S-DABITC-labeled lysines were detected. The nomenclature of peptides is indicated in the 1-h sample (under the chromatogram as a, c, d, e, and i). Numbers given on the top of the peaks are labeled lysines of h-IL-1β. The amino acid sequences are presented in Table I. Solvent A was 25 mM ammonium acetate, pH 5.0. Solvent B was acetonitrile. The gradient was 15% B isocratic from 0 to 5 min, 15%–40% B from 5 to 40 min, and 40–80% B from 40 to 45 min and was returned to 15% B from 45 to 47 min. The column was a Vydac C-18 for peptides and proteins. The column temperature was 22 °C. The flow rate was 1 mL/min.

Lys^{103} of mutant M9 (Glu^{105} to Lys), however, was diminished by about 90% (Figure 2). Two minor color peptides containing the same labeling sites at Lys^{103} and Lys^{105} were recovered—again due to the nonspecific cleavage of lysyl endopeptidase at the carboxyl end of Asn^{102} (Table I).

Biological Activities of S-DABITC-Modified h-IL-1β. Untreated h-IL-1β competed with receptor binding of ^{125}I-IL-1β as expected: 50% inhibition was achieved at about an equimolar concentration of unlabeled h-IL-1β (0.5 nM), and full inhibition was found at near 10 nM, i.e., a 20-fold molar excess over ^{125}I-IL-1β (Figure 3). In contrast, S-DABITC-labeled h-IL-1β exerted decreased binding with increasing extent of S-DABITC labeling. The 20-min modified h-IL-1β displayed a reduced binding ability by a factor of 4, i.e., 50% inhibition of ^{125}I-IL-1β binding was obtained at about 2 nM. After 3-h S-DABITC modification, only about 25% of inhibition was observed at 10 nM.

h-IL-1β modified with S-DABITC also lost its ability to induce endothelial adhesiveness as a function of the reaction time (Figure 4). The ED_{50} value was reduced approximately 100-fold after 6 h of reaction time as compared with that of the unmodified sample, whereas the slope of the dose–response curve and the maximal effect remained unchanged, as could be expected from the observation that the receptor binding affinity was also reduced in parallel (Figure 3).

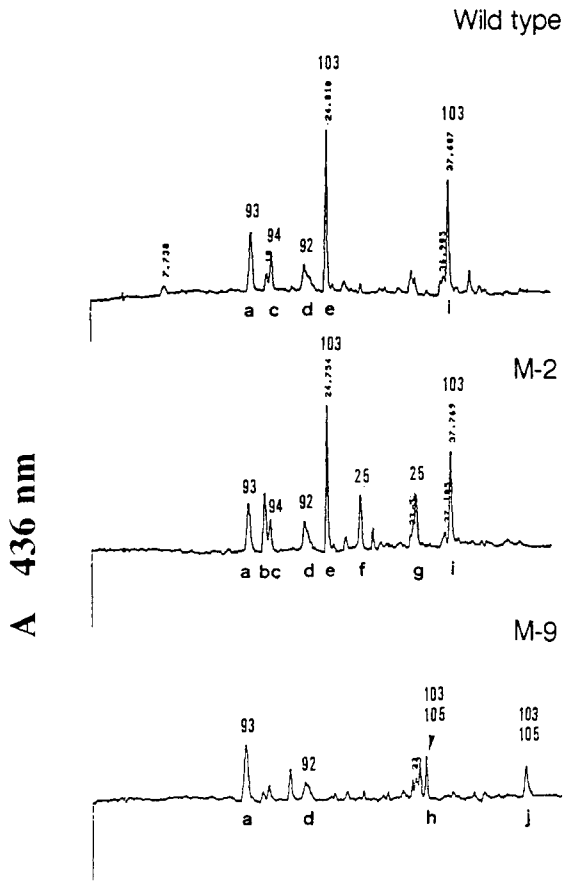

FIGURE 2: Peptide mapping of S-DABITC-derivatized h-IL-1β and its mutants M2 and M9. Mutant M2 had a single substitution at residue 25 (Glu replaced by Lys), and M9 had a substitution at residue 105 (also Glu replaced by Lys). S-DABITC derivatization was carried out for 1 h under the conditions described in the text. Peptide nomenclatures are indicated under each chromatogram, and peptide sequences are given in Table I. Chromatographic conditions are identical to those described in the legend of Figure 1.

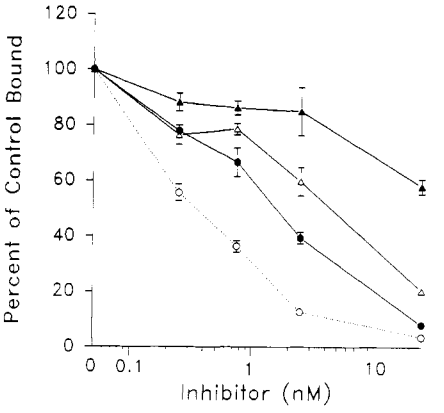

FIGURE 3: Binding of S-DABITC-labeled IL-1β to the receptor. Shown are competition binding curves using EL-4.61 cells as the source of a receptor and ^{125}I-IL-1β as a radioligand alone (control) or in the presence of increasing concentrations of h-IL-1β labeled with S-DABITC for 20 min (●), 1 h (△), and 3 h (▲) (solid lines) or untreated h-IL-1β (control, ○).

Discussion

S-DABITC is a water-soluble color reagent that selectively labels amino groups of proteins under mild conditions (Chang, 1989a). Most amino groups of proteins are, however, ϵ-amino

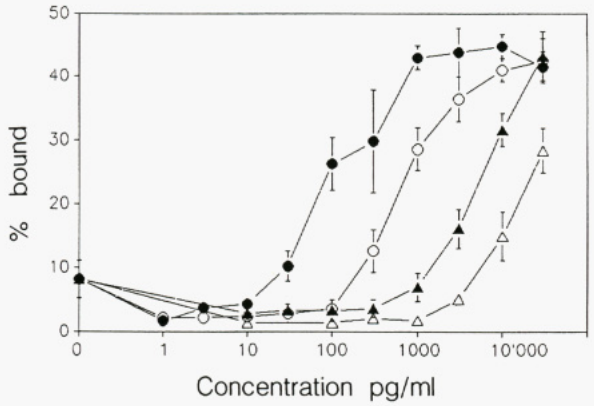

FIGURE 4: Induction of endothelial adhesiveness of h-IL-1β and S-DABITC-modified h-IL-1β. Monolayers of human umbilical vein endothelial cells were incubated for 4 h in the presence of the indicated concentrations of the unmodified control h-IL-1β (●) and of h-IL-1β modified with S-DABITC for 1 h (○), 3 h (▲), or 6 h (△) as described in the text. The adhesiveness induced by this treatment was assessed by addition of ^{51}Cr-labeled HL-60 cells for 20 min, followed by determination of the bound radioactivity. The ordinate indicates the percentage of bound cells (100% corresponding to 1×10^5 cells or 72 062 cpm). The means of quadruplicates and standard deviations are indicated.

groups of lysyl side chains which exhibit an average pK value of 10–10.5. At the pH (8.3) employed for S-DABITC derivatization, the vast majority (>99%) of the ε-amino groups are protonated and their chemical reactivities are thus understandably sluggish. Recombinant h-IL-1β contains 15 lysines, and 4 (Lys^{103}, Lys^{92}, Lys^{93}, and Lys^{94}) were shown to react preferentially with S-DABITC. The reactivity of these 4 lysines cannot be simply explained by their solvent accessibilities because they are, in fact, among the least accessible lysines of h-IL-1β (Table III and Figure 5A), particularly Lys^{103}, whose side chain is mostly buried within a hydrophobic pocket (Figure 5B) and whose ε-amino group is less exposed than any other lysine residue except that of Lys^{16} (Table III). The reactivity of Lys^{103} is also unlikely to be enhanced by the apolar binding between the S-DABITC and the hydrophobic pocket encompassing Lys^{103}, since its reaction rate is not affected by the presence of an excess amount of methyl

Table III: Solvent-Accessible Areas (Å^2) of the 15 Lysine ε-Amino Groups of h-IL-1β[a]

Lys^{16}	0.10	Lys^{74}	31.01	Lys^{94}	19.06
Lys^{27}	30.91	Lys^{77}	33.84	Lys^{97}	51.81
Lys^{55}	28.34	Lys^{88}	45.92	Lys^{103}	8.99
Lys^{63}	24.24	Lys^{92}	22.69	Lys^{109}	36.20
Lys^{65}	50.62	Lys^{93}	18.80	Lys^{138}	32.28

[a] The solvent-accessible surface of each atom was calculated according to the method of Lee and Richards (1971) from the fully refined crystal of IL-1β (Priestle et al., 1989).

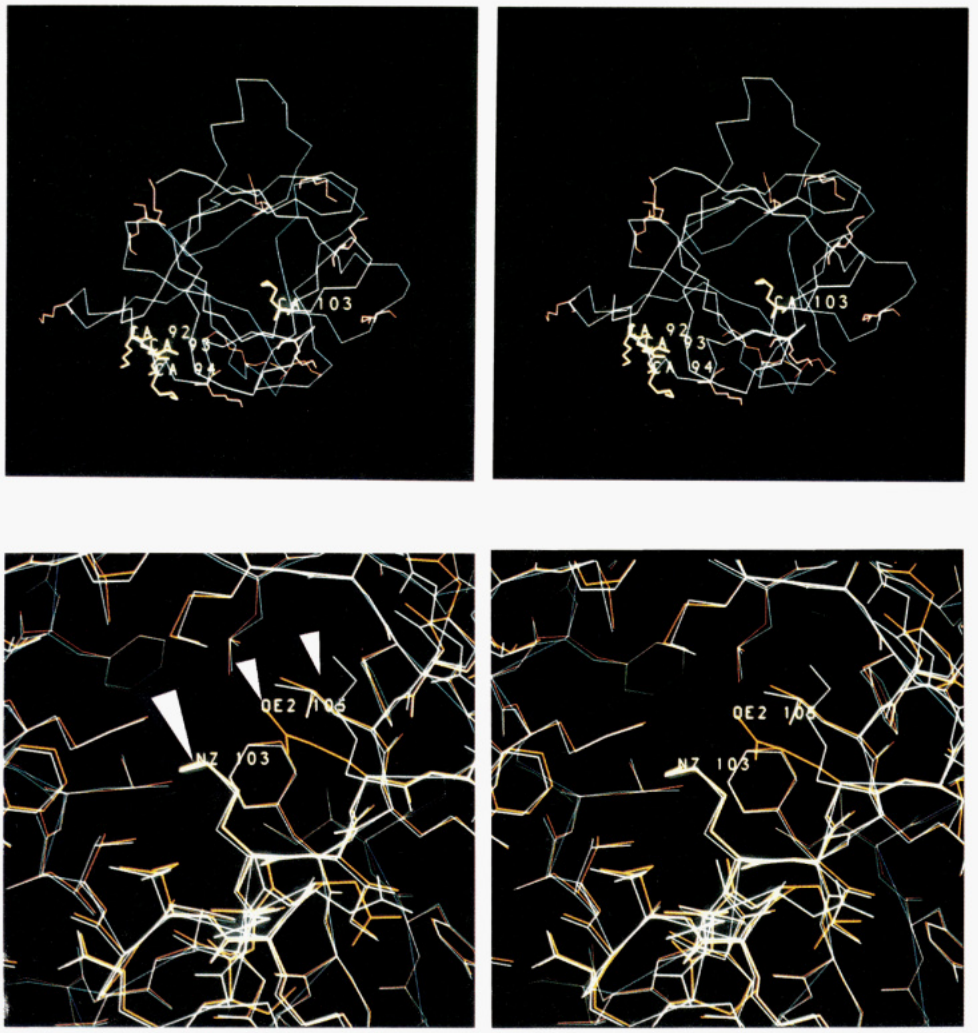

FIGURE 5: Stereoview of the C_α structure of h-IL-1β. (A, top) The entire molecule. The yellow side chains indicate the four S-DABITC reactive lysines. Positions of the remaining 11 lysines are shown in red. The relative solvent accessibility (Lee & Richards, 1971) of these 15 lysyl residues is given in Table III. (B, bottom) Closeup view of the microenvironment of the side chain of Lys^{103} (indicated by the large arrow). The C_α structures of the wild-type h-IL-1β (red) and mutant M9 (blue) are overlapped to show the side chains of residue 105 in both proteins (indicated by small arrows, red side chain for Glu^{105} of the wild-type h-IL-1β and blue for Lys^{105} of mutant M9).

orange—an azo dye structurally homologous to S-DABITC. The mechanism which accounts for the robust reactivity of these preferred lysines therefore must be attributed to the microenvironments that dictate the pK value of their ϵ-amino groups. These microenvironments are apparently highly sensitive to the presence of neighboring amino acids. A substitution of Glu by Lys at residue 105 could, for example, alter the pK value of Lys^{103} and drastically reduce its S-DABITC reactivity (Figure 2).

The S-DABITC method has been applied to study the structure of the heparin binding site of antithrombin (Chang, 1989a; Sun & Chang, 1989) and the hirudin binding site of thrombin (Chang, 1989b, 1991). In both cases, it was observed that lysines which were identified as being situated within or near the ligand binding site were in fact preferentially derivatized by S-DABITC. For example, among the 35 lysines of antithrombin, about 4 were highly reactive toward S-DABITC and 3 out of these 4 reactive lysines were shown to be involved in heparin binding (Chang, 1989a). Similar results were found in the case of the hirudin/thrombin interaction (Chang, 1989b). These findings revealed an intriguing connection between the S-DABITC reactivity of a lysyl residue and its role in ligand binding. It also suggests that the reactive Lys^{103} of h-IL-1β might similarly participate in the function of ligand (receptor) interaction. This assessment is further credited by additional evidence. (a) The impairment of the biological activities of h-IL-1β, both in the receptor binding assay and in the HL-60 cell adhesion test, appears to be quantitatively related to the extent of modification at Lys^{103}. (b) The human interleukin-1 receptor antagonist (h-IL-1ra) and h-IL-1β bind to the receptor on EL-4.61 cells with comparable affinities (Eisenberg et al., 1990). The two proteins display only 26% of sequence homology, but intrestingly the basic amino acids clustered between Lys^{93} and Lys^{103} of h-IL-1β are fully conserved in the h-IL-1ra. (c) Thirty-two h-IL-1β mutants have been produced, and only four of them exhibited reduced receptor binding affinity (van Oostrum et al., manuscript in preparation). Among these four mutants, the substitution of Glu^{105} by Lys (mutant M9) caused the most drastic decrease (100-fold) of the binding affinity to the receptor on mouse EL-4.61 cells. In order to verify the structural integrity of mutant M9, the protein was crystallized using conditions described for IL-1β (Schaer et al., 1987) and its structure resolved by molecular replacement using the high-resolution structure of h-IL-1β as a trial model (Priestle et al., 1988). Mutant M9 is a stable molecule, and its three-dimensional structure is nearly identical to that of wild-type h-IL-1β. The replacement of a glutamyl side chain by a lysyl side chain at residue 105 apparently causes no additional steric hindrance for the accessibility of Lys^{103} (Figure 5B) but does change the microenvironment of Lys^{103} and sharply diminishes its S-DABITC reactivity (Figure 2)—a mechanism that might indeed account for the impaired biological function of h-IL-1β mutant M9.

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Registry No. Lysine, 56-87-1.

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