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Site directed mutants of human interleukin-1α: a ^{1}H-NMR and receptor binding study

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Mutant human interleukin-1α proteins were constructed by oligonucleotide directed mutagenesis. Six different mutants were tested for receptor binding activity and showed no alteration with respect to the wild-type protein. Analysis of these mutants by nuclear magnetic resonance spectroscopy confirmed the structural integrity of the mutant proteins and permitted the sequence specific assignment of the histidine and tryptophan residues.

Interleukin-1; Receptor binding; Mutagenesis; NMR

1. INTRODUCTION

Interleukin-1 (IL-1) activity (reviews [1,2]) is mediated by two distinct 17 kDa proteins, IL-1α and IL-1β, which, despite their limited amino acid homology, both bind to the same receptor [3,4]. The purification and characterization of recombinant human IL-1α and IL-1β have been described [5,6]. To determine which regions of the protein are important for receptor interaction, a series of point mutations has been introduced into both proteins by site directed mutagenesis. In the case of IL-1β, these mutant proteins have been investigated by nuclear magnetic resonance (NMR) [7], and receptor binding studies have been carried out in conjunction with an assessment of the mutant conformations in comparison with the wild-type protein [8].

In this paper we describe an analogous study on IL-1α. Using two-dimensional NMR spectroscopy the conformation of two histidine mutants (at positions 46 and 127) and one tryptophan mutant (at position 139) is probed and sequence specific assignments for all the histidine and tryptophan resonances are obtained. In addition, we show that all mutants exhibit the same binding affinity for the IL-1 receptor as wild-type IL-1α.

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2. MATERIALS AND METHODS

2.1. *Preparation of wild-type and mutant IL-1α*

Site-specific mutagenesis was used to introduce the point mutations in IL-1α. The template was the phage M13 derivative mp8 carrying the coding region for mature IL-1α. Mutagenesis was carried out by the double-primer procedure [9] with oligonucleotides carrying one or two mismatches. After verification of the mutation by the dideoxy sequencing method [10], the modified IL-1α genes were transferred to a plasmid to be expressed under control of the λP_L promotor and the bacteriophage Mu *ner* gene ribosome binding site. The *Escherichia coli* host was W3110 cI857, carrying the gene for the temperature sensitive repressor of phage λ. Fermentation and protein purification procedures were as described [6].

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2.2. *NMR spectroscopy*

Samples for NMR spectroscopy contained 1 mM protein in D_2O containing 250 mM sodium phosphate buffer, 5 mM sodium azide, pH 7.5. All spectra were recorded on a Bruker AM 500 spectrometer equipped with digital phase shifters at 25°C. Pure phase absorption two-dimensional Hartman-Hahn (HOHAHA) spectra [11,12] were recorded using the time proportional incrementation method [13,14]. 128 transients were collected for each of the 512 increments with a relaxation delay of 1 s between successive transients. The sweep width employed was 7042 Hz and the digital resolution in both dimensions, after zero-filling in the t_1 dimension only, was 6.88 Hz/point.

2.3. *IL-1 receptor binding assay*

Competition binding was carried out using radioiodinated IL-1α as described in detail [4,8].

3. RESULTS AND DISCUSSION

The various mutants of human IL-1α used in the present study are summarized in table 1 alongside the receptor binding data. The choice of mutants was based on the earlier observation that a His mutant of IL-1β, namely the His 30 $\longrightarrow$ Arg substitution, exhibited a marked decrease in receptor binding activity. We therefore carried out substitutions for all histidines present in the sequence of IL-1α. As can easily be appreciated from the inhibition data, there was no detectable difference between wild type IL-1α and any of the mutant proteins. Thus, although IL-1α and IL-1β bind to the same receptor, different amino acids may play a role in this interaction.

In addition to the receptor binding studies we

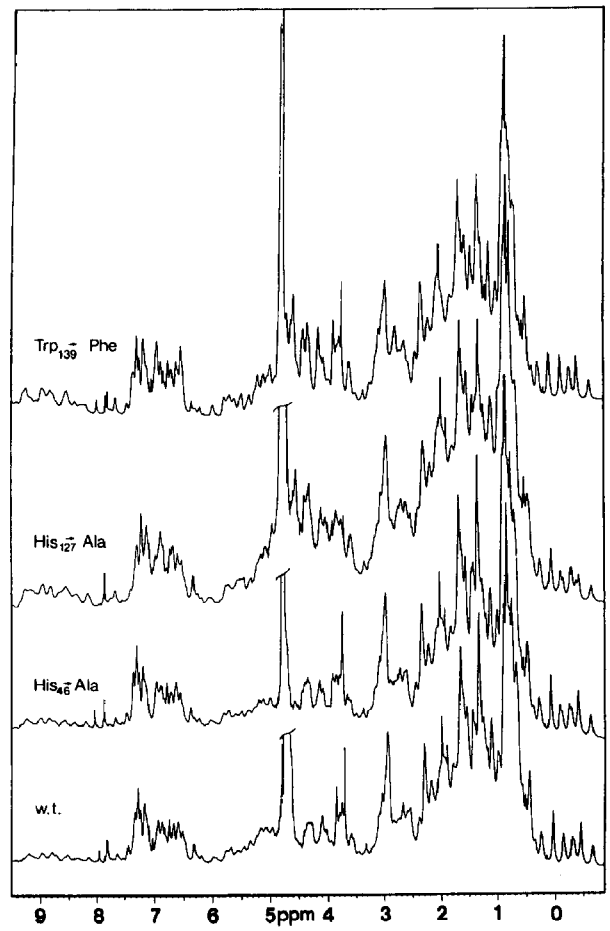

Fig.1. 500 MHz ^{1}H-NMR spectra of IL-1α and single amino acid substitution mutants at 25°C.

Table 1

Competition binding analysis of IL-1α point mutants

Mutant	Substitution variant	% inhibition of ^{125}I-IL-1α binding			
		0.1 ng/ml	1 ng/ml	10 ng/ml	100 ng/ml
Wild type	none	7.5 ± 1.8	38.2 ± 3.8	85.3 ± 2.2	91.8 ± 1.2
M15	His_{46} $\longrightarrow$ Ala	7.9	45.3	77.2	85.7
M24	His_{46} $\longrightarrow$ Arg	0	40.5	72	82.4
M27	His_{116} $\longrightarrow$ Ala	8.5	15.4	77.5	91.0
M29	His_{127} $\longrightarrow$ Ala	16.0	41.7	86.2	91.0
M30	His_{127} $\longrightarrow$ Arg	20.1	44.4	86.3	91.6
M16	Trp_{139} $\longrightarrow$ Phe	0	40.8	83.8	90.0

The indicated concentrations of unlabeled IL-1α or IL-1α point mutants were mixed with ^{125}I-IL-1α (1 ng/ml) prior to addition of 0.5×10^6 EL4-6.1 thymoma cells. After 3 h at 4°C, bound ^{125}I-IL-1α was measured by spinning the cells through an oil gradient. Data are presented as % inhibition of duplicate determinations relative to controls in the absence of competitor. Data for wild type IL-1α represent the mean (± 1 SD) of 3 independent experiments, whereas the mutants represent the mean of 2 experiments

carried out an NMR spectroscopic investigation to probe the conformation of the various mutants. Fig.1 shows the one-dimensional 500 MHz ^{1}H-NMR spectra of the wild-type protein and some mutants. With the exception of the expected disappearance of the His resonances in the aromatic region of the spectra for the His mutants and some shifts in the aromatic region for the Trp mutant, the overall appearance of the spectra is very similar. This clearly demonstrates the structural integrity of all mutant proteins investigated. In particular, the high field methyl region (0.5–1.0 ppm), which contains upfield shifted resonances arising mainly from ring currents due to the proximity of these protons to aromatic residues, is almost identical for all spectra. This region is regarded as one of the fingerprints of local and global conformation and therefore suggests that no large conformational differences exist between the wild-type and mutant proteins. The sequence specific assignment of the three aromatic histidine resonance spin systems was easily deduced from the missing resonances in the various mutant spectra.

pH-titration of the wild-type and mutant proteins permitted the determination of pK_a values for the three histidines. His 127, which gives rise to the lowest field resonances, has a fairly high pK_a of 7.5, while His 46 and 116 have pK_a values of 6.9. The chemical shifts of the $C_\epsilon H$ and $C_\delta H$ of the imidazole ring for all the histidines at pH 7.5 are indicated in the two dimensional HOHAHA spectrum shown in fig.2. This figure illustrates the aromatic region of the wild-type spectrum (a) compared to the spectrum of the Trp 139 ⟶ Phe mutant (b). The cross-peaks arise from direct and relayed through-bond (scalar) connectivities. In the wild-type spectrum the spin system of Trp 139 is marked below the diagonal and the cross-peaks belonging to this amino acid are clearly missing in the mutant spectrum. Above the diagonal in fig.2a all nine Tyr spin systems are indicated. Apart from the missing Trp resonances in fig.2b, one can clearly observe two cross-peaks which are shifted (marked by an asterisk), one of them belonging to a Tyr residue. Thus, the Trp 139 ⟶ Phe substitution has a very localized deshielding effect on a structurally neighbouring Tyr residue which

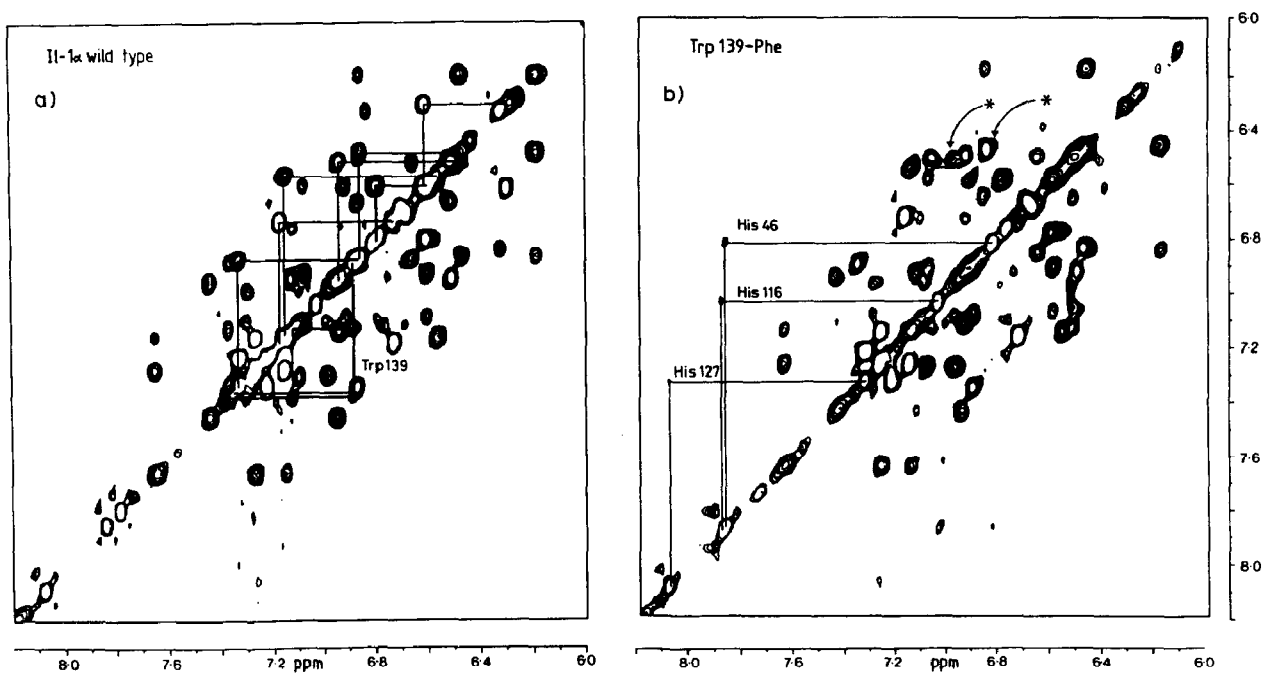

Fig.2. Aromatic region of the pure phase absorption HOHAHA spectra of wild-type IL-1α (a) and the Trp 139 ⟶ Phe mutant (b) at 25°C. Some spin system connectivities are indicated by connecting lines. In panel a Tyr spin systems are indicated above the diagonal and the Trp 139 spin system below. The His spin systems are indicated in panel b. The mixing times were 31 ms (a) and 39 ms (b).

manifests itself in the downfield shift of its resonances. All other resonances, however, show no detectable shifts relative to the wild-type spectrum.

In conclusion, our present study on IL-1α and mutants thereof clearly demonstrates that the variants investigated exhibit identical receptor binding activity to the wild-type protein and also show no or very small conformational changes. A full structural investigation of IL-1α by NMR is presently underway and will provide the basis for future mutational strategies to locate the receptor binding site.

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