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A ^{1}H-NMR study of human interleukin-1β

Sequence-specific assignment of aromatic residues using site-directed mutant proteins

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Complete identification of spin systems in the aromatic region of recombinant human interleukin-1β has been achieved using two-dimensional homonuclear Hartmann-Hahn spectroscopy. In addition, sequence-specific assignments for the four tyrosine residues have been carried out with the help of a series of mutant proteins, obtained by site-directed mutagenesis of the cloned gene. It is shown that, for the mutant proteins investigated, either none or only local structural changes occur. The use of NMR spectroscopy to determine the structural identity of site-directed mutant proteins with respect to the wild-type protein is discussed.

Interleukin-1 (Il-1) belongs to a class of cellular mediators known as cytokines which participate in the regulation of immunological and inflammatory processes (for recent review see [1]). Virtually every nucleated cell type can produce Il-1 activities, activated monocyte and macrophage lines being the major source. Il-1 exhibits a wide range of activities including thymocyte proliferation via the induction of Il-2 release, stimulation of B-lymphocyte proliferation, prostaglandin and collagenase release as well as fibroblast growth factor activity. The immunological importance of these effects and the additional observation that purified Il-1 is directly cytocidal for certain tumor target cells, has stimulated research efforts into the cloning of the gene(s) responsible for Il-1 activities and purification of the encoded protein(s). To date the cloning of four Il-1 cDNAs has been reported: a murine sequence [2], a human one isolated from peripheral blood monocytes [3] and two human macrophage-derived ones [4]. The two macrophage-derived human sequences have been termed Il-1α and Il-1β, the murine one being homologous to the human Il-1α and the monocyte-encoded sequence being closely related to the macrophage Il-1β sequence [4]. Both, Il-1α and Il-1β genes code for precursor polypeptides of approximately 31 kDa which are processed to the mature 17-kDa extracellular proteins. Expression in *Escherichia coli* of the mature Il-1α (coding for 159 amino acids) and Il-1β (coding for 153 amino acids) produces proteins which, after purification, are active in commonly used Il-1 assays [4]. Thus, through the use of expression vectors in *E. coli*, large amounts of highly purified recombinant Il-1 are potentially available for detailed biochemical and structural characterization.

So far, very little is known about the detailed mechanism of action of Il-1; it binds to its receptor sites on a variety of cell types, but nothing is known about the events following the Il-1–receptor interaction. This has prompted us to embark on a study concerning the structure of the purified protein as a first step towards understanding the structure/activity relationships of Il-1. We have chosen recombinant Il-1β for our studies, since it can be produced as a soluble protein in large quantities in *E. coli* and purified to homogeneity. In this paper we report on a two-dimensional ^{1}H nuclear magnetic resonance (NMR) investigation on purified recombinant wild-type Il-1β and on a variety of mutant proteins which were constructed using site-directed mutagenesis on the cloned gene.

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Abbreviations. Il-1, interleukin-1; Il-1α, interleukin-1α; Il-1β, interleukin-1β; COSY, homonuclear two-dimensional correlated spectroscopy; HOHAHA, homonuclear two-dimensional Hartmann-Hahn spectroscopy; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

MATERIALS AND METHODS

Mutagenesis of Il-1β

Il-1β mutants were constructed by oligonucleotide-directed mutagenesis using the two-primer approach [5, 6]. The template was single-stranded M13mp108, a derivative of M13mp8 which posseses an additional *Nco*I site next to the multiple cloning site, containing the coding region for mature Il-1β. As mutagenic primers we used 15–20-bases-long oligonucleotides containing either single mismatches (for all Tyr mutants), or multiple mismatches (two for the His mutant and three for the Trp/Tyr double mutant) approximately in the middle of each oligonucleotide.

Phage harbouring the mutations were initially identified by plaque hybridization using the ^{32}P-labelled mutagenic oligonucleotide as a probe [7]. The frequency of mutation was between 0.3% and 3%. After plaque purification, the single strand of a mutant clone was sequenced by the dideoxy method [8], verifying the desired mutation as the sole one within the entire coding sequence. The mutated Il-1β gene was subsequently cloned into the expression vector derived from pPLc24 [9] in which Il-1β is expressed under control of the bacteriophage λ P_L promotor and the ribosome binding site of the bacteriophage Mu *ner* gene [10]. After transformation in *E. coli*, strains harbouring the mutant genes on the plasmid were used for protein production.

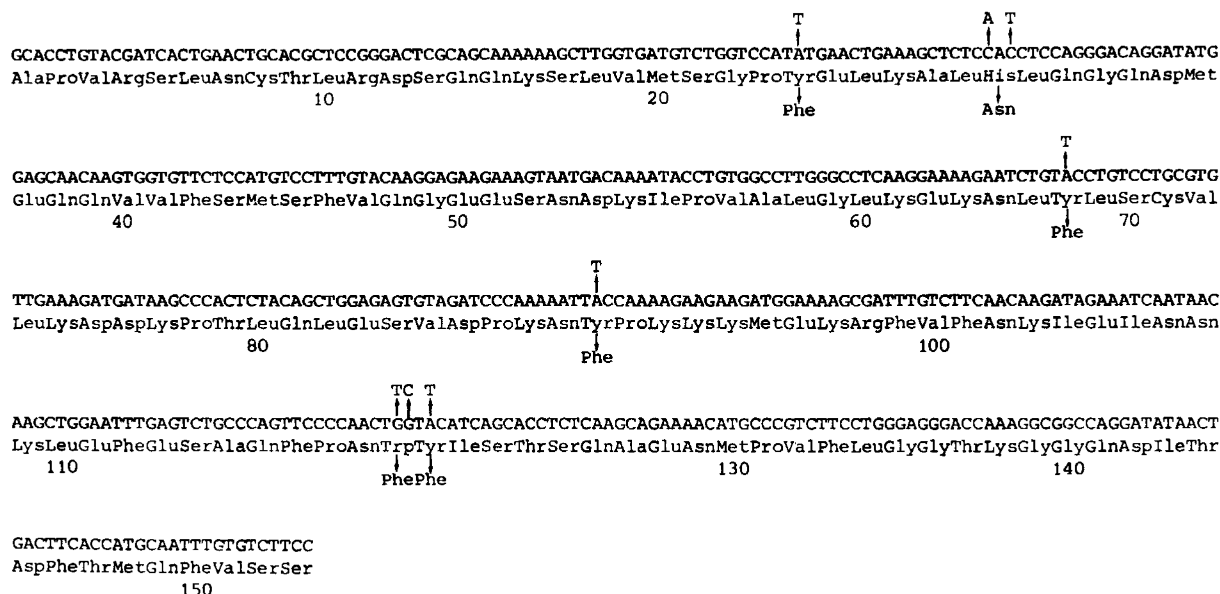

Fig. 1. *Nucleotide sequence coding for mature Il-1β and the corresponding amino acid sequence.* For all mutants studied, nucleotide changes are indicated by arrows while the resulting amino acid changes are indicated on the lower line

Purification of wild-type and mutant recombinant-derived Il1-1β

Mutant Il-1β proteins were purified as previously described for the wild-type protein [10]. The isolated proteins were shown to be pure by SDS/polyacrylamide gel electrophoresis and reversed-phase HPLC. Physical homogeneity was demonstrated by gel permeation chromatography on Ultrogel AcA54 and sedimentation equilibrium analysis. Results identical to those described for the wild-type protein were obtained [10].

The biological activity of the aromatic substituted mutants was tested by the lymphocyte activating factor assay and shown to be similar to that previously described for the wild-type recombinant-derived protein [10] (and J. M. Dayer, personal communication).

NMR spectroscopy

Samples for NMR spectroscopy contained 1.4 – 1.6 mM protein in D_2O/phosphate buffer (150 – 300 mM), sodium azide (3 – 6 mM), pH 7.5. All NMR spectra were recorded on a Bruker AM500 spectrometer equipped with an Aspect 3000 computer and digital phase shifters. Direct through-bond connectivities were demonstrated by means of absolute-value COSY spectra [11]. Direct and relayed through-bond connectivities were determined by pure phase absorption two-dimensional MLEV-17 Hartmann-Hahn (HOHAHA) spectroscopy [12, 13] recorded at several mixing times ranging over 20 – 60 ms. Appropriate phase cycling was used to eliminate axial peaks and the pure phase absorption spectra were recorded using the time-proportional incrementation method [14 – 16]. For all two-dimensional spectra 128 transients were collected for each of 512 increments with a relaxation delay of 1 s between successive transients. The sweep width employed was 7042 Hz. Apodization was carried out in both dimensions using an unshifted sine-bell squared for the COSY spectrum and a $\pi/6$ shifted one for the HOHAHA spectra. A square real 1K × 1K frequency domain spectrum was obtained by zero-filling in the t_1 dimension to yield a digital resolution of 6.88 Hz per point. After transformation all spectra were symmetrized [17].

RESULTS

Identification of aromatic spin systems

The nucleotide sequence of the native Il-1β gene and the corresponding amino acid sequence is shown in Fig. 1. Il-1β contains one histidine, one tryptophan, four tyrosine and nine phenylalanine residues. All 168 aromatic protons give rise to resonances in the region between 6.4 – 8.2 ppm. The $H^{\delta1}$ proton resonance of the tryptophan and both $H^{\varepsilon1}$ and $H^{\delta2}$ proton resonances of the histidine were identified in a one-dimensional spin-echo spectrum in which only resonances with long spin-spin relaxation times are observed [18]. In addition, a pH titration was carried out, which also allowed the identification of the histidine $H^{\varepsilon1}$ and $H^{\delta2}$ resonances. At 40 °C the single histidine titrates with a pK_a of 7.45, the chemical shift of the $H^{\varepsilon1}$ resonance moving from 8.56 ppm in the protonated form upfield to 7.80 ppm in the deprotonated form and exhibiting a classical Henderson-Hasselbalch titration curve. Similarly the $H^{\delta2}$ resonance titrates between 7.15 ppm and 6.95 ppm. The observed pK_a of 7.45 for His-30 is significantly higher than that found for free or solvent-accessible histidines. For example, the pK_a for *N*-acetyl-L-histidine is 6.54 [19] and that for the surface histidine of ribonuclease is 6.72 [20].

All coupled resonances in the aromatic region were identified using two-dimensional COSY and homonuclear Hartmann-Hahn (HOHAHA) spectra [12, 13]. A comparison between an absolute-value COSY spectrum and a pure-phase absorption HOHAHA spectrum for wild-type Il-1β at 25 °C is shown in Fig. 2. Whereas the COSY spectrum contains only information about through-bond connectivities between resonances belonging to protons separated by three bonds (i.e. magnetization transfer across one C-C bond), it is also possible to observe connectivities across two or more C-C bonds in the HOHAHA spectra. Moreover by increasing the mixing time in the HOHAHA experiment, additional single

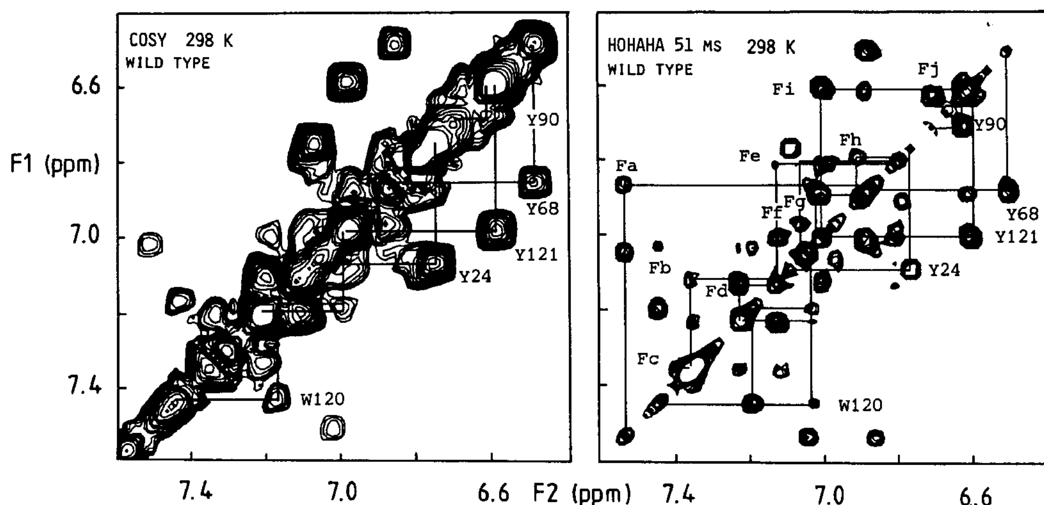

Fig. 2. *Aromatic region of the absolute-value COSY and pure phase absorption HOHAHA spectra of wild-type Il-1β at 25°C.* Amino acids are labelled by the one-letter code and the spin system connectivities are indicated

and multiple relayed cross peaks appear in succession which can then be used to identify complete coupled spin systems for each aromatic residue. This simplifies the assignments of spin systems considerably as can easily be assessed from Fig. 2. The complete spin systems for the four Tyr residues and the single Trp residue are labelled in the bottom half of the HOHAHA spectrum whereas the nine Phe spin systems are labelled Fa to Fj above the diagonal. The His cross peak between the $H^{\varepsilon 1}$ and $H^{\delta 2}$ is very weak and only detectable at short mixing times, and therefore not included in the spectrum shown in Fig. 1. Each of the four tyrosine residues gives rise to resonances characteristic of an aromatic ring flipping rapidly about the C^{β}-C^{γ} bond such that the $H^{\delta 1}$ and $H^{\delta 2}$ resonances and the $H^{\varepsilon 1}$ and $H^{\varepsilon 2}$ resonances are superimposed. Similarly the Phe resonances exhibit a degenerate spin pattern due to rapid ring flips. For the Phe resonances no assignments were made to either H^{δ}, H^{ε}or H^{ζ} resonances within each set, except for those which clearly displayed three resonances. In such cases the H^{ε} resonances are easily identifiable (see Table 1). The distinction between the H^{δ} and H^{ε} resonances for the tyrosine spin system was based on a 2D-NOESY spectrum which showed NOE cross peaks between H^{β} proton resonances and the H^{δ} ring proton resonances. All chemical shift values for the aromatic resonances of wild-type Il-1β are summarized in Table 1.

Identification of aliphatic spin systems

As far as NMR studies are concerned, human recombinant Il-1β is a fairly large protein which is composed of 153 amino acids. It therefore represents a formidable task to identify all aliphatic spin system; indeed, a complete identification might not even be possible. Fig. 3 illustrates the aliphatic region of a HOHAHA spectrum. In an examplary fashion several easily identifiable spin systems have been indicated; for example, five out of the total of fifteen leucines, five out of the total of eleven valines, as well as two alanine and two threonine spin systems. The chemical shift values for these aliphatic spin systems are also listed in Table 1. No attempt has been made as yet to achieve completeness. As a first step, however, towards solving the three-dimensional structure of Il-1β in solution, we are currently involved in the construction of truncated versions of Il-1β at the nucleotide level. For example, the N-terminal half of IL-1β, comprising 76 amino acids, has been expressed in *E. coli* and, if the truncated protein is folded similarly to its respective region in the intact protein, this will allow complete assignments to be made.

Sequence-specific assignment of Tyr resonances

Comparison of homologous proteins for assignment purposes has been widely used and such experiments have been carried out since the early seventies [21, 22]. The applicability of this approach depends on two prerequisites: firstly, the desired mutants (preferably point mutations) have to be available; secondly, the spectra of both wild-type and mutant protein have to be either identical or very similar except for the missing resonances. Previously, suitable proteins were therefore natural variants isolated from different species or individuals, such as cytochrome *c*, myoglobin and hemoglobin. The limitation of having to rely on the availability of naturally occurring variants has been overcome due to the progress of modern molecular biology. In principle it is now possible to produce any protein and mutants thereof by cloning of the gene and carrying out site-directed mutagenesis on the cloned gene [23]. This is the approach we have chosen for the sequence-specific assignment of the tyrosine residues in human Il-1β.

Using oligonucleotide-directed mutagenesis, each of the four triplets coding for tyrosine (TAT, TAC) was substituted in turn for triplets coding for phenylalanine (TTT, TTC) in the mutagenic oligonucleotide; thus only one base actually needed to be changed per triplet. The substitution of Tyr-121 was not carried out as a point mutation but as a double mutant in which the neighbouring Trp-120 was changed to a phenylalanine at the same time. In addition, we also investigated a point mutation comprising His-30, the only histidine in the sequence, which was changed to asparagine. All nucleotide changes, as well as the resulting amino acid changes, are indicated in Fig. 1. The sequence of the mutated genes were verified by nucleotide sequencing and the proteins were produced in *E. coli* using the expression vector described in Materials and Methods. Wild-type and mutant proteins were purified using the same protocol (see Materials and

Table 1. *Proton resonance assignments at 25°C*

Chemical shifts are expressed relative to 4,4-dimethylsilapentane-1-sulphonate. In the case of both the tyrosine and phenylalanine aromatic rings, rapid flipping occurs as only a single averaged resonance can be detected for the δ1 and δ2 protons and for the ε1 and ε2 protons. Where three resonances are indicated for the aromatic phenylalanine protons, the middle resonance represents the ε proton. For histidine, values at pH 7.42 are given

Aromatic amino acid	Protons	Chemical shift	Aliphatic amino acids	Protons	Chemical shift
		ppm			ppm
Tyrosine	δ, ε		Alanine	α, β	
Tyr-24		7.11, 6.78	Ala-a		6.20, 1.31
Tyr-68		6.90, 6.52	Ala-b		5.47, 1.51
Tyr-90		6.64, 6.72			
Tyr-121		7.03, 6.62	Threonine	α, β, γ	
			Thr-a		4.59, 3.93, 1.18
Tryptophan	δ1, ε3, ζ3, η, ζ2		Thr-b		4.41, 4.00, 0.98
Trp-120		7.31, 7.45, 7.21, 7.04, 7.21			
			Valine	α, β, γ1, γ2	
Phenylalanine	(δ, ε, ζ)		Val-a		4.37, 1.83, 0.95, 0.90
Phe-a		7.55, 7.06, 6.88	Val-b		4.33, 1.67, 0.79, 0.79
Phe-b		7.37, 7.23, 7.14	Val-c		4.17, 1.89, 0.85, 0.77
Phe-c		7.41, 7.36	Val-d		3.80, 1.56, 0.65, 0.18
Phe-d		7.25, 7.14	Val-e		4.24, 2.00, 0.72, 0.54
Phe-e		7.15, 7.01, 6.28			
Phe-f		7.08, 6.97	Leucine	γ, δ1, δ2	
Phe-g		7.06, 6.88	Leu-a		1.03, 0.11, −0.59
Phe-h		6.93, 6.81	Leu-b		1.17, 0.58, −0.58
Phe-i		6.90, 7.03, 6.63	Leu-c		1.23, 0.62, 0.41
Phe-j		6.65, 6.62	Leu-d		1.38, 0.59, 0.40
			Leu-e		2.01, 0.70, 0.54
Histidine	ε1, δ2				
His-30		8.19, 7.12			

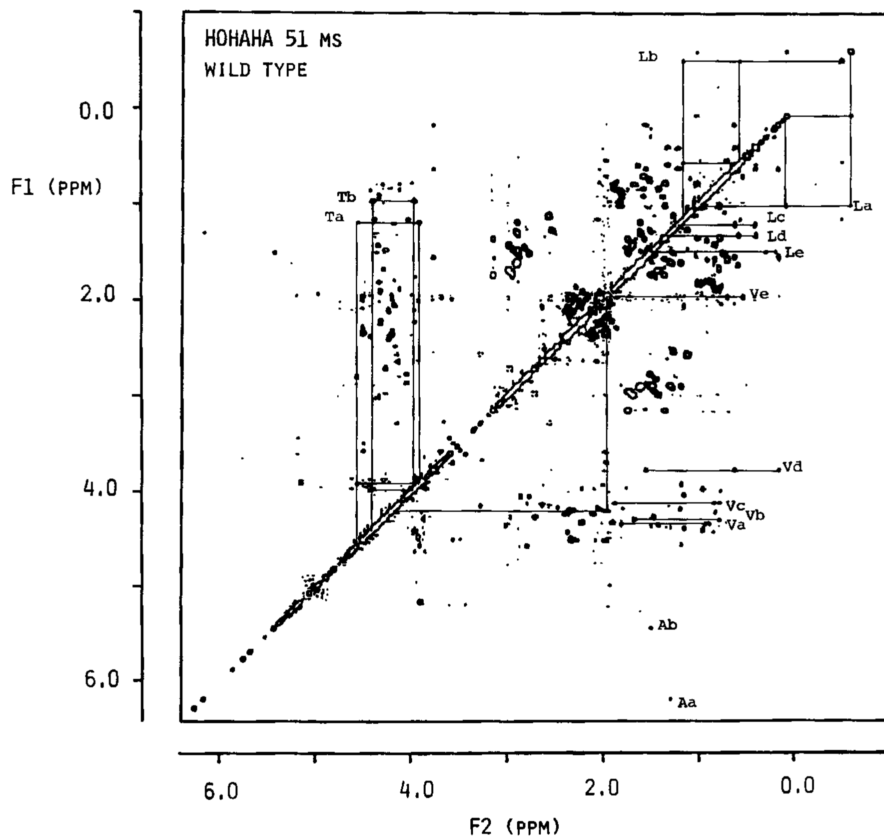

Fig. 3. *Aliphatic region of the pure phase absorption HOHAHA spectrum of wild-type Il-1β at 25°C.* Amino acids are labelled by the one-letter code and some Ala, Val, Thr and Leu spin systems are indicated

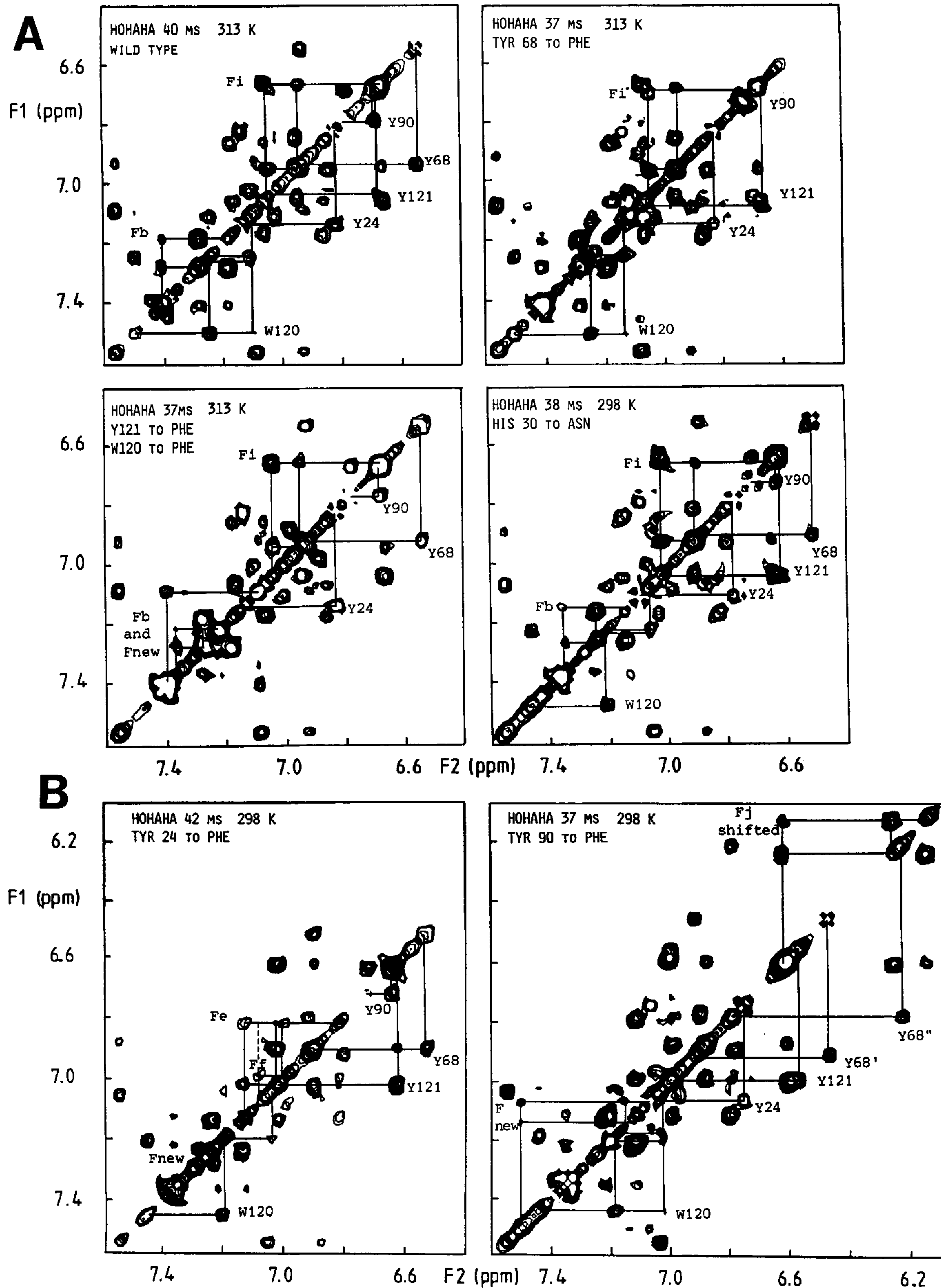

Fig. 4. *Aromatic region of the pure phase absorption HOHAHA spectra of (A) wild-type Il-1β (40°C), the Tyr-68→Phe (40°C), Trp-120/Tyr-121→Phe/Phe (40°C), and His-30→Asn (25°C) mutants, and (B) the Tyr-24→Phe (25°C) and Tyr-90→Phe (25°C) mutants*

Methods) and all mutant proteins behaved similarily to the wild-type protein during purification, indicating that the mutations did not result in extensive structural alterations. The amino acid composition of the purified mutant proteins was consistent with the intended changes (data not shown).

Fig. 4 shows the comparison of the aromatic regions of wild-type Il-1β and the five mutant proteins. The aromatic spectra of the mutant proteins can be categorized into three classes: (a) those identical to the wild-type apart from the missing resonances; (b) those similar to the wild-type but

exhibiting small shifts which, however, can be easily identified; (c) those that show obvious changes compared to the wild-type spectrum. In contrast to the aromatic region, no significant differences between the wild-type and any of the mutant proteins could be observed in the aliphatic region of the HOHAHA spectra (cf. Fig. 3) which are virtually superimposable.

Spectra belonging to category (a) arise from mutants His-30 to Asn and Tyr-24 to Phe. In these two cases the two-dimensional HOHAHA spectra are superimposable onto that of the wild-type. The only difference in the His-30→Asn mutant spectrum compared to wild-type is the disappearance of the His resonances. Similarily for the Tyr-24→Phe mutant, the cross-peak belonging to the Tyr at 7.11 ppm/6.78 ppm is no longer there and there is a set of resonances arising from the newly substituted Phe (7.24 ppm/7.28 ppm). The fact that the aromatic region of the HOHAHA spectra of these mutants are almost superimposable also extends to the aliphatic region (not shown).

The Tyr-68→Phe mutant as well as the Trp-120/Tyr-121 double mutant exhibit spectra very similar to the wild-type protein. For the spectrum of the Tyr-68 mutant, a small shift of the cross-peak belonging to the Phe resonances Fi is observed as well as a small upfield shift of the H^δ resonances of Tyr-90, such that the cross-peak Y90 is now positioned very close to the diagonal. In the spectrum of the double mutant one can observe that cross-peaks marked W120 are clearly missing; also missing is the cross-peak belonging to Y121, but since this cross-peak overlaps with the cross-peak belonging to Fi, there is still a cross-peak left in this position. The disappearance of the tyrosine cross-peak could nevertheless be ascertained from the comparison of the appropriate HOHAHA spectra at a higher temperature (40°C), at which the Y121 and Fi cross peaks are slightly shifted so that they are visible as separate peaks.

The mutant whose spectrum exhibits the most changes is the Tyr-90→Phe one. Here one observes marked upfield shifts of some aromatic resonances leading to completely new cross-peaks in the region 6.6 – 6.2 ppm. The lower field end of the aromatic region, however, remains identical to the wild-type spectrum, with an additional set of new resonances exhibiting the typical spin pattern for a phenylalanine. Inspection of the aliphatic region in the spectrum of the Tyr-90→Phe mutant does not reveal any substantial changes (data not shown), which in context with the localised changes in the aromatic region indicates that the overall folding of the mutant protein with respect to the wild type remains intact.

DISCUSSION

Identification of all the aromatic spin systems of Il-1β has been carried out as described above, using in particular two-dimensional Hartmann-Hahn spectroscopy. Although in principle this might have been possible by COSY spectroscopy, relying only on direct through-bond connectivities can lead to ambiguities. As can easily be observed from the comparison of the COSY and HOHAHA spectra shown in Fig. 2, there are several advantages in using the spin-lock Hartmann-Hahn experiment [12, 13]. (a) Since net magnetization transfer takes place, all peaks in the phase-sensitive spectrum have the same phase, being purely absorptive. This is in contrast to a phase-sensitive relayed COSY spectrum in which individual components of a cross-peak multiplet are 180° out-of-phase relative to one another. (b) The HOHAHA experiment is more sensitive than a normal COSY experiment since no cancellation of cross-peak intensity due to their anti-phase character occurs. This manifests itself in narrower and sharper cross peaks, as will be appreciated from Fig. 2. The excellent resolution of cross-peaks in the aromatic region of the spectrum of Il-1β is of particular importance close to the diagonal. Since there is extensive overlap, mainly due to the large number of phenylalanine residues whose resonances are very close to each other, only a method that allows good resolution makes it possible to identify all individual spin systems.

Sequence-specific assignment of the tyrosine spin systems was achieved using point mutants in which the tyrosines were replaced by phenylalanines. The simplest case was Tyr-24 since the spectrum of the mutant is essentially identical to the wild-type spectrum apart from the missing tyrosine signals and the additional phenylalanine signals. Tyr-68 was assigned in a similar fashion but in this case another tyrosine cross-peak appeared to be missing as well. Closer inspection of the spectrum revealed this cross-peak positioned very close to the diagonal, since one set of resonances was shifted upfield so that both the H^ε and H^δ resonances are very close to each other. The double mutant Trp-120/Tyr-121 clearly shows the tryptophan spin system missing as well as one tyrosine one, thereby identifying Tyr-121. Having three of the four tyrosine spin systems assigned allows the identification of the fourth by exclusion.

Confirmation of the assignment of Tyr-90 comes from the spectrum of the Tyr-90 mutant. Here the cross-peak assigned to Tyr-90 is indeed missing but, in addition, quite substantial changes in the high-field part of the aromatic region occur. The resonances arising from Tyr-68 are split into two sets of half-intensity compared to the wild-type spectrum with one set being shifted to higher field. Furthermore, one phenylalanine spin system exhibits a substantial upfield shift. This implies that a noticeable rearrangement of the local protein structure comprising Tyr-68, Tyr-90 and a phenylalanine has taken place. It can be concluded from the fact that only minor changes are observed in the rest of the aromatic region and the aliphatic part of the HOHAHA spectrum that the overall folding of the protein has remained intact. In other words, the observed changes are not due to a denaturation or unfolding of the entire protein. Further support for the notion of a local structural alteration in the Tyr-90 mutant can be drawn from the fact that no change in biological activity relative to the wild-type protein is observed for this mutant (J. M. Dayer, personal communication). The splitting of the H^δ and H^ε into single proton resonances for Tyr-68 may be the result of two different situations: (a) the fast flipping of the aromatic ring about the C^β-C^γ bond no longer takes place so that the H^ε and H^δ protons are no longer equivalent; or (b) Tyr-68 takes up two different conformations, each of which exhibits fast aromatic ring flipping. A distinction between these possibilities would only be possible if the two conformational states had quite different populations; a 1:1 situation can never be distinguished from a slow flipping tyrosine ring. Since in the above case the only well-resolved resonance of the two sets, mainly the high-field resonance of Tyr-68, has the intensity of one proton, we are either dealing with a slow flipping tyrosine ring or two equally populated conformations. Increasing the temperature up to 50°C did not lead to coalescence of these resonances and we did not observe any changes in intensity. (To explore a wider temperature range was not possible since the mutant protein denatured at 50°C and precipitated.) Since slowly flipping

aromatic rings have been observed repeatedly in proteins [24, 25], we tend to favour the interpretation of our data as arising from an immobilized tyrosine ring. As to the identity of the upfield-shifted phenylalanine resonances, one can only speculate. It is not unlikely that this particular phenylalanine may be involved in the relative immobilization of Tyr-68, thereby experiencing an upfield ring current shift from the aromatic tyrosine ring. Further experiments, however, are necessary to examine this point.

A further point of interest is the high pK_a of His-30. It has been observed that the formation of salt bridges (i.e. an electrostatic interaction between the positively charged imidazole moiety of histidine and carboxylate anions or phosphate anions on the protein surface) and hydrogen bonding may raise the pK_a values of imidazoles by about one unit. It therefore seems likely that the protonated His-30 in Il-1β is involved in a salt bridge or hydrogen bond which stabilizes that part of the protein structure in its immediate vicinity. This interpretation is strengthened by the finding that the stability of the His-30 mutant, as measured by urea gradient gels [26], is identical to that of the wild type at pH 9 but significantly reduced at pH 4 (P. W., unpublished data). This lower stability is reflected in an apparent 10-fold reduction in the specific activity of the mutant protein relative to that of the wild type (J. M. Dayer, personal communication).

All other mutants have the same biological activity, measured by the lymphocyte activating factor assay [27], as the native protein (J. M. Dayer, personal communication). This result supports the NMR findings that either no or only minor structural changes are associated with the mutations studied here. Furthermore, as the Tyr-90 mutant, which shows the most marked NMR changes, is also fully active, we conclude that the region of altered local structure in this mutant is most likely not located in the active centre of the protein.

As we have demonstrated in the present study, site-directed mutants may be used for sequence-specific assignment of amino acid residues in cases where the size of the protein prohibits a complete sequential assignment using two-dimensional through-bond and through-space connectivities [28, 29]. Thus, in principle, complete assignments can be obtained, albeit involving an extensive set of mutant proteins. In practice, however, this might not be a sensible way to proceed. Nevertheless, using mutant proteins may be a way to complement sequence-specific assignments in larger proteins.

It should be pointed out that NMR spectroscopy may have at least two important uses with respect to genetically engineered proteins. Firstly, the structural identity of a natural and a recombinant protein can be rapidly and easily ascertained from a comparison of the two-dimensional COSY and NOESY spectra since these can be regarded as a reflection of the three-dimensional protein structure in solution. Secondly, since the above also applies to site-directed mutant proteins, NMR can also help to establish whether the mutant protein structure is essentially unchanged. In this way 'protein engineers' may be able to correlate activity changes to the site of mutation without the danger that structural changes elsewhere in the protein are responsible for the observed effects. The only other method which is able to provide this information is X-ray crystallography and, except in selected cases, crystallizing and solving the structure of all site-directed mutants currently produced in the various systems is far too slow a process to be applicable to such a large set of proteins.

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