Identification of the Major Positional Isomer of Pegylated Interferon Alpha-2b

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ABSTRACT: Interferons display a wide range of antiviral, antiproliferative, and immunomodulatory activities on a variety of cell types and have been used to treat many diseases including hairy-cell leukemia and hepatitis B and C and have also been applied to other therapeutic areas. To improve the pharmacological properties of interferon (IFN) alpha-2b, a long-acting pegylated form (PEG-IFN) has been developed [PEG, monomethoxy poly(ethylene glycol) with average molecular mass of 12 000 Da]. PEG-IFN is a mixture of pegylated proteins with differing sites of PEG attachment. To identify the major positional isomer in the pegylated material [PEG-IFN(His-34)], NMR studies were conducted on a subtilisin-digested *N*-acetylated peptide of the major positional isomer [PEG-IFN(His-34)dig], synthetic peptide analogues containing His-34, as well as unmodified IFN and PEG-IFN(His-34). Our studies reveal a novel interferon−polymer attachment site as a histidine-linked interferon conjugate. We show that the major component of PEG-IFN is pegylated in the imidazole side chain of histidine-34. Chemical shift data suggest that pegylation occurs mainly at the $N^{\delta 1}$ position in the imidazole side chain of this residue. This positional isomer, PEG-IFN(His-34), comprises approximately 47% of the total pegylated species when PEG-IFN is synthesized under the current experimental conditions at pH 6.5 with an electrophilic derivative of PEG, succinimidyl carbonate PEG. The reversibility of the histidine modification was examined. The PEG−imidazole adduct in the intact protein, PEG-IFN(His-34), is labile but much more stable than in the peptide, PEG-IFN(His-34)dig. Apparently, the tertiary structure of the intact protein protects the His^{34}-imidazole ring from depegylation.

Interferons are naturally occurring cytokines that have a broad spectrum of antiviral, antitumor, and immunomodulatory properties (*1*−*3*). Recombinant alpha interferons (IFN-α)[1] were first approved for the treatment of hairy-cell leukemia by the United States Food and Drug Administration over a decade ago (*4*). Since then its indications have been expanded to cover a diverse range of therapeutical areas, including hepatitis B and C, dermatology, and oncology. The recommended treatment for chronic hepatitis C virus (HCV) is IFN-α three times per week (*5*). IFN-α is administered daily for most oncology indications. It has been speculated that a slow-releasing, long-acting formulation of interferon with improved pharmacokinetics may provide improved tolerability and convenience and may demonstrate activity in patients with known IFN resistance. Therefore, a long-acting pegylated form of the drug (PEG-IFN) was developed.

The PEG-IFN used in this study is a covalent conjugate of IFN with monomethoxy poly(ethylene glycol) (PEG, average molecular mass of 12 000 Da). PEG-IFN is synthesized by the reaction of IFN with an electrophilic derivative of PEG, succinimidyl carbonate PEG (SC-PEG), in 100 mM sodium phosphate (pH 6.5). During the production of PEG-IFN, in principle, pegylation can occur at any of the numerous nucleophilic sites of the 165-amino acid protein including the ϵ-amino groups of the 10 lysines, the α-amino group at the N-terminal cysteine, the imidazolyl nitrogens of the 3 histidines, and the hydroxyl groups at the 14 serines, 10 threonines, and 5 tyrosines. Various factors, including solvent accessibility, protein conformation, and local electronic and pK_a effects determine the relative reactivity of the potential pegylation sites. The resulting product is a heterogeneous population of molecules, consisting mainly of monopegylated IFN with smaller amounts of multipegylated (predominately dipegylated) and nonpegylated IFN (*6*). In addition, monopegylated PEG-IFN itself is composed of a number of species each having a different site of PEG attachment. Such positional isomers of interferon alpha-2a conjugated to single lysine residues were recently described (*7*). In that study, IFN was chemically conjugated via a urea linkage (*8*).

The process described in our research produces a novel interferon−polymer attachment profile, a histidine-linked interferon conjugate. This α-interferon−polymer conjugate demonstrates increasing biological activity as a function of time under in vivo conditions, which may be related to a labile histidine modification (*9*). We used various NMR techniques to identify and characterize the major positional isomer of PEG-IFN [PEG-IFN(His-34)], which comprises approximately 47% of the pegylated species (*6*) and has a

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[1] Abbreviations: HCV, hepatitis C virus; HPSEC, high performance size exclusion chromatography; IFN, recombinant human interferon alpha-2b; IFN-α, recombinant alpha interferon; MALDI-MS, matrix-assisted laser desorption mass spectrometry; PEG, monomethoxypoly-(ethylene glycol) with average molecular mass of 12 000 Da; PEG-IFN, covalent conjugates of IFN with PEG; PEG-IFN(His-34), major positional isomer of PEG-IFN; PEG-IFN(His-34)dig, subtilisin-digested *N*-acetylated peptide of PEG-IFN(His-34); SC-PEG, succinimidyl carbonate PEG.

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relatively high bioactivity and moderate stability. A subtilisin-digested *N*-acetylated fragment of the major positional isomer of PEG-IFN [PEG-IFN(His-34)dig], its synthetic analogues, and intact IFN and PEG-IFN(His-34) were utilized to (i) identify the pegylation site of this major positional isomer of PEG-IFN and (ii) study its stability under in vitro conditions.

EXPERIMENTAL PROCEDURES

Isolation of PEG-IFN(His-34). PEG-IFN was synthesized by the reaction of purified interferon alpha-2b with SC-PEG, an electrophilic derivative of PEG, in 100 mM sodium phosphate at pH 6.5. Following the reaction, the material was diafiltered and separated by ion exchange chromatography. The major positional isomer (approximately 47% of the total pegylated species of PEG-IFN) was isolated by preparative cation exchange chromatography using a TosoHaas strong cation exchange column (SP-5PW, 21.5 mm × 15 cm) (TosoHaas, Montgomeryville, PA). Mobile phases A and B were composed of 10 and 80 mM sodium phosphate, both at pH 5.8. The column was equilibrated in mobile phase A, and the major positional isomer was eluted with a linear gradient of increasing percentages of mobile phase B, using UV detection at 214 nm. The isolated fraction was concentrated using Centricon 10 microconcentrators (Amicon, Beverly, MA) having a molecular mass cutoff of 10 kDa.

Subtilisin Digestion of PEG-IFN(His-34) to Produce PEG-IFN(His-34)dig. A solution of 178 μL containing 640 μg of protein isolated as described above and 128 μg of subtilisin in 50 mM MES buffer (pH 5.5) was incubated at 30 °C for 45 min, 2 μL of acetic anhydride was added, and the solution was incubated for another 15 min. The reaction mixture was immediately fractionated by HPSEC (high performance size exclusion chromatography) on a Superdex 75 column (Pharmacia, Uppsala Sweden) using 10 mM sodium phosphate (pH 6.0) as the mobile phase at a flow rate of 0.7 mL/min. Detection was by UV at 214 nm. The high molecular weight fraction (containing pegylated peptide fragments) was collected and concentrated using Centricon 10 microconcentrators. MALDI-MS analysis (Voyager DE-Linear Biospectrometry Workstation, PerSeptive Biosystems) of the solution showed a broad peak with a center of mass around 13 500 Da, corresponding to a pegylated peptide. When treated with hydroxylamine, the mass spectra showed a single peptide with mass 1161.53 Da that corresponds to the theoretical average mass (1161.17) for the $[M + H]^{+1}$ ion of the acetylated segment of IFN alpha-2b, Asp^{32}−Arg−His−Asp−Phe−Gly−Phe−Pro−Gln^{40}, along with reduction in the peak at 13 500 Da. The digestion and purification procedure described above was repeated twice to obtain sufficient material for NMR analysis. The final isolates of the three runs were pooled and exchanged into 20 mM sodium acetate-d_3 in D_2O (pH 5.5) for NMR experiments.

Synthetic Peptides. The nonpegylated peptides, Ac-HDFGFPQ and Ac-DRHDFGFPQ, were chemically synthesized using a solid-phase peptide synthesizer. Their monopegylated analogues, Ac-H(PEG)DFGFPQ and Ac-DRH(PEG)DFGFPQ, were prepared by reacting the peptides with SC-PEG in 100 mM sodium phosphate buffer (pH 6.5) to pegylate the lone nucleophilic histidine residue in the peptides. Pegylated peptides were purified by HPSEC as described for the subtilisin digest purification, concentrated, and exchanged into 20 mM sodium acetate-d_3 in D_2O (pH 5.5) buffer using Centricon 10 microconcentrators.

Intact Protein Solutions. A portion of the solution of PEG-IFN(His-34), isolated as described above by preparative cation exchange chromatography, was concentrated and exchanged into D_2O using Centricon 10 microconcentrators. The final solution, 1 mM protein concentration, was acidified to pH 3.5 with deuterium chloride. IFN drug substance was similarly exchanged, concentrated to 1 mM, and acidified to pH 3.5.

NMR Measurements. All NMR experiments were acquired on a Varian *UnityPlus* 600-MHz NMR spectrometer. Proton NMR spectra of PEG-IFN(His-34)dig and its synthetic peptide analogues were collected at 4 °C. NMR studies on intact IFN protein samples included 1D proton NMR, 2D homonuclear TOCSYs (*10*, *11*) at various mixing times, and 2D heteronuclear gradient ^1H-^{13}C HSQC (*12*) NMR methods. NMR data for the proteins were acquired at 25 °C.

RESULTS

The Major Positional Isomer of PEG-IFN is Pegylated at His-34. Figure 1 shows the aromatic regions of the proton NMR spectra of four model peptides Ac-HDFGFPQ, Ac-H(PEG)DFGFPQ, Ac-DRHDFGFPQ, and Ac-DRH(PEG)-DFGFPQ, and PEG-IFN(His-34)dig obtained from the major positional isomer of PEG-IFN. It was known from earlier data that digestion of a major fraction of PEG-IFN (purified with cation exchange chromatography) with subtilisin resulted in the generation of two hydroxylamine-sensitive pegylated fragments corresponding to Asp^{32}−Gln^{40} and His^{34}−Gln^{40} (data not shown). The four model peptides represent pegylated and nonpegylated peptides with these two amino acid sequences. They were designed to probe the effect of pegylation at the histidine residue on the chemical shifts of the $H^{\epsilon 1}$ and $H^{\delta 2}$ protons of the imidazole ring to aid in the analysis of the subtilisin digest of the major positional isomer of PEG-IFN [PEG-IFN(His-34)dig]. By experimenting with different enzyme/substrate ratios, conditions were found that resulted in the production of only a single pegylated fragment. NMR studies had shown a 0.30 ppm upfield and 0.17 ppm downfield shift for the $H^{\epsilon 1}$ and $H^{\delta 2}$ imidazolyl protons, respectively, of tBoc-His as a result of imidazole pegylation (data not shown). Comparison of the proton spectra of peptide Ac-HDFGFPQ and Ac-H(PEG)DFGFPQ shows that two new peaks appear at 7.97 and 6.64 ppm due to pegylation, which correspond to the $H^{\epsilon 1}$ and $H^{\delta 2}$ protons of the pegylated histidine residue, respectively. Therefore, pegylation of Ac-HDFGFPQ causes similar shift changes to those observed for tBoc-His. From the peak intensities, it is easily estimated that about 70% of the Ac-H(PEG)DFGFPQ peptide is pegylated (Figure 1). Comparison of the NMR spectrum of Ac-DRH(PEG)-DFGFPQ with that of Ac-DRHDFGFPQ demonstrates that pegylation has only a small effect on the chemical shift of the His $H^{\delta 2}$ proton, but it causes the His $H^{\epsilon 1}$ proton to shift upfield by 0.34 ppm (from 8.32 to 7.98 ppm). On the basis of the intensity of the peaks, the ratio of pegylated to nonpegylated Ac-DRHDFGFPQ peptide is about 60 to 40%.

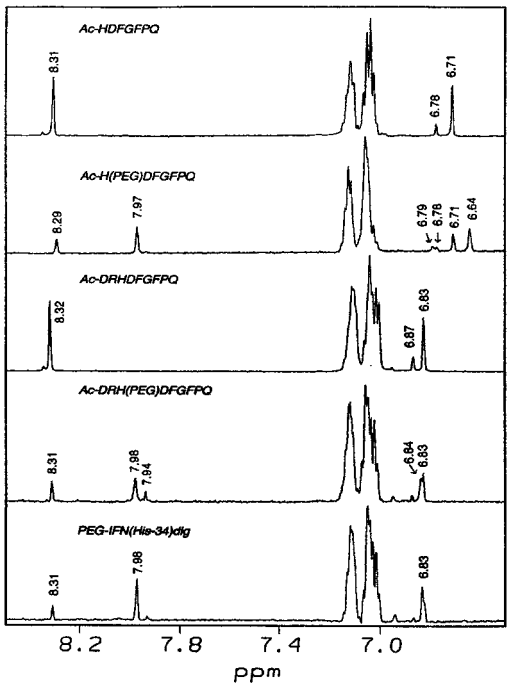


FIGURE 1: Aromatic region of the proton NMR spectra of (top to bottom) four synthetic peptides, Ac-HDFGFPQ, Ac-H(PEG)-DFGFPQ, Ac-DRHDFGFPQ, and Ac-DRH(PEG)DFGFPQ, and PEG-IFN(His-34)dig in D_2O at pH 5.5 and 4 °C.

The bottom spectrum of Figure 1 shows the aromatic region of the proton NMR spectrum of PEG-IFN(His-34)-dig. A detailed comparison of the spectra between the subtilisin digest sample and the model peptides reveals that the former is composed of mostly Ac-DRH(PEG)DFGFPQ and a small amount of depegylated Ac-DRHDFGFPQ with a ratio of about 80 to 20%. The absence of peaks in the region between 6.6 and 6.8 ppm indicates that the subtilisin digest sample contains neither Ac-HDFGFPQ nor the pegylated Ac-H(PEG)DFGFPQ. These data taken together indicate that the major positional isomer of PEG-IFN is pegylated at the imidazole ring of His-34. The assignments of imidazolyl protons of His-34 in the various peptides are summarized in Table 1.

The PEG−imidazole bond of Ac-DRH(PEG)DFGFPQ in the subtilisin digest sample is very labile as can be seen from Figure 2. The $H^{\epsilon 1}$ proton signal of pegylated (7.98 ppm) and nonpegylated (8.31 ppm) peptide significantly decreases and increases, respectively, within 49 h of the sample preparation. The rate constant of depegylation is 0.028 $hour^{-1}$ at 4 °C (Figure 3).

NMR Studies on Intact IFN and PEG-IFN(His-34). Dynamic light-scattering and NMR studies showed that the two proteins are monomeric below pH 4 (data not shown). The NMR resonance lines, however, became reversibly broader at higher pH. Figure 4 shows the aromatic region of a proton spectra of IFN (top) and the major positional isomer of PEG-IFN, PEG-IFN(His-34) (bottom), under conditions in which the proteins are monomeric. The sharp resonances of IFN at 8.79 and 8.62 ppm belong to the $H^{\epsilon 1}$ protons of the three histidines (see below). On the basis of their relative intensities, the peaks at 8.79 and 8.62 ppm originate from one and two His $H^{\epsilon 1}$ protons, respectively. In the spectrum of PEG-IFN, a new sharp peak at 8.28 ppm appeared, whereas the intensity of the peak at 8.62 ppm was reduced by half when compared to the spectrum of non-pegylated IFN. Therefore, the new peak at 8.28 ppm was assigned to the $H^{\epsilon 1}$ proton of pegylated His-34. This is supported by the following observation: because of depegylation over the course of a few weeks, the intensity of the signal at 8.28 ppm decreased gradually and a new signal at 8.65 ppm appeared with increasing intensity. This can be explained by depegylation of His-34 analogous to the depegylation seen for Ac-DRH(PEG)DFGFPQ peptide that was generated by subtilisin digestion and *N*-acetylation of PEG-IFN(His-34) (see above). In the peptide, pegylation caused the $H^{\epsilon 1}$ resonance to shift upfield by 0.33 ppm from 8.31 to 7.98 ppm. Here, a similar upfield shift is observed due to pegylation of His-34 $H^{\epsilon 1}$ (0.37 ppm). These data strongly suggest that the peaks at 8.28 and 8.65 ppm correspond to pegylated and depegylated His-34 $H^{\epsilon 1}$, respectively.

Table 1: Assignments of the Imidazolyl Protons of His-34 in the NMR Spectra of Figure 1

sample	peak position (ppm)	assignments
Ac-HDFGFPQ	8.31	$H^{\epsilon}1$ (nonpegylated)
	6.71 (6.78)[a]	$H^{\delta}2$ (nonpegylated)
Ac-H(PEG)DFGFPQ	8.29	$H^{\epsilon}1$ (nonpegylated)
	7.97	$H^{\epsilon}1$ (pegylated)
	6.71 (6.78)[a]	$H^{\delta}2$ (nonpegylated)
	6.64 (6.79)[a]	$H^{\delta}2$ (pegylated)
Ac-DRHDFGFPQ	8.32	$H^{\epsilon}1$ (nonpegylated)
	6.83 (6.87)[a]	$H^{\delta}2$ (nonpegylated)
Ac-DRH(PEG)DFGFPQ	8.31	$H^{\epsilon}1$ (nonpegylated)
	7.98 (7.94)[a]	$H^{\epsilon}1$ (pegylated)
	6.84	$H^{\delta}2$ (pegylated)
	6.83	$H^{\delta}2$ (nonpegylated)
PEG-IFN(His-34)dig	8.31	$H^{\epsilon}1$ (nonpegylated) Ac-DRHDFGFPQ
	7.98 (7.94)[a]	$H^{\epsilon}1$ (pegylated) Ac-DRH(PEG)DFGFPQ
	6.83 (6.88)[a]	$H^{\delta}2$ (non-, pegylated) of two species above

[a] These signals were also observed in the two nonacetylated nonpegylated model peptides and are likely due to the cis-isomer of the Phe−Pro peptide bond.

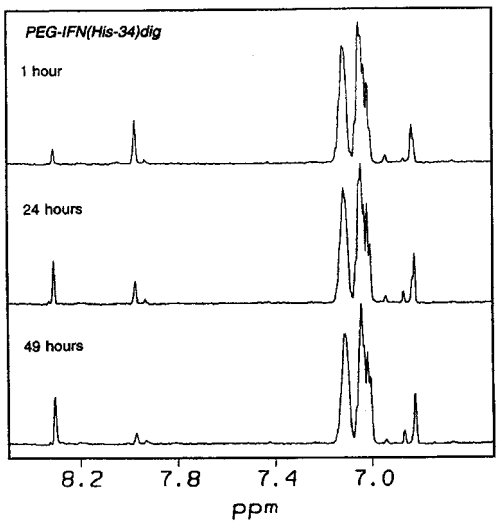


FIGURE 2: Time dependence of the imidazolyl proton NMR signals of Ac-DRH(PEG)DFGFPQ observed in the NMR sample of PEG-IFN(His-34)dig due to depegylation (pH 5.5, 4 °C).

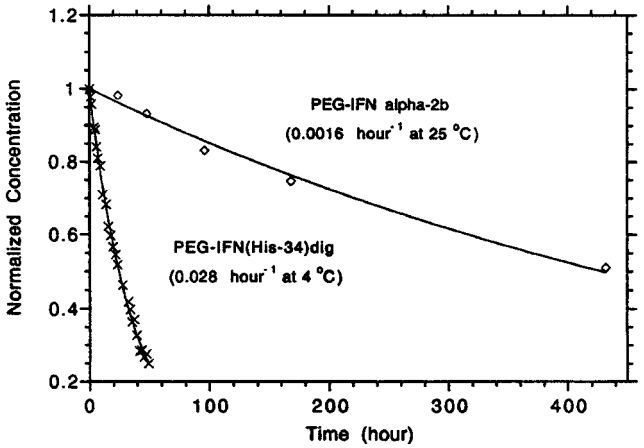


FIGURE 3: Decrease of the signal intensity of His-34 $H^{\epsilon 1}$ due to depegylation of (1) Ac-DRH(PEG)DFGFPQ in the NMR sample of PEG-IFN(His-34)dig (pH 5.5, 4 °C) and (2) intact PEG-IFN (pH 3.5, 25 °C) showing that the PEG-imidazole bond is significantly more stable in the intact protein even at room temperature and lower pH.

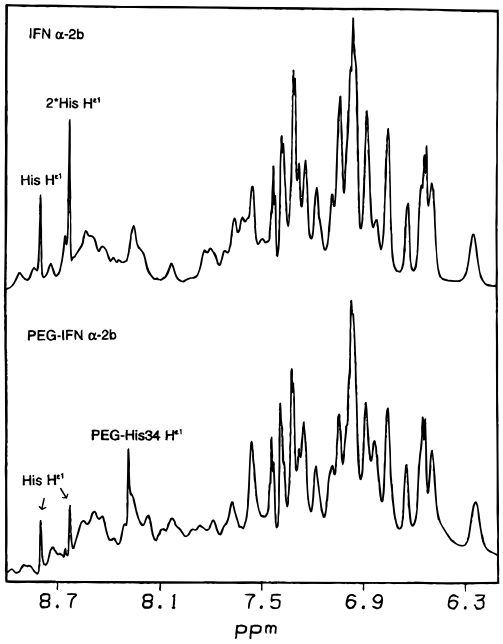


FIGURE 4: Aromatic region of 1D proton NMR spectra of IFN (top) and PEG-IFN(His-34) (bottom) in D_2O at pH 3.5 and 25 °C, with the $H^{\epsilon 1}$ signals of the three histidines indicated. Because of pegylation, the $H^{\epsilon 1}$ signal of His-34 is shifted upfield by 0.36 ppm in PEG-IFN.

Additional 2D homonuclear and heteronuclear NMR experiments were conducted to confirm assignments of the imidazolyl protons of His-34 in PEG-IFN(His-34). In a 2D TOCSY of IFN with a mixing time of 45 ms, two sharp cross-peaks were observed at (8.79/7.26 ppm) and (8.62/7.38 ppm) with a relative ratio of 1:2 (data not shown). Because of their characteristic chemical shifts, these cross-peaks were unambiguously recognized as ($H^{\epsilon 1}/H^{\delta 2}$) cross-peaks of histidine residues. In the 2D TOCSY of PEG-IFN(His-34), the same two sharp cross-peaks were observed corresponding to the two nonpegylated histidine residues (Figure 5). The cross-peak at (8.62/7.38 ppm), however, was only half the intensity when compared to the TOCSY spectrum of IFN. Because of pegylation of His-34, we expected that half of this cross-peak will shift to a characteristic new position. As a result of depegylation, an additional weak cross-peak was observed at (8.65/7.35 ppm), which was assigned to the depegylated portion of His-34 of PEG-IFN(His-34). The cross-peak for pegylated His-34, however, was not observed in the TOCSY experiments even at multiple mixing times. We speculate that this is likely due to a reduction of the small four-bond coupling constant caused by pegylation.

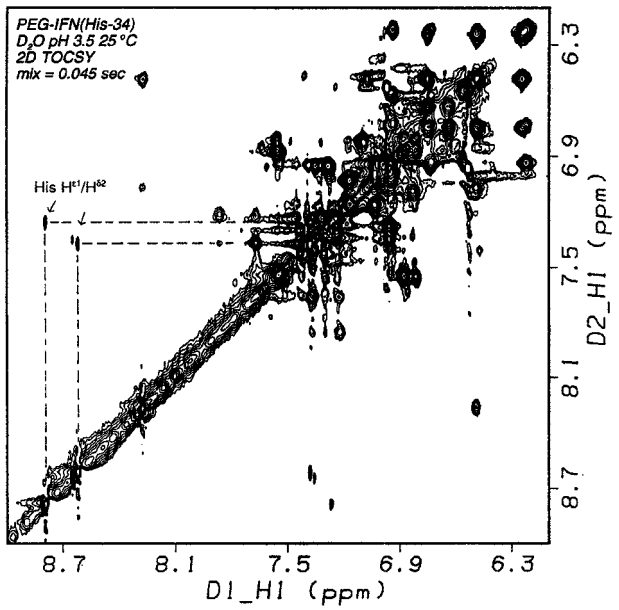


FIGURE 5: Aromatic region of a 2D TOCSY spectrum of PEG-IFN(His-34) in D_2O at pH 3.5 and 25 °C. Two ($H^{\epsilon 1}/H^{\delta 2}$) cross-peaks and an additional weak ($H^{\epsilon 1}/H^{\delta 2}$) cross-peak are observed for the two nonpegylated histidines and depegylated His-34, respectively. No ($H^{\epsilon 1}/H^{\delta 2}$) cross-peak could be detected for pegylated His-34, presumably due to a reduction of the small four-bond coupling by pegylation.

Since histidines have characteristic $^{13}C^{\epsilon 1}$ chemical shifts, a 2D heteronuclear gradient ^1H-^{13}C HSQC spectrum was used to further characterize pegylation of His-34 in PEG-IFN-(His-34) (Figure 6). Two strong $^1H/^{13}C$ cross-peaks were observed at (8.79/135.81 ppm) and (8.62/134.49 ppm), correlating the $H^{\epsilon 1}$ and $^{13}C^{\epsilon 1}$ resonances of the two nonpegylated histidines. Two additional weak cross-peaks were detected at (8.28/138.89 ppm) and (8.65/134.58 ppm), which are from the pegylated and depegylated portions of His-34, respectively, as judged from their ^1H chemical shifts. The chemical shift assignments for the histidines in IFN and PEG-IFN(His-34) are summarized in Table 2.

The stability of the PEG−imidazole conjugate of PEG-IFN(His-34) was measured by monitoring the signal intensity of the pegylated His-34 $H^{\epsilon 1}$ over time. Figure 3 shows that depegylation occurs much more slowly in intact PEG-IFN-(His-34) than in the subtilisin-digested *N*-acetylated peptide of PEG-IFN(His-34), Ac-DRH(PEG)DFGFPQ, even at higher temperature and lower pH. Apparently, the protein structure considerably protects the PEG−imidazole bond of His-34 from hydrolysis.

NMR studies on intact PEG-IFN(His-34), the major positional isomer of PEG-IFN, provide further evidence that the PEG group is bound to the imidazole ring of one histidine residue. Together with the results from the NMR analysis on the pegylated−interferon fragments (see previous section),

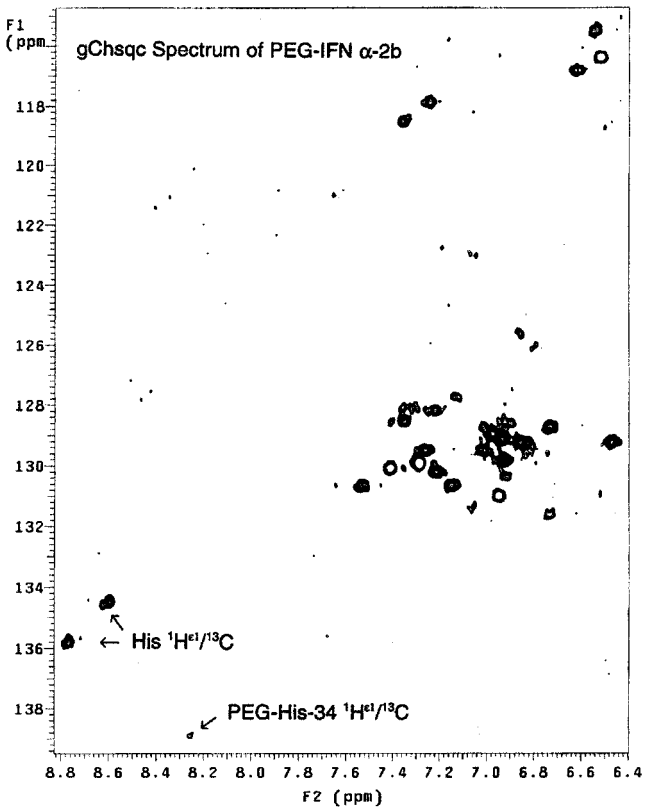


FIGURE 6: 2D heteronuclear gradient ^1H-^{13}C HSQC spectrum of PEG-IFN(His-34) in D_2O at pH 3.5 and 25 °C acquired 4 days after sample preparation.

Table 2: Chemical Shift Assignments[a] of Histidines in IFN and PEG-IFN(His-34)

assignments	$H^{\epsilon}1$	$H^{\delta}2$	$13C\epsilon1$
His #1	8.79	7.26	135.81
His #2	8.62	7.38	134.49
His-34 (depegylated)	8.65	7.35	134.58
His-34 (pegylated)	8.28		138.89

[a] Chemical shifts are in ppm. Proton chemical shifts are referenced to the residual H_2O signal at 4.80 ppm at 25 °C, and carbon chemical shifts are referenced to 10% dioxane in D_2O at 69.46 ppm (*18*).

it can be concluded that PEG-IFN(His-34) is pegylated at the imidazole ring of His-34.

DISCUSSION

The Extent of Pegylation at His-34 is pH Dependent. PEG-IFN was synthesized by the reaction of IFN with SC-PEG, an electrophilic derivative of PEG, in 100 mM sodium phosphate (pH 6.5). Characterization studies of PEG-IFN indicated that PEG-derivatized His-34 comprised ~47% of the pegylated species when produced at pH 6.5. The relative distribution of the positional isomers, however, varies dramatically with pH (*6*). The crystal structure of IFN alpha-2b was recently determined to a resolution of 2.9 Å (*13*). A high-resolution solution structure of IFN alpha-2a was also reported (*14*). The two structures are similar and reveal a four-helix bundle global fold. Figure 7 shows the location of the three histidines of IFN in the three-dimensional solution structure of IFN alpha-2a. His-57 is located in helix B, which forms a left-handed four-helix bundle with α-helices A, C, and E and, therefore, is shielded from the solvent. His-34 is in a long loop region of 30 amino acids connecting

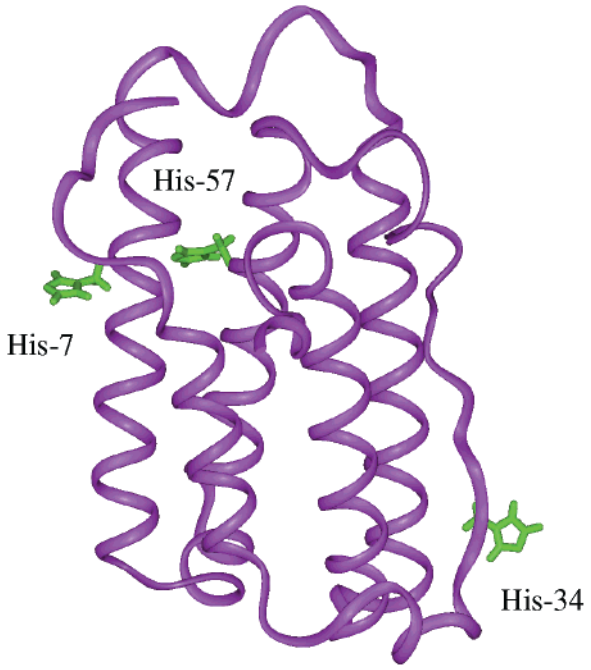
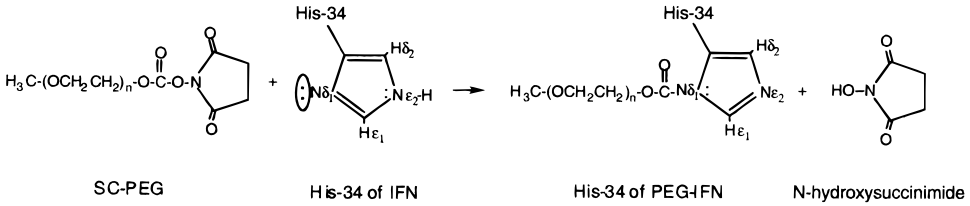


FIGURE 7: Ribbon diagram of the average solution structure (*14*) of IFN displaying the location of the three histidine residues.

helix A and helix B and is exposed to the solvent, which makes His-34 a readily available pegylation site. His-7 is located at the N-terminus before helix A and is also exposed to the solvent in the solution structure. We speculate, however, that His-7 is shielded from the solvent due to the formation of oligomers of IFN under the pegylation conditions normally used to produce PEG-IFN (pH 6.5). Dynamic light-scattering and NMR studies show that IFN forms oligomeric structures above pH 4. The formation of oligomers at pH 6.5 may reduce the solvent accessibility of His-7. Therefore, the high yield of PEG-IFN(His-34) is due to the solvent accessibility of His-34 in the interferon molecule at pH 6.5.

Chemical Shift Data Suggest that PEG-IFN(His-34) is Mainly Pegylated at $N^{\delta 1}$ in the Imidazole of Histidine-34. The side chain of histidine is an aromatic acid−base. The acid−base reaction between the imidazolium cation and the neutral imidazole makes this amino acid so important in many biological processes. The local surroundings of each histidine residue of IFN will determine its acid−base behavior and, therefore, both the extent of pegylation and the site of attachment of the PEG group in the imidazole ring. At the reaction pH of 6.5, the side chain of His-34 is expected to be in equilibrium between the imidazolium cation and two tautomers of the neutral imidazole. The neutral imidazole is an aromatic heterocycle containing one pyrrole nitrogen (N-H) and one pyridine nitrogen (N:), which together contribute three valence electrons to an aromatic ring formed from five *p* orbitals. Three additional π electrons are contributed from the three carbons in the five-membered ring. As a result, a total of six π electrons remain in an aromatic π molecular orbital system at all times but fluidly redistribute in response to changes in the coulomb effects as the nitrogens gain or lose protons at their σ lone pairs. In the neutral imidazole, the two nitrogens are necessarily nonequivalent because the pyrrole-like nitrogen (N-H) has a proton attached to it. The base is the σ lone pair on the

Scheme 1



pyridine-like nitrogen (N:), and it gains a proton in an acid−base reaction with a macroscopic pK_a of ∼6.4 when the base is histidine in a polypeptide (*15*). Moreover, the two ring nitrogens are not stereochemically equivalent to each other because of the substitution at the C4 carbon in the ring. On the basis of studies of model compounds, the ratio between the two neutral tautomers, the $N^{\delta 1}$-H tautomer and the $N^{\epsilon 2}$-H tautomer, is 1:4 in aqueous solution (*16*). The SC-PEG can react with either tautomer through the "pyridine-like" nitrogen (N:) to form different pegylated products. The following discussion suggests that PEG-IFN(His-34) is mainly pegylated at the $N^{\delta 1}$ ring nitrogen of His-34 (Scheme 1), which is consistent with the prevalence of the $N^{\epsilon 2}$-H tautomer over the $N^{\delta 1}$-H tautomer in aqueous solution.

The chemical shifts of the $H^{\epsilon 1}$ and $H^{\delta 2}$ ring protons are determined by the summation of the inductive effect of the electronegative nitrogens within the ring and the anisotropic effect of the aromatic ring current. In a nonpegylated peptide, such as Ac-HDFGFPQ and Ac-DRHDFGFPQ (Figure 1), both $H^{\epsilon 1}$ and $H^{\delta 2}$ resonate in the downfield region because of the aromatic ring current that is delocalized and, therefore, uniform for both protons. However, the inductive effects from the two electronegative nitrogens in the ring are different for $H^{\epsilon 1}$ and $H^{\delta 2}$. The inductive effect of an electronegative atom falls off rapidly as it passes through a number of bonds. $H^{\epsilon 1}$ is only two bonds away from both ring nitrogens, whereas $H^{\delta 2}$ is an additional bond away from $N^{\delta 1}$. Hence, $H^{\epsilon 1}$ resonates further downfield than $H^{\delta 2}$.

Pegylation will change the electronegativity of the nitrogen that is pegylated in the ring. Consequently, the inductive effect experienced by the $H^{\epsilon 1}$ and $H^{\delta 2}$ ring protons will change. The extent of the inductive effect on the $H^{\delta 2}$ will strongly depend on which of the two ring nitrogens is pegylated. If pegylated at $N^{\epsilon 2}$, the inductive effect from $N^{\epsilon 2}$ would be similar for both $H^{\epsilon 1}$ and $H^{\delta 2}$. In contrast, pegylation at $N^{\delta 1}$ is expected to cause a larger chemical shift change for $H^{\epsilon 1}$. Although pegylation may also affect the aromatic ring current, this effect is delocalized, and hence, the chemical shift change will be uniform for $H^{\epsilon 1}$ and $H^{\delta 2}$ regardless of which nitrogen in the ring is pegylated.

In the pegylated peptides [Ac-H(PEG)DFGFPQ, Ac-DRH-(PEG)DFGFPQ, and PEG-IFN(His-34)dig], the $H^{\epsilon 1}$ and $H^{\delta 2}$ protons shift both upfield when compared to the corresponding nonpegylated peptides (Table 1). The chemical shift changes are however nonuniform and much larger for $H^{\epsilon 1}$ than $H^{\delta 2}$. According to the discussion above, this suggests that the PEG group be mainly attached to the $N^{\delta 1}$ nitrogen in the model peptides as well as in PEG-IFN(His-34)dig. A similar upfield shift of $H^{\epsilon 1}$ was also observed for PEG-IFN-(His-34) upon pegylation, whereas the chemical shift of $H^{\delta 2}$ did not change in the intact protein (Table 2). As discussed in the previous section, the imidazole of His-34 is on the surface of IFN. Hence, its acid−base behavior is expected to be similar to that of the model peptides. Additional factors to those discussed above that could affect the chemical shifts of $H^{\epsilon 1}$ and $H^{\delta 2}$ appear negligible in PEG-IFN(His-34). It is therefore reasonable to conclude that the imidazole $N^{\delta 1}$ nitrogen of His-34 is pegylated in PEG-IFN(His-34) (Scheme 1). Attempts to support this conclusion by collecting ^1H-^{13}C HMBC spectra (*17*) on the pegylated peptide, Ac-DRH-(PEG)DFGFPQ (molecular mass ∼13.5 kDa), were not successful. In principle, with this experiment one could differentiate if pegylation occurred at the $N^{\delta 1}$ or the $N^{\epsilon 2}$ position by observation of the three-bond carbon−proton correlations between the carbonyl carbon of the PEG-substituent and the corresponding ring protons. Although other two-bond and three-bond carbon−proton correlations within the imidazole ring of the histidine were detected, no such correlation to the carbonyl carbon of the PEG-substituent was observed suggesting that the underlying coupling constant is too small.

CONCLUSIONS

We have presented the identification of the major positional isomer of PEG-IFN, which comprises approximately 47% of the pegylated products, using various NMR techniques. Detailed NMR studies of a subtilisin-digested *N*-acetylated peptide of this major positional isomer, synthetic peptide analogues, as well as intact protein samples of both IFN and this major positional isomer prove that the major component of PEG-IFN is pegylated in the imidazole side chain of histidine-34. Chemical shift data suggest that pegylation occurs mainly at the $N^{\delta 1}$ position in the imidazole side chain of this residue. The tertiary structure of the protein considerably protects the PEG-imidazole bond of His-34 from hydrolysis. The relatively slow hydrolysis of the His-PEG bond and subsequent release of free IFN may be related to the increase in activity observed for this positional isomer over time (*9*).

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