Carboxyalkylated Histidine Is a pH-Dependent Product of Pegylation with SC-PEG

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Purpose. Pegylation of therapeutic protein usually results in a mixture of monopegylated proteins with differing sites of modification. With rh-interferon-α2A pegylation, we have found that this heterogeneity includes two classes of pegylation site chemistry, the relative proportions of which can be adjusted by reaction pH.

Methods. The effect of pegylation reaction pH on the relative proportion of three peaks produced was investigated. Products were purified and characterized by peptide mapping, chemical stability to neutral hydroxylamine, and biologic activity.

Results. Reactions at basic pH levels produced a mixture of products pegylated at lysine residues as has been observed elsewhere. However, the dominant product of reactions at mildly acidic levels of pH showed distinct chemistry and higher cytopathic effect activity. The primary site of modification at this pH was His34. We developed a quantitative assay using sensitivity to neutral hydroxylamine to measure the proportion of urethane bonds involving carboxyalkylated histidines. This assay showed that histidine was pegylated preferentially at low pH levels with another protein, rh-Interleukin-10.

Conclusions. Reaction pH can be used to select the preferred pegylation site chemistry.

KEY WORDS: polyethylene glycol; chemical modification; interferon.

INTRODUCTION

The effectiveness of many cytokines *in vivo* often is limited by their rapid clearance from the bloodstream. The covalent attachment of polyethylene glycol (PEG), a large, highly soluble, nontoxic adduct, has been found to retard clearing, which has prompted its use as a modifier of several biologic drugs (1–5). The prolonged serum half-life of PEG biologics, coupled with reduced adverse effects on renal physiology and morphology, may allow a less-frequent injection schedule (6). However, the large amorphous nature of PEG can affect adversely specific activity of modified biologicals *in vitro,* presumably by obscuring receptor-binding or active sites (7). The rationale behind using PEG biologicals assumes that this reduced activity is offset by increased availability *in vivo.*

Many chemistries have been developed for attaching this polymer to amino, carboxylate, and thiol groups under mild conditions, but pharmaceutical products developed to date largely have used urethane or amide linkages between protein amines and PEG. The product of these reactions is usually a mixture of pegylated proteins with differing sites of PEG attachment. The relative proportions of these positional isomers reflect local variances in amino group exposure and pK_a and can be altered by a number of variables, including pegylation reaction pH.

The following reports in the literature suggest that pegylation reaction pH can affect the site of pegylation. Investigation of calcitonin modified with succinimidyl carbonyl PEG (SC-PEG) at a pH of 8 revealed a mixture of monopegylated positional isomers that included linkages involving all possible alpha and epsilon amino groups (8). When rh-interferon-α2A was pegylated via urea linkage at pH 10, every amino group except the N-terminal alpha amine group was modified in the resulting mixture of monopegylated positional isomers (9). In cytopathic effect assays, the activity of these positional isomers ranged from 6–40% of the activity of unmodified interferon. In contrast, pegylation performed under acidic conditions (pH 5) was used to achieve site-specific modification at the N-terminus of recombinant human granulocyte stimulating factor with N-hydroxysuccinimidyl carboxymethyl PEG by an amide linkage. This reaction pH greatly diminished the nucleophilic character of epsilon groups relative to the alpha amino group. Additionally, the product was treated with hydroxylamine to remove nonamide linkages to improve product homogeneity (5).

In this study, we investigated the role of pH in the pegylation of rh-interferon-α2B using SC-PEG, expanding this parameter to include mildly acidic conditions. This reagent has been reported as having high selectivity for amines in mild base (10), although in a stronger base (pH 9 and higher), it can be degraded via Lossen rearrangement, causing lowered yields and possibly low-level modifications of amine, carboxylate, and hydroxyl groups in a protein by a β-alanine derivative (11). When the effects of reaction pH were correlated with product structure and activity, it was observed that the composition of positional isomers of pegylated interferon changed substantially across the range of pH 5.4 to 10. Furthermore, *in vitro* cytopathic effect activity of monopegylated interferon was found to increase with decreasing pH. The increased biologic activity was associated with the enrichment of a specific pegylated species possessing a distinctive chemical lability in the presence of neutral hydroxylamine. The major positional isomers of monopegylated interferon produced at pH 6.5 were characterized. We have discovered that the hydroxylamine-sensitive isomer of PEG-rh-interferon-α2B is pegylated at histidine 34. The relative abundance of this highly active isomer was increased with decreasing pH. Conversely, the proportion of isomers pegylated primarily at epsilon amino groups was increased with increasing pH. This study was extended to examine the effect of reaction pH on pegylation of rh-interleukin-10; we have determined that the preference for histidine pegylation at mildly acidic pH is a general property that is largely independent of protein.

MATERIALS AND METHODS

Bovine pancreatic trypsin, endoproteinase Glu-C and endoproteinase Lys-C were purchased from Roche Molecular

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Biochemicals (Indianapolis, IN). Purified rh-interferon-α2B and rh-interleukin-10 were supplied by Schering–Plough (Union, NJ). Succinimidyl carbonyl PEG_{12000} was obtained from Shearwater Polymers (Huntsville, AL). Molecular weight standards for SDS-PAGE and size exclusion were obtained from Amersham–Pharmacia Biotech (Piscataway, NJ). Other chemicals were of the highest purity available. Protein N-terminal sequence analysis was performed by Argo Bioanalytica (Morris Plains, NJ) using a Hewlett–Packard model G1005A protein sequencer. MALDI-MS was performed using a Perseptive DE-STAR mass spectrometer. SDS-PAGE was performed by method of Laemmli (12).

Reaction of rh-Interferon-α2B and rh-Interleukin-10 with SC-PEG

Pegylated-Interferon was produced by agitating pure interferon (1.88 mg/mL) with SC-PEG (4 moles per mole interferon) in 100 mM sodium phosphate, pH 6.5 or 8.0, at 22°C for 2 h. The reaction was terminated by addition of glycine (100 mM) to a final concentration of 0.62 mM. For reactions at pH 5.4 and 10, sodium acetate and sodium borate, respectively, were substituted for sodium phosphate. Monopegylated interferon was resolved from dipegylated and unmodified interferon by ion exchange chromatography. By size-exclusion high-performance liquid chromatography (SE-HPLC), this material was over 90% monopegylated with dipegylated and unmodified interferon each present in levels below 5%.

Pegylation of rh-interleukin-10 was performed in identical manner with the following changes: the reaction was performed at pH 6.2 in 100 mM sodium phosphate for 10 min or pH 8.6 in sodium borate for 50 min. The molar ratio of SC-PEG to rh-interleukin-10 monomer was 4:1.

Purification of Monopegylated Interferon Positional Isomers

Pools of purified positional isomers were obtained by cation exchange chromatography. The monopegylated-interferon product was adjusted to pH 4.7 and 6 mS and applied to a column prepacked with Mono-S (HR 10/10, Amersham-Pharmacia) and equilibrated with 40 mM sodium acetate, pH 4.7, 25 mM sodium chloride at 4°C. After washing with equilibration buffer (5 cv), three peaks of monopegylated interferon were eluted with a gradient (20 cv) to 250 mM NaCl. Unmodified interferon was then eluted with a gradient (3 cv) to 750 mM NaCl. Flow was maintained at 30 cm/h. Fractions (0.25 cv) were collected. Pools made on the basis of offline absorbance measurements were dialyzed against equilibration buffer and each was applied to a second Mono-S column equilibrated as described previously. After washing with equilibration buffer (5 cv), a single major peak of monopegylated interferon was eluted with a gradient (30 cv) to 200 mM NaCl.

To determine relative abundance of positional isomers as well as unmodified interferon, this cation exchange chromatography was performed routinely on an analytical scale using a Mini-S column (HR 5/5, Amersham-Pharmacia). Buffers, post-injection wash length, and linear flow rate were identical to that used on a preparative scale with Mono-S. Elution was achieved with a gradient (30 cv) to 200 mM NaCl.

Enzymatic Digestion of Purified Monopegylated Interferon Positional Isomers

Purified positional isomers pools were digested as follows: the substrate (100 μg) was adjusted to pH 7.8 by the addition of sodium phosphate, pH 8, to a final concentration of 100 mM. The reaction was initiated by adding trypsin (5 μL of 1 mg/mL solution in 0.1 mM HCl) and incubating at 37°C for 2 h. At this point, a second identical aliquot of trypsin was added together with an aliquot of endoproteinase Glu-C (10 μL of 1 mg/mL solution in phosphate-buffered saline; Sigma Chemical Co., St. Louis, Mo). Incubation was continued overnight. The reaction was terminated by purification of pegylated peptides by SE-HPLC.

For MALDI-MS, PEG peptides (L2-PEG, residues 32–49) and unmodified peptides (L2, residues 32–49) were generated by proteolysis as follows. A purified positional isomer pool (A) in 100 mM sodium phosphate, pH 8, was digested using protease Lys-C (1:20 mass:mass) at 37°C for 16 h. The peptides were isolated by reverse-phase (RP)-HPLC on C-4 (Supelco). The isolates were immediately analyzed by MALDI-MS.

Purification of Pegylated Peptides

Pegylated peptides were isolated from protease digests by size exclusion high-performance liquid chromatography (HPLC) on Superdex 200 (HR 10/30, Amersham–Pharmacia) in 100 mM sodium phosphate, pH 5, 150 mM sodium chloride at 0.5 mL/min and collected (0.5-mL fractions).

Incubation with Hydroxylamine

Monopegylated interferon was diluted to 0.4 mg/mL into 0, 100, 200, 400, or 600 mM hydroxylamine at pH 7. Solutions were incubated for 1 h at ambient temperature, after which the products were dialyzed against 40 mM sodium acetate, pH 5 (1:1000 v/v) at 8°C for 6 h. The dialysates were then analyzed for relative abundance of positional isomers and unmodified interferon on Mini-S by cation exchange chromatography.

Monopegylated rh-interleukin-10 was incubated in the same manner, except that the hydroxylamine concentration was 450 mM and the incubation period was extended to 16 h.

Cytopathic Effect Assay of Interferon

The ability of interferon to prevent cell death *in vitro* by infection with encephalomyocarditis virus was performed as per Mossman (13).

RESULTS

Effect of Reaction pH on Pegylated Interferon Composition and Biologic Activity

In four parallel experiments, recombinant human interferon-α2B was pegylated with SC-PEG at a pH of 5.4, 6.5, 8, and 10. When the monopegylated products were resolved by small-scale ion exchange chromatography (Mono-S), a number of positional isomers were observed (Fig. 1, upper panel). Three major peaks, as well as several smaller incompletely resolved shoulders, were evident. The chromatographic pattern changed with the reaction pH. The relative abundance of the major peaks shifted significantly (Fig. 1, upper panel). For

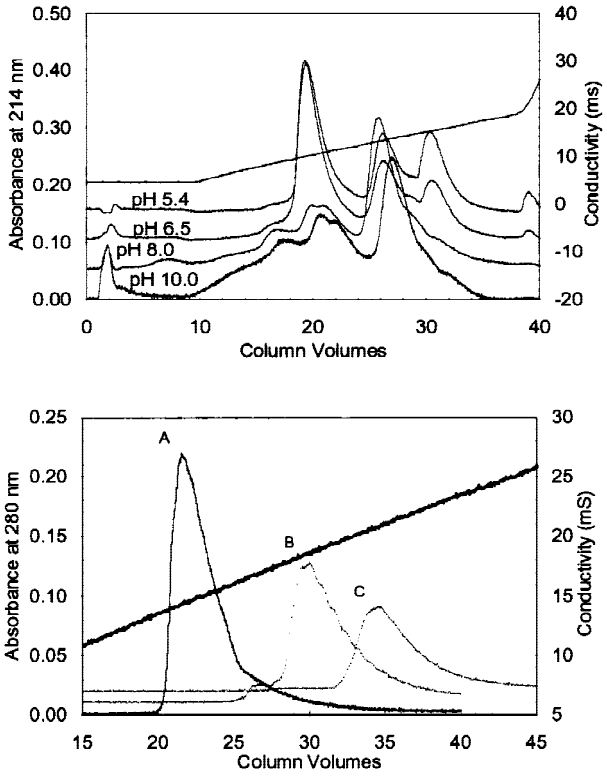

Fig. 1. Upper Panel: Ion exchange chromatography (Mono-S) of monopegylated products of pegylation performed under different pH conditions. The chromatograms of four reactions performed at pH 5.4, 6.5, 8, and 10 are overlaid. The conductivity gradient (pH 6.5) is also shown. Each chromatogram is labeled with the reaction pH. Lower Panel: Ion exchange chromatography of purified peaks A–C after two cycles of preparative ion exchange chromatography (overlaid).

the three major peaks, eluting at 20 (A), 26 (B), and 31 (C) column volumes, the peak height ratio, A:B:C, ranged from 47%:27%:26% at pH 5.4 to 25%:65%:10% at pH 8, respectively. The largest shift in the chromatographic pattern occurred between pH 6.5 and 8.

Pegylation reaction pH also affected the biologic activity (cytopathic effect assay) of the products. Products of acidic reactions were more active: 61.8 MIU/mg (39% of unmodified interferon activity) at pH 5.4 and 74.5 MIU/mg (48%) at pH 6.5. In contrast, products of basic reactions showed lower activity: 33.3 MIU/mg (21%) at pH 8 and 27.8 MIU/mg (18%) at pH 10.

Because the products of the pH 6.5 reaction possessed the highest specific activity and the number of peaks was similar for all reaction conditions, the pH 6.5 reaction condition was used as the source for subsequent characterization efforts.

Peptide Mapping of Positional Isomers of Monopegylated Interferon

The three major products of pegylation at pH 6.5, peaks A, B, and C, were purified by two successive cycles of exchange chromatography on Mono-S. Peak fractions from the second round were used as the source material for peptide mapping. Analytical ion exchange chromatography on Mini-S indicated that each of these fractions was highly enriched in a single species, which eluted at a conductivity identical to that observed in the two rounds of preparative chromatography (Fig. 1, lower panel).

Aliquots of the three pools of purified positional isomers containing equal amounts of monopegylated interferon by SE-HPLC were cleaved proteolytically in parallel studies. Proteolytic digestion was accomplished by simultaneous incubation with both trypsin (1:10 m/m) and endoproteinase Glu-C (1:10 m/m), a condition intended to cause complete digestion with minimal missed cleavages. The resulting pegylated peptides were isolated from proteins and nonpegylated peptides by size exclusion chromatography on Superdex 200, a mild procedure that exploits the unique molecular weight of peptides pegylated with SC-PEG_{12000}.

Each of the isolated pegylated peptides from the positional isomer pools A–C was subjected to N-terminal sequence analysis. The primary sequence in both peaks B and C could be clearly identified (Table I, Fig. 2). The majority (>65%) of pool B was identified as KYFQR (residues 121–125, Fig. 2), suggesting pegylation of Lys121. This was also supported by the lack of cleavage by trypsin after Lys121 (assuming that pegylation blocks cleavage) and by the phenylthio-hydration (PTH) derivatives formed by Edman degradation. The retention time of PTH-PEG-Lysine was expected to differ from that of PTH-Lysine. In the first cycle of the pegylated peptides from peak B, a PTH amino acid derivative that eluted slightly before PTH-Leucine was observed. Because the level of PTH-Lysine was much lower than expected, this new peak probably represented the PTH derivative of PEG-Lysine. Furthermore, this unique peak was observed in all pools only at cycles for which PTH-PEG-Lysine was expected. The major peptide sequence identified in pool C, representing over 80% of the signal, was ACVIQGVGVTE (residues 97–107, Fig. 2). Although there are no potential sites for stable pegylation on this peptide, it is linked by disulfide bond to the N-terminal peptide, which contains an alpha amino group that represents a suitable pegylation site (Fig. 2). It was assumed that the urethane linkage between PEG and the alpha amino group blocked direct identification of the N-terminal peptide by Edman degradation. When the pegylated peptides of pool C were reduced by 1 mM DTT prior to SE-HPLC, the signal for the sequence from residues 97–107 was reduced to less than 10% of the nonreduced signal. After subtracting out the primary signal, minor sequences were detected in both pools B and C (Table I). Although these assignments were not unambiguous, they do suggest that most lysyl side chains in interferon were modified to at least a small extent.

Equal amounts of pegylated interferon from pools A–C were subjected to digestion, but the recovery of PTH-amino acids from the sequence analysis of Pool A was much lower than the recoveries observed with the other pools, suggesting that peptides(s) were depegylated during processing. No single dominant peptide was evident (Table I), but a small amount of histidine was present in cycles 1 (0.6 pmol) and 3 (0.4 pmol), which was twice as high as levels observed in cycles 2 and 4. This suggested that overlapping peptides beginning with residues 32 and 34 (Fig. 2) were minor components of this pool, caused by incomplete cleavage at Arg33. Although this assignment is equivocal, no other peptides generated by these conditions contain histidine at positions 1 and 3.

TABLE I. Sequences Observed in Peptides Generated from Pools A–C

Pool	Sequence	Location	Site	Amount analyzed (pmol)	Proportion of pool
A	HDFGFPQEE	34–41	H34	1.2	7%
	DRHDFGFPQEE	32–41	H34	0.9	6%
	YSPCAWE	135–141	K31*	3.5	22%
	*K*YSPCAWE	134–141	K134	2.5	15%
	ISLFSCLK	24–31/33	K134* or	4.2	26%
	ISLFSCL*K*DR		K31		
	*K*YFQR	121–125	K121	2.5	15%
	ACVIQGVGVTE	97–107	C1*	1.6	10%
B	*K*YFQR	121–125	K121	28.7	68%
	ACVIQGVGVTE	97–107	C1*	7.1	17%
	*K*FYTE	83–87	K83	6.1	15%
C	ACVIQGVGVTE	97–107	C1*	64.5	85%
	FGNQFQ*K*AE	43–51	K49	6.7	9%
	*K*YFQR	121–125	K121	3.0	4%

Sequences with clear-cut assignments (the signal difference for assigned phenylthiohydration(PTH)-amino acid in current vs. leading and trailing cycles is greater than twofold) are underlined. Remaining assignments were discerned after other sequences were subtracted from the data. PTH-PEG-lysine residues, which elute in a position close to PTH-leucine, are listed in Greek lower case (κ). Asterisks denote pegylated residues that were assigned indirectly by disulfide bridging (see text).

The low recovery of pegylated peptides from peak A and the incomplete cleavage by trypsin at Arg33 suggested the possibility of an unexpected chemistry: a urethane bond involving the imidazole group of histidine (i.e., a carboxyalkylated histidine). This hypothesis was supported by the observation that another carboxyalkylating agent, diethylpyrocarbonate (DEPC), is a highly specific histidine-modifying reagent used for enzyme active site characterization when reacted at acidic pH (14–16). If a species pegylated by carboxyalkylation at His34 were present in Peak A, then steric hindrance of trypsin binding at Arg33 would explain the partial cleavage observed at this residue. In addition, pegylation by histidine carboxyalkylation, in contrast to lysine carboxyalkylation, would be more prone to base-catalyzed hydrolysis at elevated temperatures during tryptic digestion, causing lower recovery of histidine-pegylated peptides. Complete hydrolysis should occur during the coupling phase of Edman degradation (16), resulting in the observation of PTH-His, not PTH-PEG-His, after Edman degradation.

Studies of histidine modified with DEPC have shown that the carboxyethylated imidazole is uniquely susceptible to aminolysis by hydroxylamine. In contrast, products formed by the reaction of DEPC with primary amines are resistant to such cleavage (14–16). To confirm that His34 is pegylated, the effect of hydroxylamine treatment on pegylated peptides from peak A was examined using MALDI-MS. Pool A was digested with protease Lys-C, the resulting peptides were resolved by reverse-phase (RP)-HPLC on C4, and the peak exhibiting the mass distribution characteristic of a pegylated peptide was collected (Fig. 3, upper panel). The pool was incubated with hydroxylamine, and analyzed again by

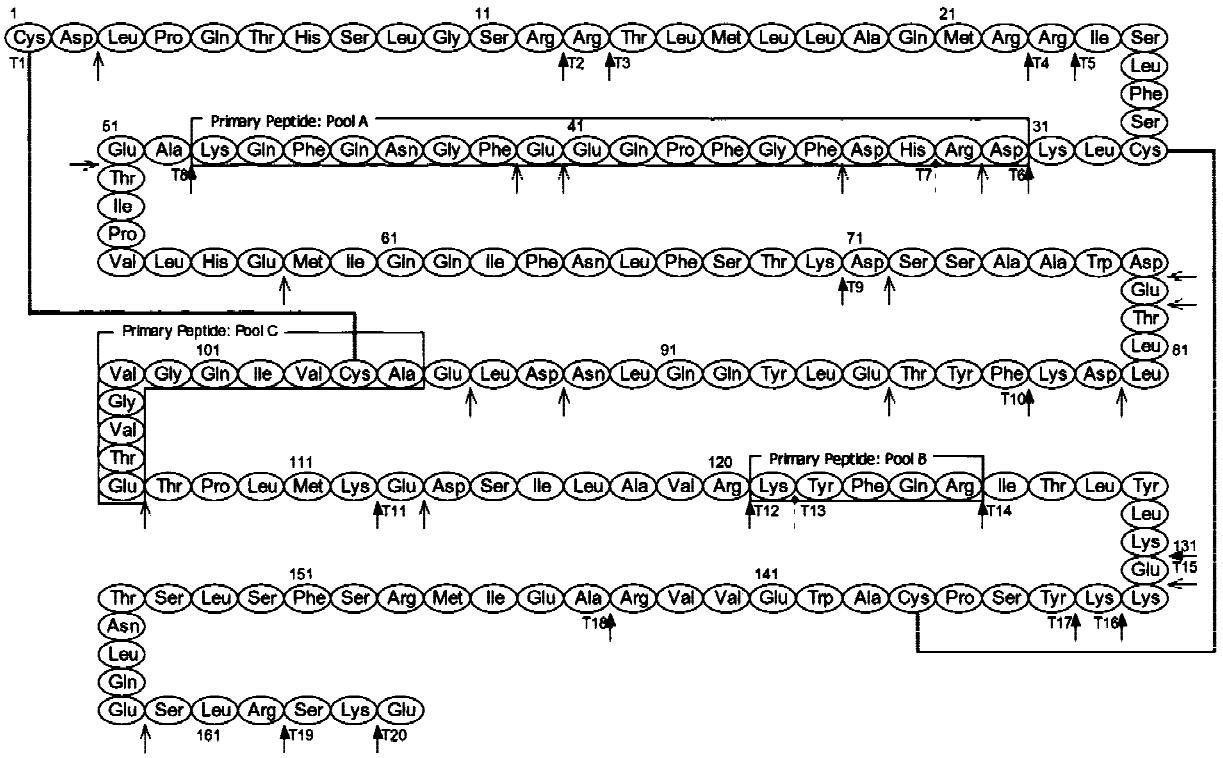

Fig. 2. Sequence of rh-interferon-α2B. Disulfide bonds are shown as solid lines between cystine residues. Predicted cleavage sites for trypsin and endoproteinase Glu-C are shown as arrows pointing between appropriate residues. Two observed missed cleavage sites are shown as diamond-studded wands between Arg33/His34 and Lys121/Tyr122. The three framed sequences identify the primary pegylated peptides obtained from digests of the three ion exchange pools.

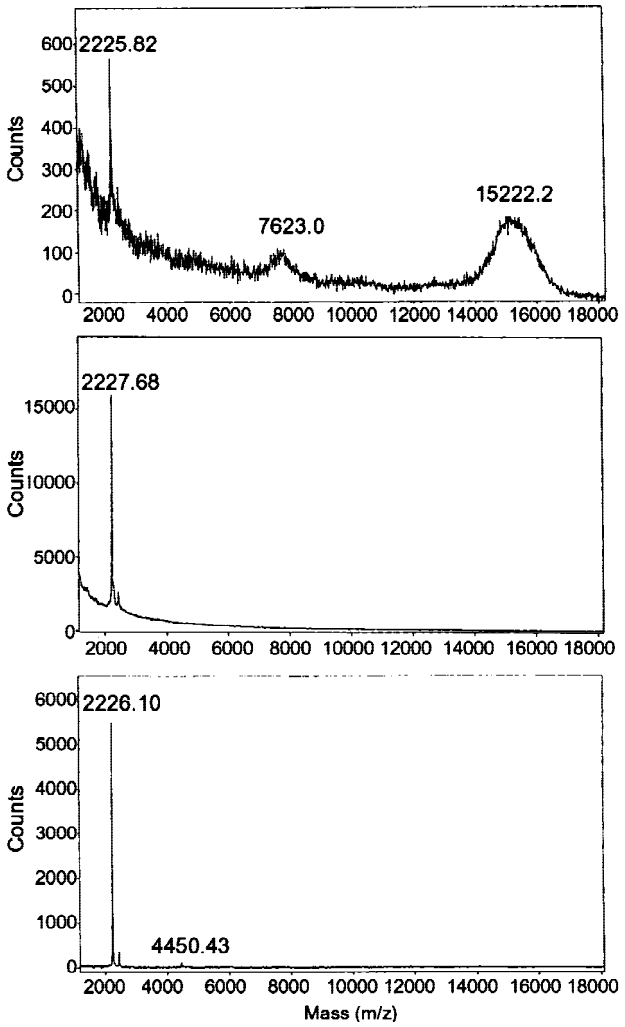

Fig. 3. MALDI-MS of pool A peptides generated by digestion with protease Lys-C before and after treatment with hydroxylamine. Top panel: untreated pegylated peptides from pool A. The pegylated peptide is characterized by a broad peak containing many discrete masses that represent a heterogeneous PEG population of slightly differing chain lengths. Middle panel: pegylated peptides from pool A after hydroxylamine treatment. Bottom panel: control peptide spanning residues 32–49 isolated from a tryptic digest of unmodified interferon.

MALDI-MS. The resulting depegylated peptide mass (2,227.7 ± 7, Fig. 3, middle panel) matched that of the peptide spanning residues 32–49 (L2, average isotopic mass of 2,226.1 Da, Fig. 3, lower panel). These studies with hydroxylamine confirmed that His34 was a site of pegylation in peak A.

Chemical Characterization of Positional Isomers of Pegylated Interferon

The MALDI-MS studies of hydroxylamine sensitivity were qualitative. For quantification, we developed an HPLC-based assay to determine the proportion of peak A containing the His-pegylated isomer, by measuring hydroxylamine-induced depegylation. Monopegylated interferon containing a mixture of all positional isomers produced at pH 6.5 was incubated for 1 h at ambient temperature with neutral hydroxylamine (0–400 mM). The products were assayed by cation exchange HPLC on Mini-S. As shown in Fig. 4, peak A was almost completely depegylated to form unmodified interferon, whereas peaks B and C were largely unaffected. Comparison of the 400 mM hydroxylamine reaction with the control indicated that over 85% of peak A was eliminated with a commensurate increase in the unmodified interferon peak. These studies not only confirmed that histidine 34 was a site of pegylation, but also established that the positional isomer with this modification was the major constituent of pool A.

A comparison of hydroxylamine (600 mM, 1 h) sensitivity between interferons created by reaction at pH 5.4, 6.5, 8, and 10 was also performed. The fraction of monopegylated product hydrolyzed to interferon was 56, 47, 8, and <1%, respectively, which correlated well with the levels of peak A in the products produced at these pH conditions (Fig. 1, upper panel). This underscored the distinctive chemistry of peak A relative to peaks B and C, as well as the effect of pegylation reaction pH on its relative abundance.

Biologic Activity of Pools A–C

The cytopathic effect activity of pools A–C was measured. The specific activity of pool A (73.2 MIU/mg, 47% vs. unmodified interferon purified on Mono-S) was significantly higher than the activity of either pool B (20.3 MIU/mg, 13% vs. control) or C (18.0 MIU/mg, 12% vs. control). By SE-HPLC, the level of unmodified interferon in pools A–C prior to CPE assay was below 5%.

Stability of Pegylated Interferon

Monopegylated interferon created by reaction at pH 6.5 was stored at pH 6.8 and ambient temperature for 30 days. By SE-HPLC, only 4% was depegylated to unmodified interferon. At pH 8.6, however, 22% of the monopegylated interferon was depegylated after only 24 h. Thus, whereas the His-pegylated isomer is more labile under basic conditions, it is stable at physiologic pH, retaining its usefulness as a therapeutic drug.

Pegylation of rh-Interleukin-10: pH Dependence of Hydroxylamine Sensitivity

The pH dependence of pegylation with a different protein, rh-interleukin-10 (IL-10), was investigated to determine whether histidine pegylation at low reaction pH was a general property or one specific to interferon. In parallel experiments, IL-10 was pegylated with SC-PEG at pH 6.5 and 8.6. The products were assayed for stability in the presence of neutral hydroxylamine. For the pH 6.5 reaction, 50–60% of the product was cleaved by aminolysis with neutral hydroxylamine, but for the reaction at pH 8.6, only 10% was labile to this reagent.

DISCUSSION

Previous work by Monkarsh *et al.* on the pegylation of interferon α2A with PC-PEG (9) led to the expectation that interferon α2B would react with SC-PEG at basic pH to yield a heterogeneous population of several different positional isomers, each pegylated at a lysine residue. This was found largely to be true. Interferon pegylated at Lysine 121 (Peak B) was the major component of pegylation under basic conditions, but pegylation was also evident at cysteine 1 (α amino

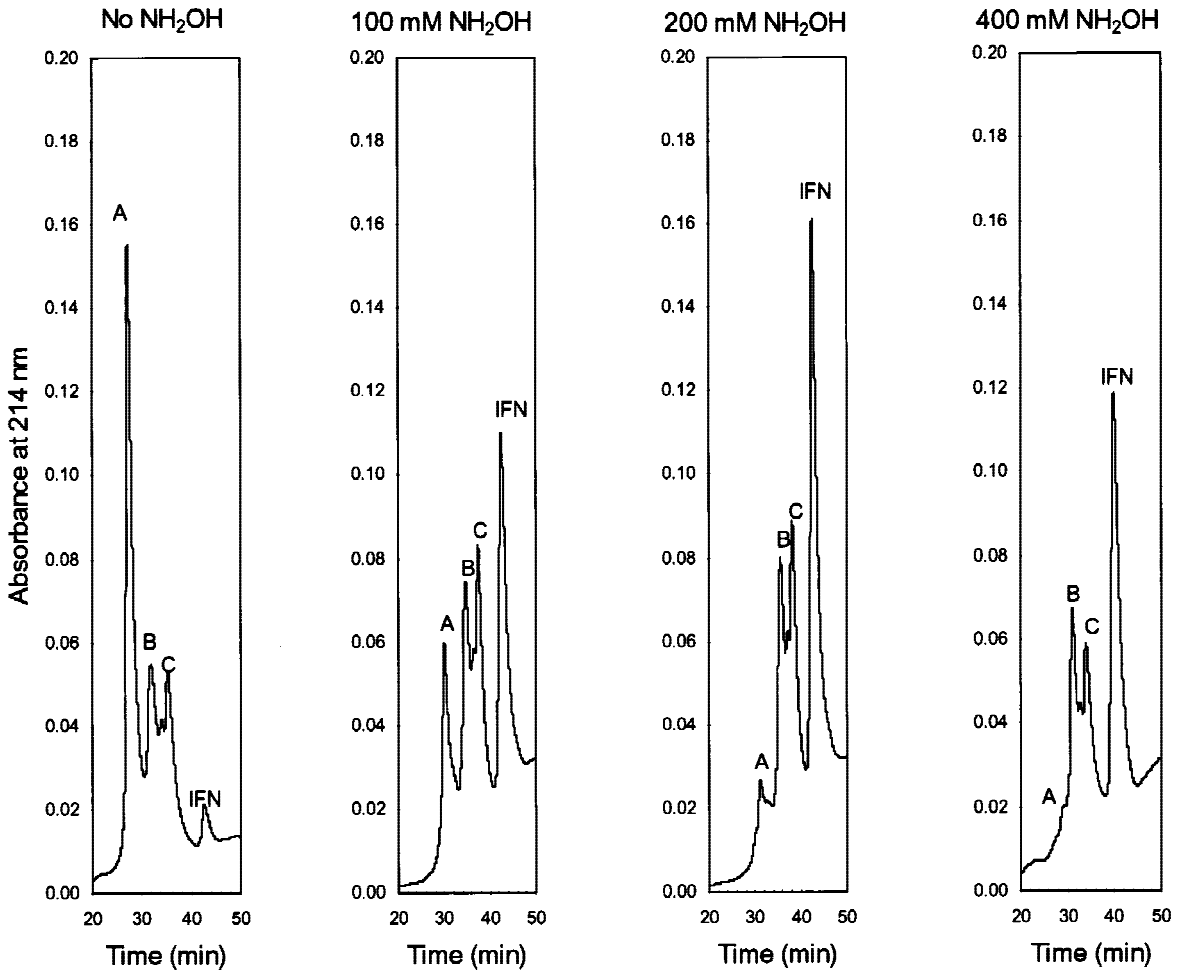

Fig. 4. Sensitivity of monopegylated interferon to hydroxylamine. Ion exchange (Mini-S) chromatograms of monopegylated interferon resolved after incubation in hydroxylamine are shown. Peaks are labeled A–C (representing the major subpopulations of monopegylated interferon) and interferon (depegylated). The concentration of hydroxylamine present during the incubation is listed at the top of each panel.

group) and lysines 31, 49, 83, and 134. It is possible that lysines 70, 111, 131, 133, and 166 were also pegylated but not detected for reasons of assay sensitivity or resolution. Uniform distribution of all possible positional isomers was not expected because variations in solvent access and local electrostatic potential can substantially alter an amine's suitability for pegylation. Interestingly, peak C (predominantly pegylated at the N-terminus) was a minor component at pH 8 and became a significant product only at lower reaction pH, a result not inconsistent with the reported absence of N-terminally pegylated interferon α2A produced at pH 10 (9). All products of reactions under basic conditions were stable during various analytical procedures, including incubation with neutral hydroxylamine.

However, acidic reactions (pH 5.4–6.5) produced a pegylated interferon characterized by a carboxyalkylated histidine. Although peak A, the dominant peak generated at this pH, was stable at neutral pH, peptide mapping studies of this pool were hindered by depegylation during the mapping procedure. N-terminal sequence analysis of pegylated peptides generated from peak A by proteolytic digestion gave no clear major sequence, but a close examination suggested the presence of a peptide pegylated at histidine 34, which was confirmed by MALDI-MS. Interferon contains three histidines, but carboxyalkylation at Histidines 7 and 57 were not observed in these studies, suggesting that Histidine 34 represents a better substrate for pegylation. This is consistent with the theoretical calculations that indicate that this histidine possesses greater solvent access in the crystalline structure. It is possible, however, that the product contains small amounts of isomers pegylated at the other histidines, albeit at levels below the threshold of detection. These peptide mapping studies clearly identified the presence of a carboxyalkylated histidine, but quantification was missing.

Quantification was obtained by using neutral hydroxylamine, which established that over 80% of peak A, but less than 10% of peaks B and C, was pegylated at histidine. This reagent has been traditionally used as a diagnostic to restore histidines carboxyalkylated with DEPC in active site blocking studies. Although peptide mapping demonstrated the existence of a PEG-His34-interferon isomer in pool A, sensitivity to neutral hydroxylamine established that a carboxyalkylated histidine isomer was the dominant component at this reaction pH.

Imidazoles are substantially more nucleophilic than primary amines at mildly acidic pH, so it is likely that histidines

represent the preferred pegylation sites under these conditions in virtually all proteins. Initial studies in this laboratory using another protein, rh-interleukin 10 (IL-10), support this. As with interferon pegylation, roughly half of monopegylated IL-10 pegylated at pH 6.5 was sensitive to hydroxylamine, whereas only 10% of the product produced at pH 10 was sensitive to hydroxylamine. The combined results of pegylated interferon and IL-10 suggest that the effect of reaction pH is largely unaffected by protein structure.

Positional isomers of interferon pegylated at histidine were distinguishable from ones pegylated at primary amines in terms of *in vitro* cytopathic activity as well. Studies with Mono-S pools established that all monopegylated interferon isomers possessed reduced activity compared to unmodified interferon, but that the reduction was significantly less for PEG-His34-interferon. This higher activity of PEG-His34-interferon, relative to PEG-Lys121-interferon and PEG-Cyst1-interferon, could possibly be caused by better receptor binding to this isomer, although the mechanism is unclear.

As summarized by Radhakrishnan *et al.,* two putative receptor-binding sites have been proposed for interferon α2B: a site each for interferon receptors α/βR (primary low-affinity receptor) and αR1 (complementary high-affinity receptor) (17). The core of the α/βR site involves the AB loop (22–51), as well as the D (112–132) and the E (137–156) helices, whereas the aR1 site is centered on helix C (78–100) (17). Arg33 is a particularly important residue for receptor interaction (18). By this model, pegylation of His34, Lys31, Lys112, Lys121, or Lys131 should all interfere with receptor binding whereas the N-terminal cysteine should not. Because PEG-Cys1-interferon (Peak C) has low activity relative to PEG-His34-interferon, the proximity of the pegylation site to the binding sites does not explain the difference in biologic activities. Other factors related to the unusual chemistry of the carboxyalkylated histidine may contribute to this effect, such as slow depegylation during the assay, possibly catalyzed by receptor binding. Alternately, the charge loss that accompanies pegylation with SC-PEG may perturb the electrostatic potential at or near the receptor binding site less when histidine is modified than is the case when an amine is pegylated.

This study of interferon pegylation with SC-PEG provides an additional approach to targeted pegylation. The chemistry of choice depends, at least partially, on protein structure and function. By examining the relative proximity of lysine and histidine residues to biologically important domains, one can design reaction conditions to yield a product with optimal activity. However, location of the binding site may not be the only benefit of histidine pegylation. There appears to be a benefit derived from the unusual chemistry of carboxyalkylation. PEG-His34-interferon possesses higher specific bioactivity than interferons modified at amino groups. Although the cause for this is uncertain, the location of the histidine is clearly not the only factor. Regardless of the reason for this relatively higher activity, histidine pegylation clearly merits consideration with receptor-binding proteins. Overall, control of reaction pH provides a simple yet effective means for rational design of pegylated biological drugs.

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