Isolation and Characterization of an Acetylated Impurity in *Escherichia coli*-Derived Recombinant Human Interleukin-10 (IL-10) Drug Substance

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INTRODUCTION

Interleukin-10 (IL-10) plays a crucial and complex role in the cell mediated immune response, displaying immunostimulatory as well as immunosuppressive properties (1). Both murine and human IL-10 extend the viability of mouse mast cells in culture (2) and play a role in stimulating their proliferation. They also enhance the expression of the class II major histocompatibility complex on small, dense resting mouse spleen B cells (3) although similar effects have not yet been observed with human B cells. Human IL-10 costimulates B lymphocyte proliferation that is induced by cross-linking surface immunoglobulin with immobilized IgM or by culture with *S. aureus Cowan* for 48 hours. IL-10 also increases the numbers of immunoglobulin-producing cells in SAC- and anti-CD40-activated B cells, causing them to produce large amounts of IgA, IgG and IgM (4).

The potential usefulness of IL-10 as a therapeutic entity stems from its ability to down-regulate the immune system; that property allows it to function as an anti-inflammatory agent or as a suppresser in T cell-mediated autoimmune diseases. At its discovery, IL-10 was called the Cytokine Secretion Inhibition Factor because it was identified as the agent in T_{H2} cells that inhibits the proliferation and function of T_{H1} cells and blocks the production of cytokines such as interferon-γ (5). It is now known that IL-10 reduces the antigen-presenting capacity of human monocytes (6) and inhibits antigen presentation on T_{H1} cells, down-regulating the class II major histocompatibility complex in macrophages. IL-10 also suppresses the production of a number of inflammatory mediators including GM-CSF, G-CSF, TNF-α and the interleukins-1, 6 and 8. Human IL-10 has been shown to inhibit the synthesis of IL-4 and IL-5, cytokines that are produced by T_{H2} and T_{H0}-like clones (7–8). IL-10 may also prove to be a useful therapeutic agent for prolonging the survival of allograft recipients, perhaps in combination with IL-4 since T_{H2} cytokines were found to induce allograft tolerance in animal models (9).

Many therapeutically active proteins are now produced in bacterial cells and are commonly screened for the presence of impurities. The variants that we observe most frequently include oxidized methionines, deamidation products, and truncated or clipped chains. One of the major problems associated with synthesizing mammalian proteins in bacterial cells rests in the fact that many proteins are post-translationally modified and the mechanisms involved in post-translational modification vary greatly among organisms (10). In this paper, we describe an impurity that appears to involve the unexpected modification of an amino acid residue.

Acetyl acyltransferases are found in both prokaryotic and eukaryotic organisms. Recently, a great deal of effort has focused on defining the role that transacetylases play in controlling transcription in eukaryotic cells by acting on the four nucleosomal histones. Histones contain relatively unstructured positively charged segments that protrude from a globular core and are deemed essential for interaction with DNA and with other proteins. Acetylation occurs reversibly at specific residues in these highly ionic histone domains, reducing their charge and their affinity for DNA. The reduction in histone charge makes it easier for the DNA to interact with other proteins including those that regulate transcription (11). The acetyl donor is acetylCoA and there are both acetylating and de-acetylating enzymes (11,12). Other eukaryotic proteins, particularly those that have N-terminal serine or threonine residues are acetylated at their N-terminus (13). The lens proteins α- and β-crystallin are N-terminally acetylated and are subject to internal acetylation at a number of lysyl groups although it is not known if the side chains are modified *in vivo* (14). N-terminally acetylated variants of recombinant interferon-α-2 produced in *E. coli* cells have been identified by mass spectrometry (15,16). The present report provides evidence for the presence of an ε-N-acetylated lysine impurity in batches of rhu-IL-10 produced in *E. coli*. Acetylated lysine residues have recently been found in recombinant human Neurotrophin-3 (17).

MATERIAL AND METHODS

Protein Isolation and Purification

Recombinant human IL-10 was produced in an *E. coli* K-12 strain using standard methods for plasmid insertion (18). The pFMR2 plasmid carries a tac promoter for the expression of IL-10 and a tetracycline resistance element for plasmid maintenance (International Patent Application: WO 91/13160, Published 5 Sept. 1991, Schering Corporation). Following fermentation the cells were broken and inactivated and rhuIL-10-containing inclusion bodies recovered. The inclusion bodies were extracted with a Tris-guanidine hydrochloride unfolding buffer containing dithiothreitol at slightly alkaline pH. After refolding, the protein was brought to greater than 95% purity by conventional methods including ion exchange and hydroxyapatite chromatography (U.S. Patent No. 5,328,989. Granted

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1994.). The amino acid sequence and monomer molecular weight of the protein were confirmed by N-terminal sequencing and SDS-polyacrylamide gel electrophoresis, respectively. Size exclusion chromatography showed that IL-10 exists as a dimer under non-denaturing conditions as expected.

HPLC Methods

RP-HPLC Chromatography was performed on a Polymer Labs PLRP-S column (4.6 mm × 50 mm; 8μ; 1000 Å). The chromatographic system consisted of a Waters Model 712 WISP automatic sample injector, two Waters model 510 high pressure pumps and a Waters Model 680 temperature controller and TCM column heater. Samples were dissolved in 10 mM Tris, pH 6.8 containing 150 mM NaCl and 0.1 mg/mL polyethylene glycol-8000. The column was eluted at 37 °C using a linear gradient comprising two mobile phases, A: 0.1% aqueous trifluoroacetic acid (TFA) and B: 90% acetonitrile containing 0.1% TFA, and monitored at 214 nm using a Waters 441 fixed wavelength detector. The gradient varied from 42% B at initial to 54% B at 24 minutes.

Protein Characterization

Peptide Mapping

Samples were dissolved in 0.1 M potassium phosphate, pH 8.0 and digested with *S. aureus* V-8 Protease (enzyme:substrate ratio: 1:20) for 18 hours at 37°C. Under these conditions, the enzyme cleaves at the C-terminal end of both glutamyl and aspartyl residues (19,20). Peptide maps were run using reverse-phase chromatography with a TFA: acetonitrile gradient on a Vydac Protein and Peptide C-18 column (2.1 × 150 mm) maintained at 40°C. The linear gradient rose from 0% B at 5 minutes to 60% B at 65 minutes.

N-Terminal Sequencing was performed using a Porton Instruments Protein Sequencer following the manufacturer's recommended protocol. ε-acetyllysine was obtained from Bachem (Catalog number: E-2705) and PITC-derivatized on the sequencer. Synthetic peptides were obtained from QCB Biochemicals (Hopkinton, MA).

Mass Spectrometry

Plasma desorption mass spectrometry was performed using a BIOION 20 californium-252 time-of-flight mass spectrometer set at an acceleration voltage of 18 kV (21–22).

Bioassay

The assay is performed using MC/9 cells cultured in 10% FBS containing 2.5% spleen conditioned medium as a growth factor. After harvesting, the cells are grown in the presence of rhuIL-10 for two days. Cell proliferation is measured by reduction of the tetrazolium salt, MTT. Samples that were isolated by reverse-phase HPLC were evacuated to dryness on a Speed Vac (Savant), redissolved in an amount of 0.1 M potassium phosphate, pH 8.0 sufficient to make a 1 mg/mL solution and left overnight at 4°C. They were then stored at −80°C until they were assayed.

RESULTS

Isolation of the Impurity

Figure 1 shows the chromatogram obtained when rhu-IL-10 was analyzed by reverse-phase chromatography on PLRP-S (a polystyrene divinylbenzene polymeric column). The partially resolved species, labeled "Peak B," that elute after the main peak, were collected and evaporated to dryness on a Speed Vac (Savant) vacuum concentrator.

Characterization of the Impurity

Aliquots of the fraction were subjected to reverse-phase and size exclusion chromatography, SDS-PAGE electrophoresis, mass spectrometry, bioassay, N-terminal analysis and peptide mapping. Rechromatography by reverse phase revealed one major peak co-eluting with Peak B. Size exclusion chromatography showed that the impurity is a dimer. SDS-PAGE and N-terminal sequencing of the first 16 amino acids similarly failed to show any differences between the impurity and native IL-10. The bioactivity of Peak B, measured by the ability of IL-10 to stimulate the proliferation of the murine mast cell line MC/9 cells, was diminished: Peak B showed only half the

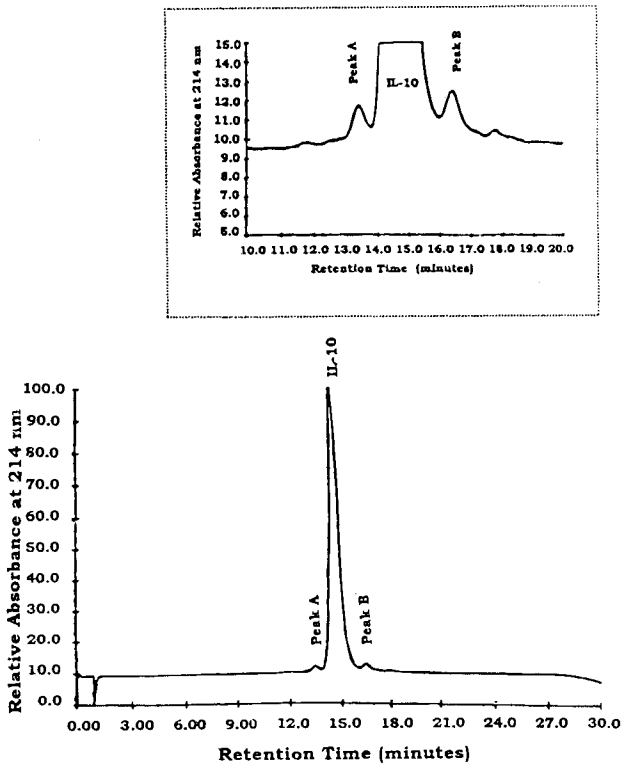

Fig. 1. Reverse-phase chromatography of IL-10 showing the impurity peaks. The inset shows an expanded view of the chromatogram. Column: Polymer Labs PLRP-S (4.6 × 50 mm, 8μ; 1000Å), thermostatted at 37°C. Injection volume: 10 μL. Protein concentration: 1 mg/mL. Mobile Phase A: 0.1% trifluoroacetic acid in water, Mobile Phase B: 90% acetonitrile containing 0.1% trifluoroacetic acid in water. The sample was eluted with a linear gradient varying from 42% B at initial to 54% B at 24 minutes. The column was then washed for 15 minutes with 100% B and re-equilibrated before injecting the next sample.

activity of an IL-10 control that had been isolated by reverse-phase and evaporated to dryness on the Speed Vac.

The most pronounced differences between IL-10 and the late-eluting impurity were found by comparative peptide mapping and by mass spectrometry.

Identification of Modified Residues

Analysis of the impurity Peak B by electrospray mass spectrometry yielded a single species with a mass 42 Da higher than that of the native protein. To locate the modified residues, both the isolated IL-10 peak and the impurity peak were digested with *S. aureus* V-8 Protease and analyzed by comparative peptide mapping (Figure 2). Four peaks found uniquely in the map of the impurity fraction were analyzed by plasma desorption mass spectrometry and N-terminal sequencing. The observed protonated molecular ion (MH^+) mass values of these peaks, designated V_1, V_2, V_3 and V_4, indicated an increase of 42 Da over the expected values. A significant amount of oxidation was also observed as evidenced by the presence of additional signals 16 Da higher than the MH^+ signals. The oxidation most likely occurred during sample preparation. The mass spectrometry results are presented in Table I.

Confirmation of the Presence of N-Acetyllysine by N-Terminal Sequencing

Figure 3 shows a chromatogram of the PTH-amino acid derivatives generated during N-terminal protein sequencing. The PTH-derivative of N-acetyllysine emerges at approximately 10.5 minutes while that of lysine appears much later, at about 22 minutes. The peak at 10.5 minutes was found too at position 2 in the sequence of a synthetically prepared peptide, N-K(Ac)-S-K-A-V-E (Quality Controlled Biochemicals, Hopkinton, MA) and in the sequencing chromatograms of the IL-10 impurity peaks.

Table I. Mass Spectrometry and N-Terminal Sequencing of V-8 Peptides Unique to Impurity Peak B

Peptide	Amino acid sequence[a]	Theoretical MH^+ value	Experimental MH^+ value
V_1	N-**K**-S-K-A-V-E	775.9	818.5
V_2	**K**-G-I-Y-K-A-M-S-E	1027.2	1069.9, 1085.8[b]
V_3	K-G-I-Y-**K**-A-M-S-E	1027.2	1069.8, 1085.9[b]
V_4	A-Y-M-T-M-**K**-I-R-N	1128.4	1170.9, 1186.7[b], 1203.0[b]

[a] Modified lysines are indicated in bold type.
[b] Molecular weight suggests the presence of oxidized methionines. Since peptides containing oxidized methionines are expected to show different retention times in reverse-phase chromatography and V_1, V_2, V_3 and V_4 represent discrete peaks, we suspect that the oxidation might have occurred during sample preparation

CONCLUSIONS

IL-10 is a recombinant protein manufactured in *E. coli* cells and isolated from inclusion bodies. The late eluting impurities found in reverse-phase chromatograms of IL-10 on polymeric columns have been identified as a mixture of mono-acetylated lysine derivatives. The protein which has a molecular weight of 18,774 contains thirteen lysines. Residues 117, 135, 139 and 157 have been identified as ε-N acetyllysines. Preliminary identification of the variants as acetylated species was based on apparent mass increases of 42 Da observed for lysine-

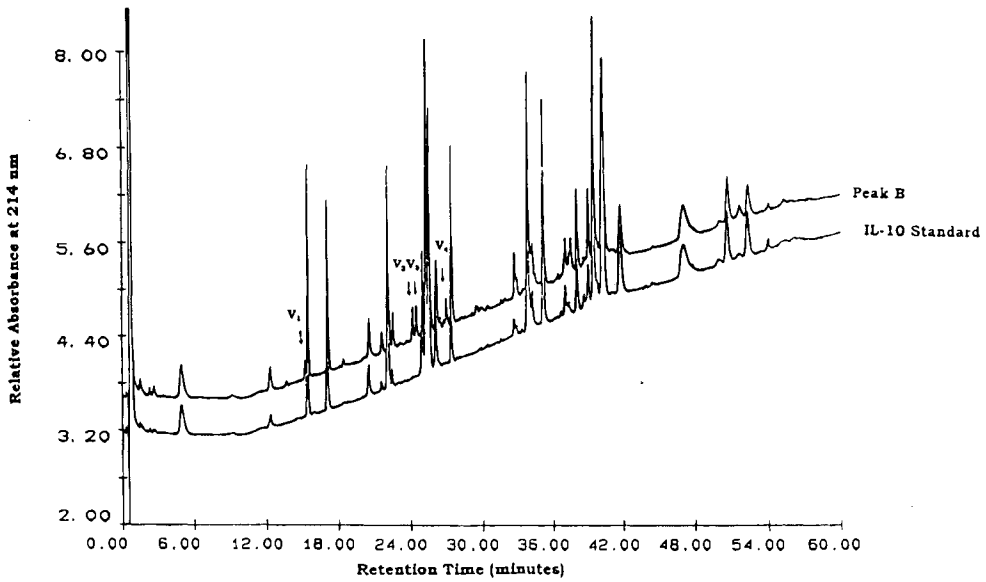

Fig. 2. Reverse-phase chromatogram showing comparative peptide maps (*S. aureus* V-8 Protease) of IL-10 and the impurity, Peak B. Both digests were performed at 37°C in 0.1 M potassium phosphate, pH 8.0 for 18 hrs with an enzyme:substrate ratio of 1:20. Column: Vydac Protein and Peptide C-18 (2.1 × 150 mm) thermostatted at 40°C. Mobile Phase A: 0.1% trifluoroacetic acid; Mobile Phase B: 0.1% trifluoroacetic acid in 90% acetonitrile. The samples were eluted isocratically for 5 minutes with 100% A and then with a linear gradient increasing from 0%–60% B in 60 minutes. The column was then washed with 100% B for 10 minutes and re-equilibrated before injecting the next sample. Arrows point to those peaks that were found only in the digest of the impurity peak.

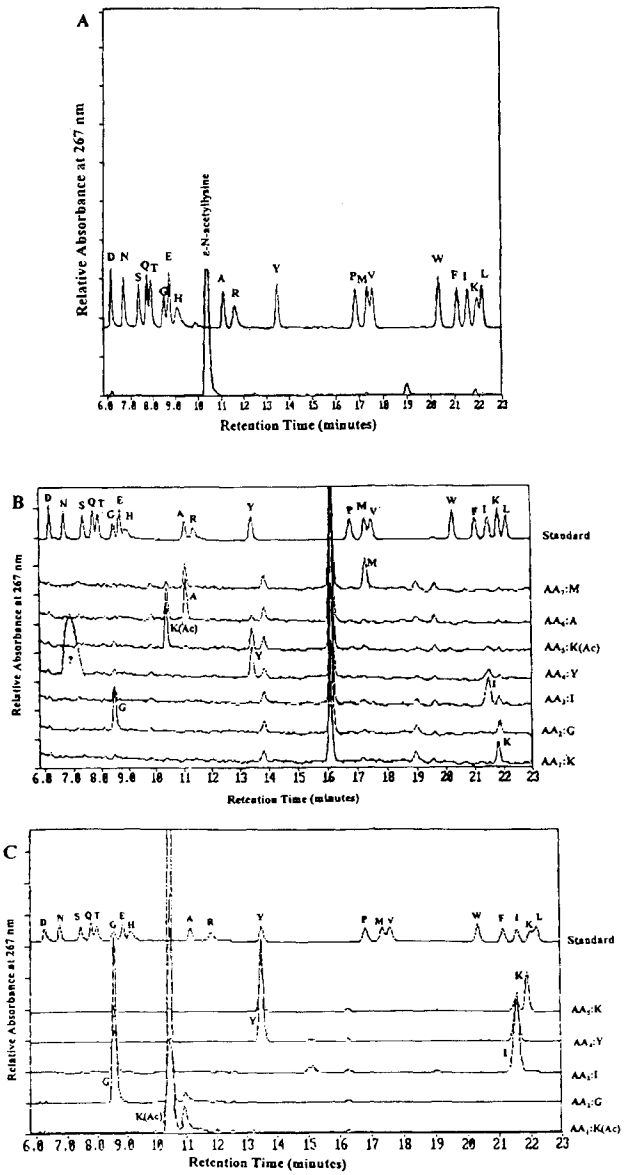

Fig. 3. N-terminal protein sequencing chromatograms showing: A. The retention time of PTH-N-M-acetyllysine. The acetylated derivative emerges at about 10.5 min. It is well separated from the other PTH-derivatives. PTH-lysine emerges at about 21.8 minutes. B. The first seven N-terminal sequencing cycles found for the peptide marked "V_3" in Figure 2. C. The first 5 five-terminal sequencing cycles found for the synthetic peptide K(Ac)-G-I-Y-K-A-M-S-E.

containing V-8 peptide fragments analyzed by mass spectrometry. Although the masses of N-acetyl and N-carbamyllysine differ by only 1 Da, the presence of carbamylated lysines was deemed unlikely since the inclusion bodies are solubilized in guanidine rather than in urea. Urea slowly decomposes to form cyanate ions that attack lysyl residues to form carbamylate derivatives but guanidine solutions are stable at neutral pH (23,24). Acetylated lysyl residues were identified positively by N-terminal sequencing.

Since the chemical methods for the acetylation of lysine require reagents such as acetic anhydride, we assume that the acetylation was driven enzymatically and occurred during fermentation. As indicated in the introduction, cellular enzymes that catalyze the ε-N-acetylation of lysyl residues exist *in vivo* (12). Although the impurity peak we characterized contained only mono-derivatized lysines, more highly derivatized species may have formed and been eliminated in the multi-step purification process. In fact, slightly raising the pH and lowering the conductivity of the mobile phase used to elute an anion exchange column completely removed the mono-acetylated impurities and they are no longer found in the IL-10 Drug Substance.

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