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CHARACTERIZATION OF RECOMBINANT HUMAN INTERLEUKIN-2 WITH MICROMETHODS

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SUMMARY

Highly purified recombinant human interleukin-2, expressed in *Escherichia coli*, was analyzed by micromethods. N-Terminal sequence analysis showed that methionine at position 0 was found in 90% of the molecules and not completely removed in post-ribosomal processing. A complete peptide map of the reduced and S-carboxymethylated protein was obtained by high-performance liquid chromatography after tryptic digestion, and the fragments were identified by amino acid analysis and automated Edman sequence analysis. Using a double-label S-carboxymethylation procedure, it was determined that there is a disulfide linkage between the cysteine residues at positions 58 and 105. The third cysteine residue at position 125 was found to be present as the free sulfhydryl.

INTRODUCTION

While recombinant DNA technology makes it possible to produce desired proteins in abundance, each new instance of a recombinant protein product must be confirmed to show that its amino acid sequence is as predicted by the nucleotide sequence in its gene. Modern micromethods make this task rapid and efficient. This report describes the use of amino acid analysis, peptide fragmentation and sequencing procedures for the characterization of recombinant human interleukin-2 (rIL-2), an immunomodulatory protein which is currently being used in clinical trials.

MATERIALS AND METHODS

Homogeneous human rIL-2 was obtained from the Biopolymer Research Department (Hoffmann-La Roche). In preliminary experiments, reduction and S-carboxymethylation of human rIL-2 was carried out as previously described[1]. For the determination of the free sulfhydryl group, a double-labeling procedure was employed. Without prior reduction, 6 nmol of the recombinant protein were dissolved in 300 μl of 6 *M* guanidine–HCl, 0.1 *M* Tris (pH 8.5) and reacted with 400 μg (2.2 μmol) of [^{3}H]iodoacetic acid (New England Nuclear, Boston, MA, U.S.A.) at 37°C for 30 min in a helium atmosphere in the dark. The mixture was treated a second

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time with 2.5 mg (13.4 μmol) unlabeled iodoacetic acid. The reaction mixture was chromatographed twice on a Supelcosil LC-308 column (20 × 4.6 mm I.D., 5 μm, 300 Å; Supelco, Bellefonte, PA, U.S.A.) in 20 m*M* trifluoroacetic acid (TFA) with an acetonitrile gradient to remove excess reagents. The purified protein was then reduced with 300 μg 1,4-dithiothreitol (DTT, Pierce, Rockford, IL, U.S.A.) in 6 *M* guanidine–HCl, 0.1 *M* Tris (pH 8.5) at 37°C for 4 h. Another S-carboxymethylation with 400 μg (2.2 μmol) of [1-^{14}C]iodoacetic acid (New England Nuclear) and high-performance liquid chromatography (HPLC) purification were performed, as described above.

The sample was readied for tryptic digestion, by evaporating the acetonitrile and TFA under a stream of helium with repeated additions of water. The sample volume was finally adjusted to 50 μl with water. Then, 300 μl of immobilized trypsin (1:30; Trypsin-30, Enzygel; Boehringer, Indianapolis, IN, U.S.A.) in 300 μl 0.1 *M* ammonium bicarbonate (pH 8) were added. After overnight incubation at 37°C the enzyme beads were pelleted and the acidified supernatant (with 10 m*M* TFA to pH 2) subjected to HPLC.

The tryptic digest was purified in a high-performance liquid chromatograph, fitted with silanized glass-lined C-8 and MC-18 Chromegabond columns (250 × 2 mm, 5 μm, 300 Å; ES Industries, Marlton, NJ, U.S.A.). The sample was chromatographed with 0.1% TFA and an acetonitrile gradient from 0% to 60%. The column effluent was monitored by sampling aliquots automatically into a fluorescamine detection system[2].

Amino acid analyses were performed on an instrument equipped for post-column reaction with fluorescamine[3]. Samples were hydrolyzed in 6 *M* hydrochloric acid, 4% thioglycolic acid at 150°C for 1 h.

Automated Edman degradation was carried out on a Model 470A sequencer from Applied Biosystems (Foster City, CA, U.S.A.). Phenylthiohydantoin (PTH) amino acid analyses were performed according to a method described by Hawke *et al.*[4], but modified for an Ultrasphere ODS column (250 × 2 mm, 5 μm) at 43°C; flow-rate 180 μl/min; solvent A, 20 m*M* TFA, 1 m*M* acetic acid, 10% acetonitrile, (pH 5.1); solvent B, 20 m*M* TFA, 75% acetonitrile (pH 3.7); gradient, 5% B from 0 to 0.4 min, from 5% B to 13% B in 0.6 min, from 13% B to 62% B in 5.5 min, from 62% B to 65% B in 7.5 min, back to initial conditions in 3 min. Reagents for sequencing were obtained from ABI (Foster City, CA, U.S.A.), whereas all other solvents and reagents were of the highest purity available from several sources. Amino acid, peptide, and protein solutions were kept exclusively in polypropylene tubes.

C-Terminal sequence analysis was carried out by use of carboxypeptidase Y. The protein, 300 pmol, was incubated with 1 μg of immobilized enzyme (Pierce) in 50 μl of 100 m*M* sodium acetate (pH 5.5). Every 20 min, 5-μl aliquots were removed for amino acid analysis.

RESULTS AND DISCUSSION

In order to compare the primary structure of human rIL-2 with the one predicted from the cDNA[5], the amino acid sequence and the location of the disulfide bridge were determined. The protein was S-carboxymethylated with tritiated iodoace-

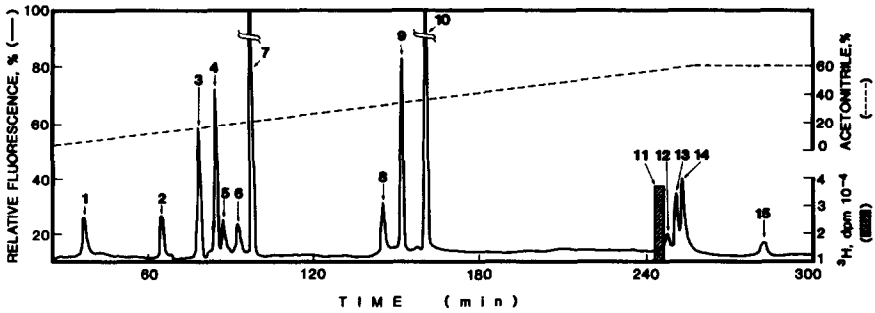

Fig. 1. Separation of tryptic peptides from recombinant human rIL-2 on Chromegabond MC-18. The numbered peaks refer to the tryptic peptides shown in Fig. 2.

tic acid prior to reduction and extensively freed from excess reagents by HPLC. The protein recovery from this desalting procedure was greater than 95%. After reduction of the remaining cystine with DTT, the newly generated sulfhydryl groups were labeled with [1-^{14}C]iodoacetic acid and the reaction mixture was purified by HPLC.

Reduction and S-carboxymethylation not only unfolds the protein and makes it more accessable for enzymatic cleavage, but also generates a radio-labeled cysteine derivative, which allows a convenient determination of the protein recovery at various steps and better quantitation during amino acid analysis and Edman degradation.

The fully reduced and S-carboxymethylated protein was fragmented with trypsin and subjected to HPLC with a gradient of acetonitrile in 0.1% TFA, as shown in Fig. 1. The tryptic peptides were collected in 3-min fractions and aliquots, corre-

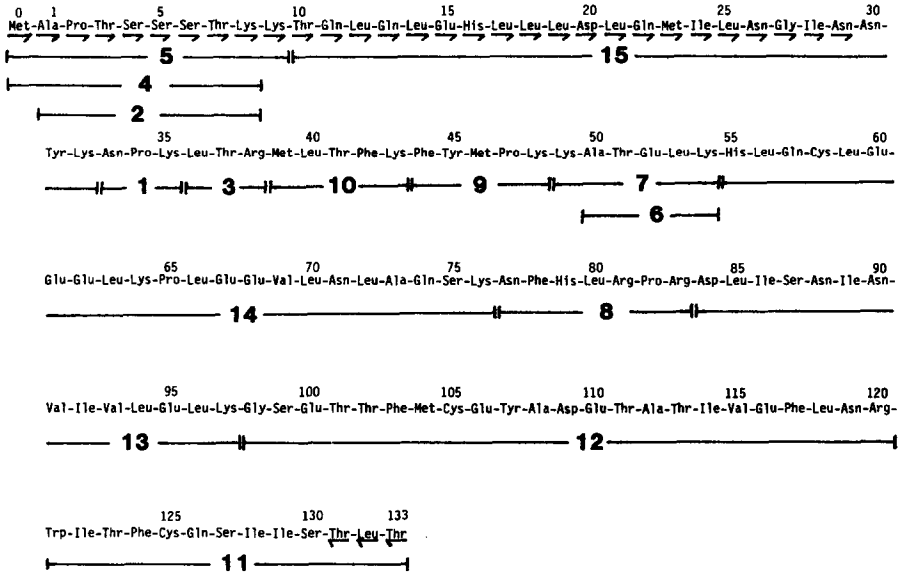

Fig. 2. Amino acid sequence of recombinant human rIL-2. The numbered regions mark tryptic peptides used for amino acid analysis and Edman degradation. Single arrows indicate N-terminal and C-terminal sequence determination from the intact protein.

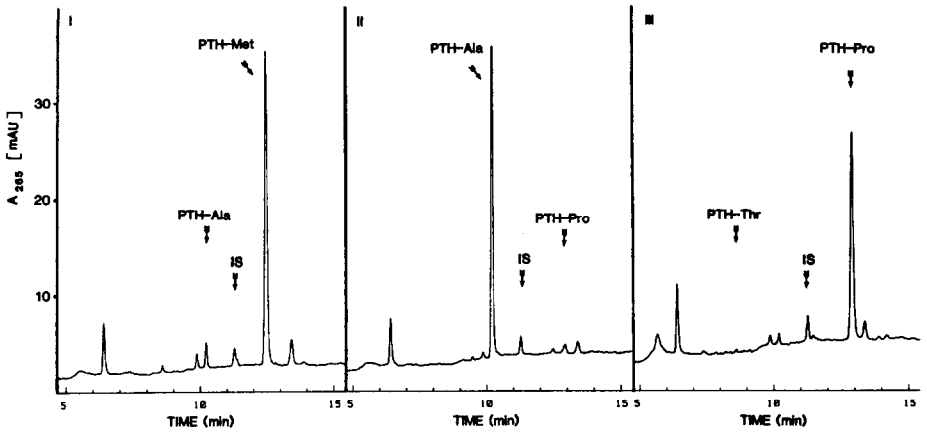

Fig. 3. HPLC separation from an N-terminal amino acid sequence analysis of 100 pmol of recombinant human rIL-2. Aliquots, representing half of each sample, were injected and separated, as described under Materials and methods. The expected PTH-amino acid derivatives and the internal standard (IS: PTH-D, L-α-aminobutyric acid) are marked by arrows. Major artifact peaks are N,N-dimethyl-N'-phenylthiourea (9.92 min) and N,N'-diphenylthiourea (13.48 min). The first three cycles are shown.

sponding to 1% of each peak (*ca.* 30 pmol), were submitted to amino acid analysis. The amino acid compositions of peptides 1 to 5, 7 to 10, and 15 corresponded to the peptides expected from complete tryptic digestion, as summarized in Fig. 2. Automated Edman sequence analysis revealed that peptide 6 was contaminated with a partial tryptic peptide composed of peptide 1 plus 3 (residues 33 to 38). Peptides 11, 12, 13 and 14 showed several discrepancies in their amino acid compositions. Sequence analysis confirmed that peptides 11 and 12 and peptides 13 and 14 were crosscontaminated with up to 10% with each other. Fragment 11, corresponding to the C-terminal end of interleukin-2, did not yield a reaction product with fluorescamine. The α-amino group expected to be present in this tryptic peptide is a part of tryptophan, which is known to yield a weak signal (unpublished results). Nevertheless, the peptide can be readily detected by its radiolabel. As this is the only tryptic peptide with a significant amount of the tritium label used for S-carboxymethylation prior to reduction, it most likely contains the cysteine residue with the free sulfhydryl group. Furthermore, peptides 12 and 14 were found predominantly labeled with [1-^{14}C]iodoacetic acid (data not shown). These results suggest that cysteine-58 and cysteine-105 form an intramolecular disulfide bridge, whereas cysteine-125 is present as the free sulfhydryl form.

The chromatogram of the tryptic digest reveals a heterogeneity of the recombinant protein at this N-terminus. Peptide 2 and peptide 4 both contain the N-terminal sequence, but differ by one amino acid. Peptide 4 contains a methionine in position 0, which was not removed completely by post-translational processing of this eucaryotic protein expressed in a procaryotic organism. A similar observation was made for human growth hormone expressed in *E. coli*[6].

N-Terminal automated Edman degradation was also carried out with the intact protein. The analysis of the PTH-amino acids was performed on an Ultrasphere ODS column with 2 mm I.D. to increase sensitivity. The analysis for the first three

amino acid cycles are shown in Fig. 3. The presence of an "out-of-phase" sequence by one residue confirms the findings of the tryptic map.

In conclusion, S-carboxymethylation with different radiolabels of human rIL-2, followed by purification, tryptic mapping with HPLC and amino acid sequencing yields detailed structural information about the protein. The amino acid sequence was found to be identical with the expected sequence from the recombinant gene.

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