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Journal of Chromatography A, 709 (1995) 135–146

JOURNAL OF CHROMATOGRAPHY A

Detection of traces of a trisulphide derivative in the preparation of a recombinant truncated interleukin-6 mutein

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Abstract

A new mutein of interleukin-6, called Δ22-IL-6 Cys 3,4, characterized by the deletion of the first 22 amino acids at the N-terminal end and by the substitution of the first two cysteines (Cys^{23} and Cys^{29}) with serine residues, was produced in *Escherichia coli* and was found to maintain the structural and functional properties of the human native form. A partially purified preparation still showed in isoelectric focusing a minor acidic component (p*I* 6.10) and a more basic component (p*I* 6.70), the native form having a p*I* of 6.56. This preparation was further fractionated in a multi-compartment electrolyser with isoelectric membranes, which allowed the collection of the more alkaline species for characterization. Mass spectra of the p*I* 6.70 form gave an additional mass of 32 atomic mass units (amu), suggesting the addition of two oxygen atoms (a potential oxidation of two methionine residues to sulphoxide). However, the five methionine residues in this higher p*I* form were identified after enzymatic hydrolysis and peptide mapping and were found to be in a reduced state. In addition, the p*I* 6.70 form was quickly converted into the native form by mild reductive treatment. On digestion and fingerprinting, the peptide from residues 50 to 65 of the p*I* 6.70 species (containing the only two cysteine residues of the molecule) exhibited a more hydrophobic behaviour in reversed-phase high-performance liquid chromatography and retained a mass increase of 32 amu. These experimental findings more likely suggest the addition of an extra sulphur atom to the only disulphide bridge to give an unusual protein trisulphide molecule.

1. Introduction

In the production of recombinant proteins, structural variations of the final product can arise at genetic and/or post-translational level or can be induced by process conditions; common changes in the covalent structure of recombinant proteins which depend on the operative conditions are, for example, deamidation of glutamine and asparagine side-chains, scrambling of disulphide bonds, generation of ragged ends by partial proteolysis or oxidation of sensitive residues [1,2]. Recently, a new post-translational modification consisting in a trisulphide cross-link (with an additional sulphur atom inserted into the Cys_2 disulphide bond) was independently discovered by two research groups as a side-

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0021-9673/95/$09.50
SSDI 0021-9673(95)00108-5

product of recombinant human growth hormone biosynthesized in *Escherichia coli* [3,4].

The identification of process-related modifications of recombinant proteins is an important issue for the characterization and validation of the final preparations. In addition, by knowing the chemico-physical nature of the induced modification it is possible to study a specific purification process which eventually results in the elimination of unwanted heterogeneous forms.

Interleukin-6 (IL-6) is a pleiotropic cytokine which can act on a wide variety of tissues. Depending on the nature of the target cells, IL-6 can exert growth promotion, growth inhibition, differentiation and induction of specific gene expression [5]. The full length form of human IL-6 consists of 185 amino acid residues starting with alanine at position 1 and contains four cysteine residues at positions 45, 51, 74 and 84 [6]. In certain cell types, IL-6 is also produced as a 184 amino acid protein with proline instead of alanine as the first amino acid [7].

Investigations of the relationship between the structure and biological function of IL-6-related proteins have demonstrated that the first 28 amino acids of mature human IL-6 are dispensable for biological activity [8] and that cysteine residues 45 and 51 could be substituted by serine residues without any impairment of the activity [9]. Bacterial expression of IL-6 derived muteins with N-terminal truncation and differential retention of the two disulfide bonds has recently been described [10]. Based on these previous results, we have studied the production of an IL-6 mutein characterized by the deletion of the first 22 amino acids at the N-terminal end and by the substitution of the first two cysteine (Cys^{23} and Cys^{29}) residues with serine residues, while the other two cysteine residues (Cys^{52} and Cys^{62}) were retained and gave a disulphide bridge (see Fig. 7: primary structure).

This new mutein, which is called Δ22-IL-6 Cys 3,4 and is under investigation for therapeutic application, was expressed in a recombinant strain of *E. coli* and was found to maintain the structural and functional properties of the human full length IL-6 [11]. In this paper, we demonstrate some unique structural modifications of a minor, higher p*I* component detected in partially purified preparations of Δ22-IL-6 Cys 3,4, suggesting the formation of a novel protein derivative with a trisulphide bridge.

2. Experimental

2.1. Equipment and chemical for electrophoresis

All analytical immobilized pH gradient (IPG) experiments were performed in a Multiphor II electrophoresis unit equipment with a Macrodrive 5 power supply and Multitemp thermostatic unit (Pharmacia Biotech, Uppsala, Sweden). Acrylamide, N,N′-methylenebisacrylamide, ammonium peroxodisulphate and N,N,N′,N′-tetramethylethylenediamine were obtained from Bio-Rad (Hercules, CA, USA). The Immobiline species used, p*K* 3.6, 4.6, 7.0 and 8.5, were from Pharmacia Biotech. HEPES [N-(2-hydroxyethyl)piperazine-N′-2-ethanesulphonic acid] and L-histidine (free base) were supplied by Sigma (St. Louis, MO, USA).

2.2. Preparation of analytical immobilized pH gradients

The gels were of 240 × 110 × 0.5 mm size. An IPG pH of 5.0–8.0 was set in a 5%T, 4%C polyacrylamide matrix [12]. After preparing the two limiting, acidic and basic mixtures, they were titrated (with weak acids and bases) to pH values close to neutrality, to ensure uniform polymerization and efficient monomer conversion throughout the pH gradient. On gel washing (4 × 30 min) in distilled water, all added titrants (and also catalysts and ungrafted monomers) were efficiently removed. The gels were then washed for 30 min in 20% glycerol, dried in air and reswollen in different additives.

2.3. Optimizing the additive concentrations

The use of dry gels allows the formation of an additive gradient at right-angles to the pH gra-

dient. This is of particular value when optimizing the additive concentrations in preliminary experiments. By applying and focusing the sample along the additive gradient, the optimum additive concentration can be determined in a single experiment.

Place the dry gel with its supporting film on a glass plate. Apply the U-frame (reswelling cassette for dry gels) on top of the gel. Clamp the glass plates together. A linear concentration gradient additive is formed by using a gradient mixer. Place 7.3 ml of rehydrating solution into each chamber of the gradient former and allow the gradient to flow from the top of the vertically standing cassette under gravity. The gels are rehydrated overnight. The protein samples (about 50 μg in 50 μl) are applied in surface wells both close to the anode and to the cathode. The electric focusing conditions are as follows: 1 h at 500 V, followed by 5000 V (maximum setting: 1 mA, 5 W) for 14 h at 10°C. Staining is carried out with Coomassie Brilliant Blue R-250 in copper sulphate solution [13].

2.4. Preparative IPGs in a liquid vein

Preparative runs were performed in a multi-compartment apparatus equipped with isolectric membranes (IsoPrime from Hoefer Sci., San Francisco, CA, USA) supported by glass-fibre filters [14,15]. After determining the p*I* values of the IL-6 isoforms in different additive solutions in analytical IPG pH 5–8 gels, eight isoelectric membranes were made with the following p*I* values: 5.50, 5.95, 6.47, 6.53, 6.57, 6.65, 6.92 and 7.35. The first and last membrane, being adjacent to anolyte and catholyte compartments, respectively, were made in a 10%T, 4%C matrix, whereas the other six were polymerized as 5%T, 4%C polyacrylamide matrices. The membranes had a diameter of 4.7 cm and a thickness of about 1 mm. After casting, the membranes were incubated 20 min in 100 m*M* ascorbic acid (pH 4.5) so as to eliminate potentially harmful N-oxides [16]. After washing and equilibrating the membranes in 20% glycerol, the multi-compartment apparatus was assembled and the protein (10 mg per 6.5 ml) was loaded into chamber 3. In order to avoid sample dilution, no reservoirs were connected to the chamber, so that the sample volume was limited to 6.45 ml per chamber [17]. After an initial, low-voltage run (maximum setting: 500 V, 1 mA, 1.5 W for 1 h) to eliminate salt in the sample, purification was continued at 3000 V, 3 mA, 1.5 W maximum for 6–16 h. The anolyte was 52 m*M* HEPES (pH 5.3, conductivity $1.5 \cdot 10^{-3}$ S m^{-1}). The supporting solution in all chambers was 20% glycerol (pH 5.3, conductivity $5 \cdot 10^{-4}$ S m^{-1}). No circulating coolant was utilized and Joule heat was dissipated in air in the cold room.

2.5. Preparation of IL-6 mutein

Δ22-IL-6 Cys 3,4 was expressed as cytoplasmic inclusion bodies by fermentation of a recombinant *E. coli* strain. Insoluble proteins recovered after mechanical cell breakage were dissolved in 6 *M* guanidine hydrochloride and refolded by tenfold dilution in pH 8 buffer solution. The presence of IL-6-related contaminants was studied using a preparation of Δ22-IL-6 Cys 3,4 partially purified starting from the refolded solution treated with 1.4 *M* ammonium sulphate and separated by column chromatography on phenyl-Sepharose (Pharmacia Biotech).

2.6. Reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC was performed using a Vydac C_4 column (250 × 4.6 mm I.D.; particle size 5 μm) at a flow-rate of 1 ml min^{-1}. Mobile phase A was 0.1% trifluoroacetic acid (TFA) (Pierce, Rockford, IL, USA) in water and mobile phase B was 0.07% TFA in 80% acetonitrile (Carlo Erba, Milan, Italy). Elution was performed with a 30-min gradient from 40 to 100% B and monitored with a UV detector at 220 nm.

2.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Discontinuous SDS-PAGE in 15% separating gel was performed according to Laemmli [18].

Samples were boiled in 2% SDS–0.025 *M* Tris (pH 6.8) with or without 5% β-mercaptoethanol. Gels were stained with Coomassie Brilliant Blue or silver stain. Immunoblotting was carried out according to the procedure of Towbin et al. [19] with some modifications. Following SDS-PAGE the proteins were electrotransferred to polyvinylidene difluoride membranes (Millipore) using a Multiphor semi-dry blotting apparatus (Pharmacia Biotech) according to the manufacturer's instructions. After blocking, overnight, the non-specific sites with 5% skim milk in Tris-buffered saline, immunostaining was performed by incubation for 1 h at room temperature with the primary antibody anti-human IL-6 (1 μg ml^{-1}) followed by incubation with the secondary antibody goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad, Richmond, CA, USA) diluted 1:1500. Detection was performed by enhanced chemiluminescence (Amersham International, Amersham, UK) according to the manufacturer's instructions.

2.8. Chemical modification of partially purified Δ22-IL-6 Cys 3,4

The oxidation was performed with 4% H_2O_2 for 1 h at room temperature and pH 7.4. The reduction was performed with 10, 5, 1 or 0.5 m*M* dithiothreitol (DTT) at pH 7.4 for 5 min at room temperature. The reaction (oxidation or reduction) was stopped by removing the reagent solution by gel filtration on disposable NAP5 columns (Pharmacia Biotech) equilibrated with 10 m*M* Tris–acetate (pH 7.4).

2.9. Peptide mapping and sequencing

Approximately 20 μg of protein were digested with 0.4 μg of sequencing grade endoproteinase-Lys C (Boehringer, Mannheim, Germany) by overnight incubation at 37°C in 1 *M* urea–0.1 *M* Tris (pH 8) buffer. The resulting peptidic fragments were separated by RP-HPLC on a C_{18} Vydac column; mobile components phase A (0.1% trifluoroacetic acid) and B (95% acetonitrile–0.07% trifluoroacetic acid) were used with linear gradient elution from 5 to 75% B in 60 min at a flow-rate of 1 ml min^{-1}. Eluted peaks were collected manually and submitted to N-terminal sequence analysis using a Model 477A pulsed-liquid phase sequencer with a Model 120A on-line analyser (Applied Biosystems, Foster City, CA, USA) for the detection of phenylthiohydantoin amino acids; standard manufacturer's programmes were used with minor modifications.

2.10. Electrospray mass spectrometry (ES-MS)

Samples collected after separation by RP-HPLC were injected at a flow-rate of 2 μl min^{-1} into a Hewlett-Packard Model 5989A single quadrupole mass spectrometer equipped with a Hewlett-Packard Model 59987A electrospray interface. Mass spectra were recorded in the positive-ion mode.

2.11. Bioassay

The biological activity of IL-6-related proteins was assayed using a proliferation test with murine hybridoma IL6-dependent cell line 7TD1 and employing spectrophotometric detection with the vital dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) as described [20].

3. Results

A new recombinant mutein of interleukin-6, Δ22-IL-6 Cys 3,4, was expressed in *E. coli* as insoluble aggregate inclusion bodies which were separated from soluble host proteins by cell breakage and centrifugation. The soluble and biologically active molecule was then obtained through a cycle of dissolution in chaotropic agent and dilution in renaturating buffer. Two simple purification steps consisting in ammonium sulphate precipitation of most of the contaminating proteins and hydrophobic interaction chromatography of the supernatant solution gave a product with a purity of about 90% when assessed by

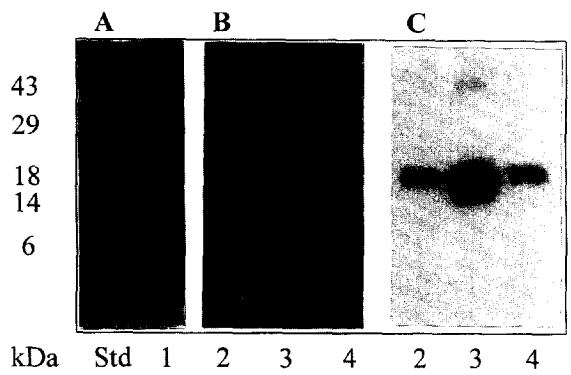

Fig. 1. Electrophoresis of truncated IL-6 muteins. 15% SDS-PAGE of partially purified Δ22-IL-6 Cys 3,4 (lane 1) and Δ22-IL-6 Cys 3,4 forms purified with the multi-compartment electrolyser: acidic form (lane 2), major form (lane 3) and basic form (lane 4). Standard protein markers (lane Std) are reported with their mass values shown on the left. Gel A was stained with Coomassie Brilliant Blue, gel B with silver stain; immunoblotting was performed on gel C using polyclonal anti-human IL-6 antibodies and chemiluminescence detection.

SDS-PAGE (Fig. 1, lane 1) and RP-HPLC analysis (Fig. 2).

However, IPG-isoelectric focusing (IEF) showed the presence of two minor product-related proteins with more basic (pI = 6.70) and more acidic (pI = 6.10) properties than the main component Δ22-IL-6 Cys 3,4 (pI = 6.56). In order to obtain some chemical evidence on the composition of the minor components with different pI values, it was necessary to set up a high-resolution preparative IEF technique. The IsoPrime equipment, allowing for the preparative purification of protein isoforms in between sets of isoelectric membranes, was adopted for this task. However, as proteins at their pI values have minimum solubility, it was necessary to explore the solubilizing power of different non-denaturing agents added to the solvent. According to Hjertén [21], compounds such as ethylene glycol and propylene glycol can have a beneficial influence in preventing protein aggregation and precipitation. According to Timasheff and Arakawa [22], glycerol also appears to be a protein-stabilizing agent, as it seems to be excluded from protein surfaces by solvophobic effects. We therefore explored a number of such solubilizers, compatible with a focusing process, by running a continuous sample streak against a concentration gradient of such additives.

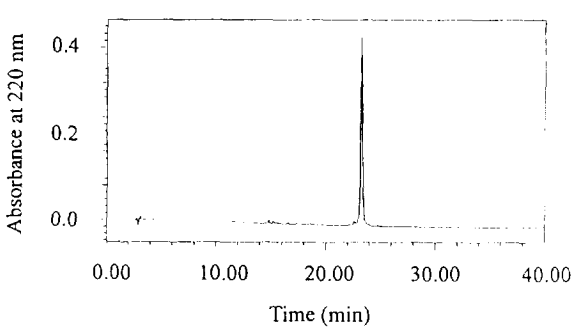

Fig. 2. RP-HPLC of a partially purified preparation of Δ22-IL-6 Cys 3,4. The sample was applied to a Vydac C_4 column and eluted with a linear gradient from 32 to 80% of acetonitrile. The chromatographic purity of the preparation was 88%, as calculated from peak areas.

As shown in Fig. 3, Δ22-IL-6 Cys 3,4 is compatible with up to about 50% additive. Above this level, two phenomena occur. If the sample has been applied at the cathodic gel side, it still moves to the pI position. However, the protein seems to shift its pI to a more alkaline value (possibly by partial unfolding) and the zone is blurred, indicating sample precipitation in the proximity of the pI region. The second phenomenon, which occurs on anodic sample application, is massive precipitation of the protein and denaturation directly at the application site, just around and above the 50% (v/v) ethylene glycol concentration threshold.

A similar phenomenon occurs in a propylene glycol concentration gradient: on anodic sample application, above 45–50% additive concentration, protein denaturation and precipitation at the deposition site take place (Fig. 4B). In contrast, Δ22-IL-6 Cys 3,4 seems to be compatible with up to 70% (v/v) glycerol level in the solvent (Fig. 4C). Dimethyl sulphoxide shows a similar behaviour to ethylene glycol and propylene glycol (Fig. 4A): on anodic application, Δ22-IL-6 Cys 3,4 begins to change its pI and starts to precipitate at the deposition site, although the phenomenon is much less pronounced than in the case of the two glycols.

Based on the above information, preparative

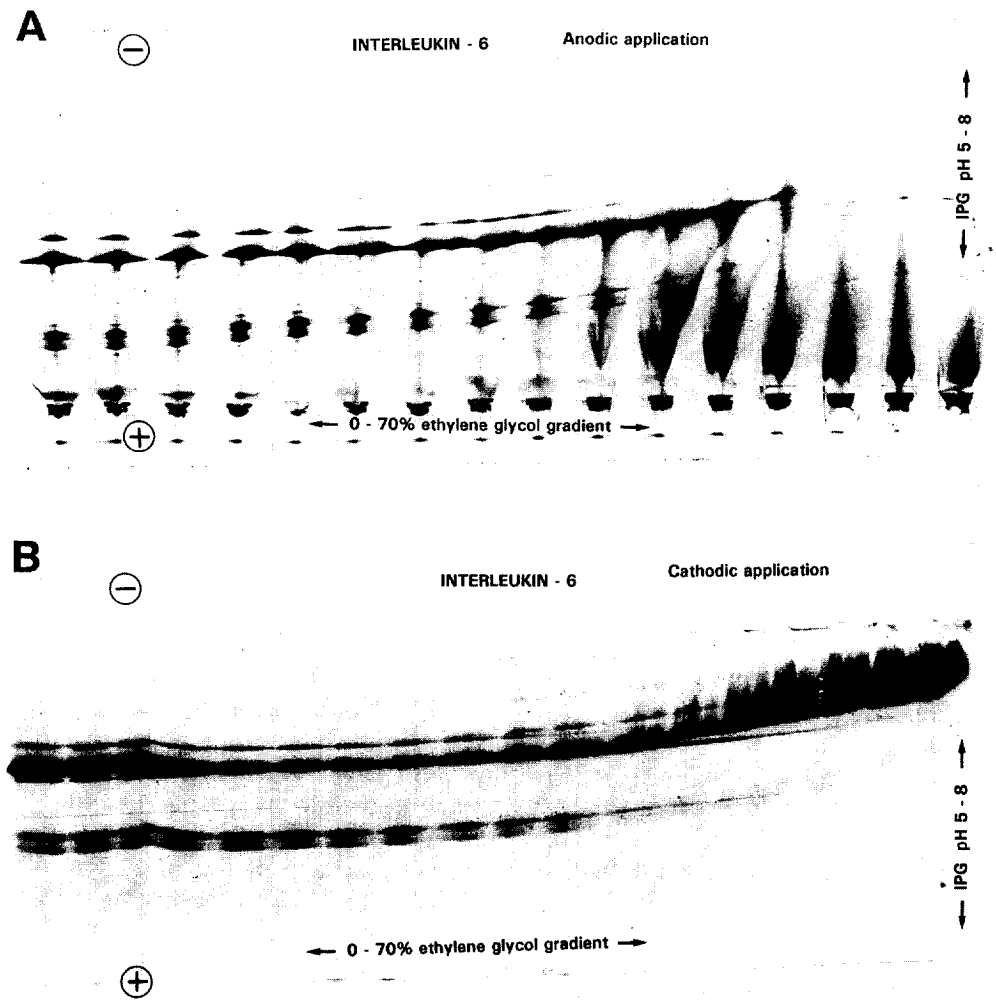

Fig. 3. Effect of ethylene glycol as solubilizer of Δ22-IL-6 Cys 3,4 isoforms at their p*I* values. IPG pH 5.0–8.0 linear gradient, reswollen in an ethylene glycol gradient (from 0 to 70%) perpendicular to the pH gradient. Both gels are made as 5%T, 4%C matrices. Sixteen samples (50 μg per sample) were applied along the acidic gel side (A) or along the basic gel side (B) and focused to the steady state (run at 5000 V for a total of 70 000 V h). Staining with Coomassie Brilliant Blue R-250 in the presence of Cu^{2+}.

runs in the IsoPrime electrolyser were conducted in presence of 20% glycerol as a solubilizing agent. Fig. 5 shows the results of such a preparative run. The main component (p*I* 6.56) was trapped in chamber 3, between two membranes having p*I*s of 6.53 (anodic) and 6.57 (cathodic). The p*I* 6.10 isoform was collected in chamber 2 (between a p*I* 5.95 and a p*I* 6.47 membrane) and the p*I* 6.70 in chamber 4 (between the p*I* 6.65 and p*I* 6.92 membranes). When analysed by SDS-PAGE (Fig. 1) both of these two minor components exhibited the same relative molecular mass (M_r) as the major Δ22-IL-6 Cys 3,4 form and were recognized by the same antibody, suggesting that they are modified Δ22-IL-6 Cys 3,4 species. Moreover, both minor components were eluted with virtually the same retention time in RP-HPLC (see Table 1). It can also be appreciated (Fig. 1, lane 1) that endogenous contaminants not related to IL-6 and having a

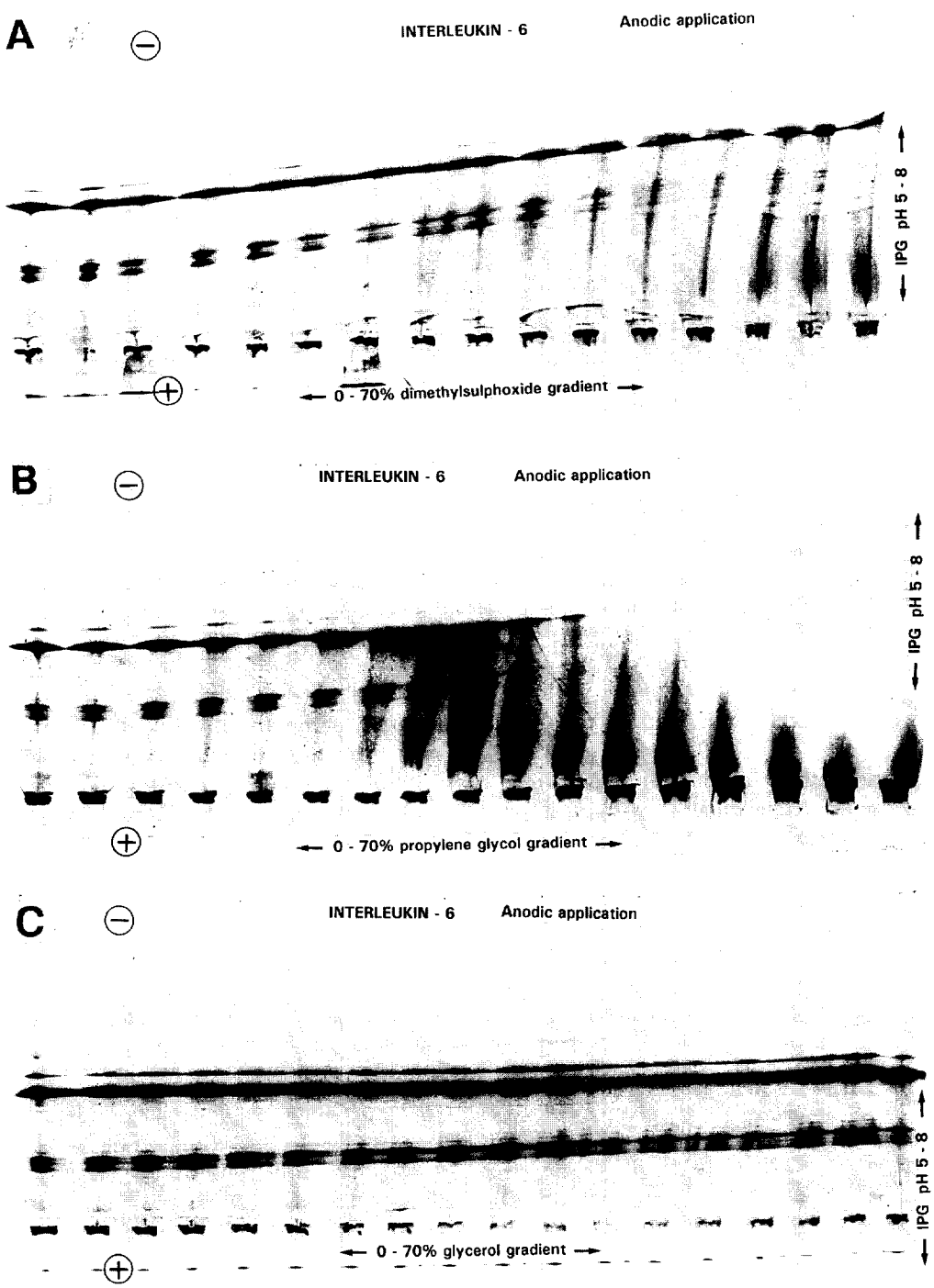

Fig. 4. Effect of different solubilizers on Δ22-IL-6 Cys 3,4 isoforms at their p*I* values. IPG pH 5.0–8.0 linear gradient, reswollen in an (A) dimethyl sulphoxide, (B) propylene glycol or (C) glycerol gradient (from 0 to 70%) perpendicular to the pH gradient. All gels are made as 5%T, 4%C matrices. Sixteen samples (50 μg per sample) were applied along the acidic gel side and focused to the steady state (run at 5000 V for a total of 70 000 V h). Staining with Coomassie Brilliant Blue R-250 in the presence of Cu^{2+}.

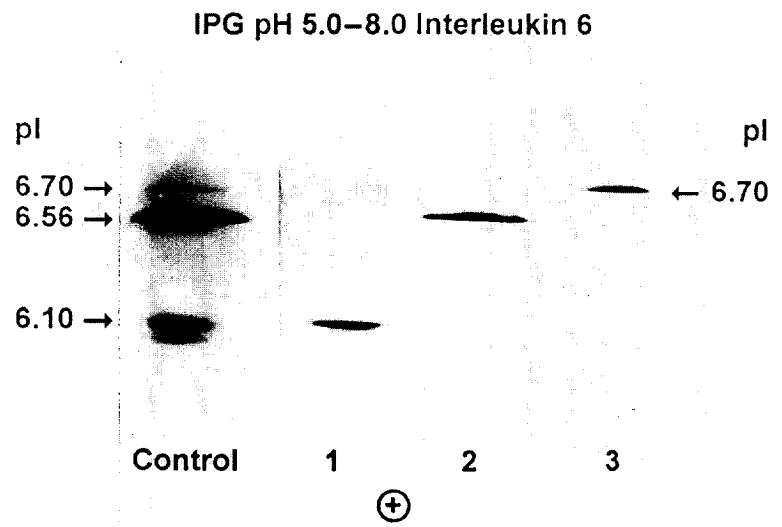

Fig. 5. Analytical IPG-IEF of isoforms of Δ22-IL-6 Cys 3,4 as recovered from the IsoPrime purification process. Analytical gel: IPG pH 5–8 linear gradient, in a 5%T, 4%C matrix, reswollen in 20% glycerol. All samples were applied in surface well (cathodic load) as 70-μl droplets (with variable amounts of proteins, from 10 to 50 μg). Focusing at 10°C, 5000 V (after an initial period of 1 h at 500 V) for 12 h. The cathode is uppermost. Staining with Coomassie Brilliant Blue R-250 in the presence of Cu^{2+}. Control = unfractionated, starting material; 1 = content of chamber 2 (p*I* 6.10 isoform); 2 = content of chamber 3 (p*I* 6.56, major component); 3 = content of chamber 4 (basic isoform, with p*I* 6.70).

lower M_r were efficiently removed in the IsoPrime run.

The biological activity of the three Δ22-IL-6 Cys 3,4 forms was determined by measuring their ability to induce proliferation of the IL-6-dependent 7TD1 cells. The results showed the same high specific activity for Δ22-IL-6 Cys 3,4 and the acidic form, while the activity of the basic form was about 75% lower (Table 1).

It has been widely reported that acidic derivatives of proteins can be formed by deamidation of the side-chain of glutamine or asparagine, as a spontaneous slow reaction or induced by environmental conditions [23]; however, the occur-

Table 1
Characterization of Δ22-IL-6 Cys 3,4 and its derivatives separated by preparative immobilized pH gradient isoelectric focusing (IPG-IEF)

Sample	RP-HPLC retention time (min)	IPG-IEF, p*I*	Mass spectrometry, M_r	Bioassay relative potency (%)
Starting preparation	23.2	–	18 695	100
Acidic form	23.1	6.10	n.d.[a]	100
Main form	23.9	6.56	18 695	100
Basic form	23.7	6.70	18 727 (+32)	25

The three Δ22-IL-6 Cys 3,4 forms with different p*I*, purified with the multi-compartment electrolyser, showed the same retention time in RP-HPLC. The more basic form showed a lower in vitro biological activity than the other forms (EC_{50} for the starting material was about 10 pg ml^{-1}). The relative molecular mass (M_r) of the main form matched the calculated value of 18 696, while the basic form showed a 32 amu increase.

[a] Not determined.

rence of a protein derivative with more basic properties is much less documented.

For this reason, we focused our interest on the elucidation of the modification of the basic form. Determination of relative molecular mass by ES-MS analysis of the major component prepared by IPG-IEF gave an M_r value of 18 695, which corresponds to the calculated value of 18 696 for Δ22-IL-6 Cys 3,4, while the basic form gave a shift of +32 atomic mass units (amu), suggesting the addition of two oxygen atoms. These data, at first, seemed compatible with the oxidation of one methionine residue to methionine sulphone or alternatively with the oxidation of two residues to methionine sulphoxide, which is a covalent modification likely to occur during the production of recombinant proteins [24,25].

Peptide fragments of Δ22-IL-6 Cys 3,4 containing the five methionine residues of the molecule were therefore identified by sequence analysis after endoproteinase-Lys-C digestion and peptide mapping; the same was done on a sample of Δ22-IL-6 Cys 3,4 extensively oxidized in vitro by incubation with H_2O_2. As reported in Table 2, the peptide fragments containing the oxidized methionine residues were eluted with a slightly shorter retention time owing to the decrease in hydrophobicity induced by the formation of methionine sulphoxide. However,

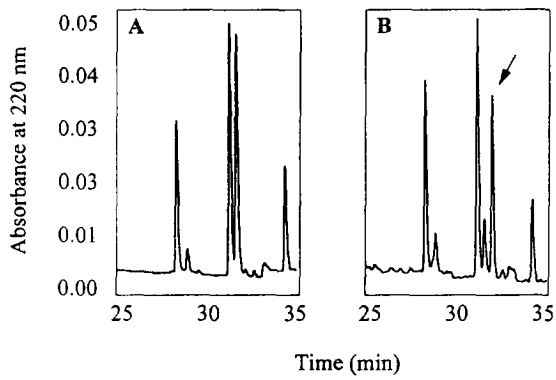

Fig. 6. Comparison of peptide mapping of Δ22-IL-6 Cys 3,4 and its more basic form. Partial elution profile (25–35 min) from a C_{18} HPLC column of endoproteinase-Lys-C digestion products of Δ22-IL-6 Cys 3,4 (A) and of the higher p*I* 6.70 isoform (B). At time 32.0 min [as indicated by the arrow in (B)] is shown the peak which is not present in native Δ22-IL-6-Cys 3,4 or in in vitro oxidized Δ22-IL-6 Cys 3,4 digest.

enzymatic digestion and peptide mapping of the basic form only showed the presence of the five peptides containing non-oxidized methionine residues (Table 2), ruling out the possibility that the 32 amu increase of this form was due to methionine oxidation.

Interestingly, the only difference between the peptide mapping of Δ22-IL-6 Cys 3,4 and its more basic form was a shift in the RP-HPLC

Table 2
Identification of oxidized methionine residues (Met-SO) in Δ22-IL-6 Cys 3,4 after in vitro treatment with H_2O_2 and in the basic form separated by preparative IPG

Peptide	RP-HPLC retention time (min)	Δ22-IL-6 Cys 3,4[a]		
		Control	H_2O_2 treated	Basic isoform
46–49 (Met^{46})	4.9	+	[b]	+
26–33 (Met^{28})	5.9	+	[b]	+
151–163 (Met^{163}-SO)	33.0	–	+	–
151–163 (Met^{163})	36.8	+	–	+
130–150 (Met^{140}-SO)	37.6	–	+	–
130–150 (Met^{140})	42.0	+	–	+
66–69 (Met^{96}-SO)	47.0	–	+	–
66–69 (Met^{96})	47.5	+	–	+

[a] + and – indicate the detection or not, respectively, of the corresponding peak in peptide mapping.
[b] After H_2O_2 treatment peptides containing Met^{28}-SO and Met^{46}-SO are not detectable because they are eluted in the flow-through.

1	SERIDKQIRY	ILDGISALRK	ETSNKSNMSE	SSKEALAENN
41	LNLPKMAEKD	GCFQSGFNEE	TCLVKIITGL	LEFEVYLEYL
81	QNRFESSEEQ	ARAVQMSTKV	LIQFLQKKAK	NLDAITTPDP
121	TTNASLLTKL	QAQNQWLQDM	TTHLILRSFK	EFLQSSLRAL
161	RQM			

Fig. 7. Primary structure of Δ22-IL-6 Cys 3,4. The amino acid sequence is reported as one-letter codes. The location of the single disulphide bond between cysteine 52 and 62 is shown. Underlined residues, from amino acid residues 50–65, represent the modified site found in the more basic form of Δ22-IL-6 Cys 3,4.

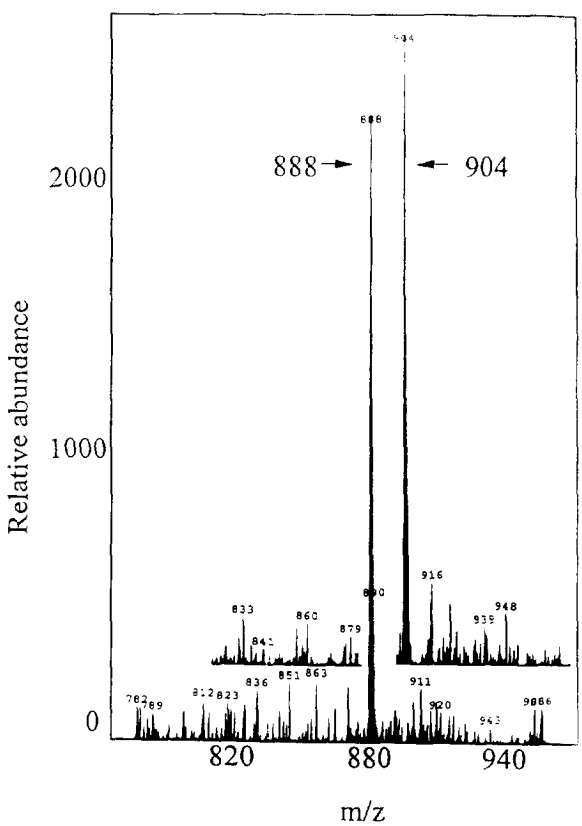

Fig. 8. Mass spectrometry of peptide 50–65. Overlay mass spectra of peptide 50–65 from Δ22-IL-6 Cys 3,4 showing a peak at $(m + 2H)^{2+} = 888$, corresponding to 1774 amu and from the higher p*I* derivative (p*I* 6.70) showing a peak shift to $(m + 2H)^{2+} = 904$, corresponding to 1806 amu.

elution profile of the peak corresponding to the peptidic fragment from residue 50 to 65 (Fig. 6), with the peptide of p*I* 6.70 species exhibiting a more hydrophobic behaviour. The sequence of the peptide 50–65 is as follows: H_2N-D-G-X-F-Q-S-G-F-N-E-E-T-X-L-V-K-OH (where X represents a cysteine residue which is not detectable by direct sequencing) and in Δ22-IL-6 Cys 3,4 a disulphide bond links the two cysteine residues present in positions 52 and 62 (Fig. 7, primary sequence).

Sequence analysis of the peptide 50–65 derived from Δ22-IL-6 Cys 3,4 and its corresponding shifted peptide from the more basic form confirmed the sequence identity. ES-MS analysis gave a single signal at *m/z* 888 and 904 for the peptidic fragment 50–65 from Δ22-IL-6 Cys 3,4 and from the more basic form, respectively (Fig. 8); these peaks can be assigned to a doubly charged molecule $(m + 2H)^{2+}$ corresponding to 1774 amu for peptide 50–65 from Δ22-IL-6 Cys 3,4 (whose calculated mass is 1775 amu) and to 1806 (+32 amu) for the corresponding peptide 50–65 from the higher p*I* (6.70) form.

Inspection of the amino acid sequence of peptide 50–65 and the fact that the more basic p*I* 6.70 form of Δ22-IL-6 Cys 3,4 can be easily transformed into the main p*I* 6.56 form by mild

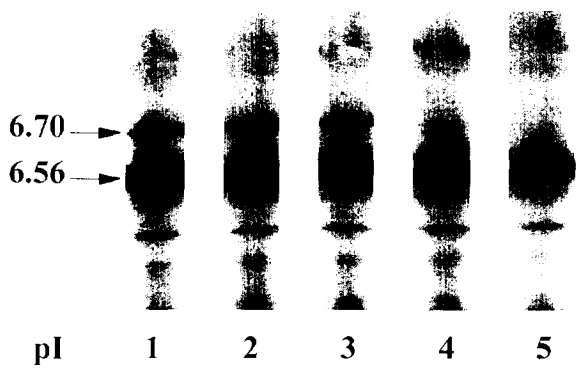

Fig. 9. Reduction of Δ-IL6 Cys 3,4 more basic form. Analytical IPG pH 6.2–7.1 linear gradient of partially purified Δ22-IL-6 Cys 3,4 not incubated (lane 1), and incubated for 5 min with 0.5 m*M* (lane 2), 1 m*M* (lane 3), 5 m*M* (lane 4) and 10 m*M* dithiothreitol (lane 5). Isoelectric points are indicated on the left. The more basic form p*I* 6.70 was reduced with 5 m*M* dithiothreitol.

reductive treatment (Fig. 9) strongly suggest a modification on the Cys_2 cross-link.

4. Discussion

Expanding commercial applications of genetic engineering techniques in the 1980s have led to the manufacture of a large number of protein products in recombinant systems. The production process of recombinant proteins can expose them to conditions which potentially are able to induce conformational alterations or covalent modifications of amino acid side-chains.

The identification of minor protein modifications usually requires the application of different analytical methodologies with high resolving power, e.g., SDS-PAGE, RP-HPLC, peptide mapping and mass spectrometry. Isolectric focusing techniques are increasingly being used for these analyses owing to their capability to detect even very minor charge heterogeneity of proteins. It has been relatively simple to attribute lower p*I* components to modifications such as deamidation, glycosylation, sulphation, phosphorylation and to all the array of surface charge modifiers that lower the net surface charge. However, higher p*I* components have gone unexplained up to the present time. The higher p*I* form of the IL-6 mutein described in this paper displayed a measured mass increase of 32 amu, which, in principle, could arise from the addition of two oxygen atoms (32.00) or of a single sulphur atom (32.07). The peptide fragment 50–65 isolated from the p*I* 6.70 form retained the 32 amu mass increase; the lack of any methionine residue in this fragment excluded the oxidative modification of methionine and restricted the mass modification to the Cys^{52}–Cys^{62} bridge.

A vast literature exists on the oxidation of thiols in aliphatic and aromatic compounds [26]. Whereas in protein chemistry we usually deal with only three oxidation states of cysteine (free thiol, disulphide and, in extreme cases, cysteic acid), in reality thiols are oxidized by a variety of reagents to a whole series of intermediate oxidation products, depending on the specific reaction conditions. Perhaps the best known oxidation pathway for thiols is via molecular oxygen. The ease of oxidation of thiols on exposure to air is well known, as is the sensitivity of this reaction to catalysts, such as metal ions, UV radiation and other initiators of radical reactions. It is also reported that auto-oxidation is accelerated by bases. In the case of cysteine, between the two oxidation states Cys_2 and cysteic acid, except for the very labile sulphenic acid, a number of stable intermediates exist: mono-, di-, tri- and tetrasulphone intermediate products have been reported [27].

Following this reasoning, we could speculate that the mass increase observed in the p*I* 6.70 form could be due to the addition of two oxygen atoms to give a cysteine sulphone derivative. However, the peptide 50–65 isolated from the p*I* 6.70 form exhibited a more hydrophobic behaviour than the unmodified peptide 50–65, which is not compatible with the formation of a more oxygenated form. Recently, Canova-Davis [3] and Jespersen et al. [4] described a novel post-translational modification of proteins, identified in recombinant growth hormone preparations, characterized by a 32 amu increase due to the addition of a sulphur atom to a Cy–S–S–Cy bridge (where Cy represents cysteine minus an SH group) to give the trisulphide Cy–S–S–S–Cy. The trisulphide proteins or peptide fragments are, as expected, more hydrophobic than the corresponding unmodified molecules and are easily reverted to the Cy–S–S–Cy form by mild reductive treatment [4]. Our experimental results and the above literature data allow us to propose a trisulphide modification of the single Cys^{52}–Cys^{62} bridge for the more basic Δ22-IL-6 Cys 3,4 form isolated as a minor contaminant of Δ22-IL-6 Cys 3,4 mutein produced in *E. coli*. We have excluded the possibility of an artefactual modification of cysteine residues due to the IPG technique on the grounds that the isolated bands do not redistribute on re-focusing, as is evident from the analytical gels in Fig. 5 (cf., the track of the p*I* 6.56 and 6.70 components).

The mechanism of trisulphide formation in proteins is unknown, although it has been suggested [4] that disulphide bridges of proteins produced in *E. coli* as inclusion bodies and

recovered by cell disruption could be exposed to and cleaved by HS^- ions in a disulphide exchange reaction forming a hydrogen disulphide derivative of one cysteine, which could then react with the other free cysteine to form a trisulphide bond. Trisulphide derivatives of both the growth hormone reported previously [3,4] and Δ22-IL-6 Cys 3,4, reported here, have only been identified in recombinant proteins expressed as cytoplasmic inclusion bodies in *E. coli*. We do not know at present if such an increment in p*I* values, in the case of a modified disulphide bridge to trisulphide, can be a general mechanism valid also for other proteins, but certainly it is a novel finding worth considering when confronted with higher p*I* isoforms in recombinant proteins.

Acknowledgements

This work was supported in part by a grant from Agenzia Spaziale Italiana (ASI, Rome), by the Radius Project in Biotechnology (ESA, Paris) and by CNR, Comitato Medicina e Biologia. E. Wenisch is the winner of the first Luigi Napolitano fellowship (ESA).

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