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Structure, stability and biological properties of a N-terminally truncated form of recombinant human interleukin-6 containing a single disulfide bond

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A mutant species of the 185-residue chain of human interleukin-6 lacking 22-residues at its N-terminus and with a Cys→Ser substitution at positions 45 and 51 was produced in *Escherichia coli*. The 163-residue protein des-(A1–S22)-[C45S, C51S]interleukin-6, containing a single disulfide bridge, formed inclusion bodies. Mutant interleukin-6 was solubilized in 6 M guanidine hydrochloride, subjected to oxidative refolding and purified to homogeneity by ammonium sulfate precipitation and hydrophobic chromatography. The purity of the mutant species was established by electrophoresis, isoelectrofocusing and reverse-phase HPLC and its structural identity was checked by N-terminal sequencing of both the intact protein and several of its proteolytic fragments. Electrospray mass spectrometry analysis of mutant interleukin-6 gave a molecular mass of 18695 ± 2 Da in excellent agreement with the calculated value. Circular dichroic, fluorescence emission and second-derivative ultraviolet absorption spectra indicated that mutant interleukin-6 maintains the overall secondary and tertiary structure, as well as stability characteristics, of the recombinant wild-type human interleukin-6. The urea-induced unfolding of mutant interleukin-6, monitored by circular dichroic measurements in the far-ultraviolet region, occurs as a highly cooperative process with a midpoint of denaturation at 5.5 M urea. The data of the reversible unfolding of mutant interleukin-6 mediated by urea were used to calculate a value of 20.9 ± 0.4 kJ · mol^{-1} for the thermodynamic stability of the protein at 25 °C in the absence of denaturant. The biological activity of mutant interleukin-6 was evaluated *in vitro* by the hybridoma proliferation assay, and *in vivo* by measuring thrombopoiesis in monkeys. Dose/response effects of the mutant were comparable or even higher than those of the wild-type protein. Overall the results of this study show that mutant interleukin-6 is a biologically active cytokine, which could find practical use as a therapeutic agent.

Keywords. Interleukin-6; protein engineering; disulfide bond substitution; protein stability; circular dichroism.

Human interleukin-6 (IL-6) is a multifunctional cytokine produced by a variety of cell types and, depending on the nature of the target cells, it can influence cell growth, differentiation, and the induction of specific gene expression (Kishimoto, 1989; Van Snick, 1990). Intensive research is being conducted in several laboratories in order to determine the mechanism of action of IL-6 and to investigate its possible use as a therapeutic agent in the treatment of a number of severe diseases, such as multiple myeloma and rheumatoid arthritis (Hirano et al., 1990). The secreted form of fibroblast-derived and leukocyte-derived interleukin-6 is a 185-residue polypeptide chain containing two disulfide bonds (Cys45–Cys51 and Cys74–Cys84; Van Damme et al., 1987; Clogston et al., 1989). IL-6 is subject to several post-translational modifications and thus multiple species of the protein were identified (Parekh et al., 1992). Proteolytic processing of human IL-6 leads to the removal of one or two amino acid residues at the N-terminus of the chain (Parekh et al., 1992). Other post-translational processing events of the interleukin-6 chain include phosphorylation at serine-residues and glycosylation at residues 46 and 145 (Van Damme et al., 1987; Simpson et al., 1988). Recently, the carbohydrate structure of the glycosylated human interleukin-6 was established by Parekh et al. (1992). The precise functional role of these post-translational events is not yet fully understood, but these covalent modifications of the cytokine apparently are not required for its biological activity, since recombinant wild-type human interleukin-6 produced in *Escherichia coli*, which lacks eukaryotic post-translational modifying reactions, retains the biological activities of the mature cytokine (Yasueda et al., 1990).

Structure/activity relationships of IL-6 have been studied utilizing mutants of this cytokine expressed in bacteria (Snouwaert et al., 1991a,b; Krüttgen et al., 1990; Jambou et al., 1988; Leebeek et al., 1992; Savino et al., 1993). In particular, it has been demonstrated that deletion of up to 28 amino acid residues from the N-terminus of human interleukin-6 does not impair bio-

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Abbreviations. IL-6, interleukin-6; mutant IL-6, recombinant human des-(A1–S22)-[C45S, C51S]IL-6 with a deletion of 22 amino acid residues at its N-terminus and with a Cys → Ser substitution at positions 45 and 51 of the wild-type polypeptide chain; $[\theta]$, mean residue ellipticity; Gdn · HCl, guanidine hydrochloride; $\Delta G_D^{H_2O}$, Gibbs free energy of denaturation in the absence of denaturant; EU, endotoxin units.

Enzymes. Trypsin (EC 3.4.21.4); endoprotease Glu-C from *Staphylococcus aureus* V8 (EC 3.4.21.19); endoprotease Lys-C (EC 3.4.21.50).

Note. The novel amino acid sequence data published here have been submitted to the EMBL sequence data bank(s) and are available under accession number(s) PO5231.

logical activity (Brakenhoff et al., 1989), while deletion of just a few residues from the C-terminus results in almost complete loss of biological activity (Krüttgen et al., 1990). The relative importance of the two disulfide bonds of the 185-residue chain of wild-type human IL-6 (Cys45 Cys51 and Cys74 Cys84) for the maintenance of biological activity has been examined by replacing pairs of cysteine residues with other amino acid residues (Jambou et al., 1988; Snouwaert et al., 1991b). It has been found that the first disulfide bridge Cys45 Cys51 does not play a functional role, whereas the activity of the mutant lacking the second disulfide bridge, Cys74 Cys84, was significantly reduced (Snouwaert et al., 1991b; Jean et al., 1993). In a recent study, Rock et al. (1994) reexamined the structural and functional properties of several disulfide-bond-deficient analogs of human interleukin-6 prepared both by chemical reduction and *S*-alkylation of the protein, as well as by site-directed mutagenesis employing Cys→Ala replacements. Unlike previous reports (Snouwaert et al., 1991b; Jean et al., 1993), these authors concluded that the Cys45 Cys51 disulfide bridge slightly contributes to the full biological activity of the cytokine. In agreement with these findings, Dagan et al. (1992) reported the preparation of a biologically active mutant of human interleukin-6 lacking 22 residues at the N-terminus and the first disulfide bridge.

In this study, we describe the large-scale production and the detailed characterization of a human interleukin-6 mutant with a deletion of 22 residues (APVPPGEDSKDVAAPHRQPLTS) at its N-terminus and with a Cys→Ser exchange for cysteine residues at positions 45 and 51 of the chain. The resulting 163-residue protein des-(A1−S22)-[C45S, C51S]IL-6 (hereafter mutant IL-6, see Fig. 1), containing a disulfide bond linking Cys52 and Cys62, was produced by fermentation of a recombinant *E. coli* strain as an insoluble aggregate. A suitable procedure was developed to dissolve and refold the recombinant mutant interleukin-6, which was purified to homogeneity and its structural identity was verified by several chemical and physicochemical criteria. Spectroscopic analyses indicated that the mutant protein maintains the overall folding and stability properties of the wild-type recombinant human interleukin-6 and, most importantly, both *in vitro* and *in vivo* experiments showed that the biological and pharmacological activities of the mutant cytokine were comparable or even higher than those of the wild-type species.

MATERIALS AND METHODS

Expression and purification of mutant interleukin-6. The gene coding for the full length cysteine-free IL-6 molecule was first assembled from synthetic oligonucleotides and subcloned into an appropriate expression vector (Jambou et al., 1988; Snouwaert et al., 1991b). The two residues at positions 74 and 84 were substituted by cysteine residues and the first 22 N-terminal amino acids were deleted to obtain the gene coding for the mutant species des-(A1−S22)-[C45S, C51S]IL-6. The construct was inserted into the pKK233-2 expression vector (Pharmacia) containing the Ptrc promoter and the gene for the resistance to ampicillin.

An *E. coli* HB101 strain was transformed with the vector containing the gene for mutant IL-6 and was cultivated in a 10-l fermentor with 4 l Shiloach medium containing 100 μg ampicillin/ml. During fermentation, glucose was maintained at a concentration of 1−2 g/l and oxygen was maintained at a saturation of 40%. The temperature was kept at 33 °C for the first 15 h and at 37 °C until the end of the process. Cultures were grown to reach an absorbance (measured at a wavelength of 600 nm) of 16−18 absorption units when they were induced with 10 g/l of lactose. Cells were harvested 4 h after induction by centrifugation at 7000 rpm in a A6.9 Kontron rotor for 10 min and stored at −80 °C until further processing.

Wet cellular paste (360 g, recovered from a 6-l fermentation) was suspended in 2 l buffer containing 20 mM Tris/HCl, 50 mM NaCl, 10 mM EDTA, pH 8 (buffer A) and was disrupted in a high-pressure homogenizer (Rannie-APV) by three passages at $6-8\times10^7$ Pa. Insoluble material was recovered by centrifugation at $7000\times g$ for 1 h at 4 °C. The pellet was first washed with 1.4 l 0.5% Triton X-100 in buffer A and subsequently with buffer A alone; each wash was followed by a centrifugation at $7000\times g$ for 1 h in order to recover the insoluble inclusion bodies.

Washed inclusion bodies were solubilized by overnight incubation at 4 °C in 1.4 l 6 M Gdn · HCl, 50 mM Tris/HCl, 1 mM EDTA, pH 8.5. The cloudy solution was clarified by centrifugation at $7000\times g$ for 30 min and diluted tenfold, under agitation, with 20 mM Tris/HCl, 0.1 M NaCl; 1 mM EDTA, pH 8.5. The solution was left for 2 h and solid ammonium sulfate was slowly added to obtain a final concentration of 1.5 M. The precipitated contaminating proteins were allowed to separate overnight and the supernatant was filtered using Whatman N.1 filter paper. The filtered solution was loaded at a flow rate of 600 ml/h onto a column (9 cm×12 cm; bed volume approximately 800 ml) of phenyl-Sepharose (FF resin, Pharmacia) previously equilibrated with 1.5 M ammonium sulfate in 20 mM Tris/HCl, pH 7.4. The column was washed with 1.5 l equilibration buffer and eluted at a flow rate of 750 ml/h with a decreasing gradient from 1.5 M ammonium sulfate in 20 mM Tris/HCl, pH 7.4 (1 l) to buffer alone (1 l). Eluted fractions were analyzed by reverse-phase high-performance liquid chromatography (HPLC) and those containing mutant IL-6 were pooled and concentrated to approximately 60 ml in an ultrafiltration cell equipped with a YM-10 membrane (Amicon). Buffer exchange of the concentrated solution was performed on a 4.4 cm×22 cm Sephadex G-25 column (Pharmacia) equilibrated and eluted with 20 mM sodium phosphate, 50 mM NaCl, pH 7.4. The final preparation was stored, in aliquots, in 20 mM sodium phosphate, 50 mM NaCl, pH 7.4, at −20 °C.

In order to optimize the oxidative refolding conditions of mutant IL-6 and thus to improve the overall yields of correctly folded, biologically active cytokine, washed inclusion bodies, or biologically active purified preparations of mutant IL-6, were dissolved in 50 mM Tris/HCl 1 mM EDTA, pH 8.5, containing either 6 M Gdn · HCl or 7 M urea and were left overnight at 4 °C. Refolding was accomplished by tenfold dilution in cold buffer solution containing 20 mM Tris/HCl 0.1 M NaCl, 1 mM EDTA, pH 8.5, followed by 2 h stirring at 4 °C. The extent of recovery of correctly folded and biologically active mutant IL-6 was determined by reverse-phase HPLC and by the *in vitro* proliferation assay using 7TD1 cells.

To assure a low level of bacterial endotoxin contamination, solutions of mutant IL-6 in 20 mM sodium phosphate, 50 mM NaCl, pH 7.4, were loaded onto a small Sepharose-Q column equilibrated with the same buffer and the unbound flow-through was collected in clean vials and stored at −20 °C. The endotoxin level was assayed using the *Lymulus* amebocyte lysate test kit (Whittaker) performed according to the manufacturer's instructions. The results were expressed as endotoxin unit (EU)/mg protein.

Analytical characterization of mutant IL-6. Discontinuous sodium dodecylsulfate (SDS)/polyacrylamide gel electrophoresis (PAGE) in 15% polyacrylamide separating gels was performed according to Laemmli (1970). Samples of mutant IL-6 were boiled in 0.25 M Tris/HCl, pH 6.8, containing 2% SDS with or without 2-mercaptoethanol. Gels were stained with Coomassie brilliant blue R-250.

Immobilized pH gradient isoelectrofocusing (IEF) was performed as described by Righetti et al. (1990). Gels with a pH 5.5−7.5 gradient were cast using Immobilines (Pharmacia) rehydrated with 20% glycerol prior to analysis. The sample of mutant IL-6 in 10 mM Tris acetate, pH 7.6, containing 20% glycerol was loaded at the cathodic side and run overnight at 2.5 kV and 10°C. Staining of the gels was performed with Coomassie brilliant blue.

Reverse-phase HPLC of mutant IL-6 was performed using a Vydac C_4 column (4.6 mm×250 mm, 5 μm particle size) purchased from The Separation Group (Hesperia). The column was equilibrated with water/0.1% trifluoroacetic acid and eluted with a linear gradient of 40−100% acetonitrile/0.07% trifluoroacetic acid in 30 min at a flow rate of 1 ml/min. The absorbance of eluted material was monitored by ultraviolet absorbance detection at 226 nm.

Electrospray mass spectrometry analysis of purified mutant IL-6 was performed with a Hewlett Packard model 5989A single quadrupole mass spectrometer equipped with an electrospray interface Hewlett Packard model 59987A. Prior to analysis, the sample of mutant IL-6 in buffer solution was desalted by reverse-phase HPLC on a Vydac C_4 column and the eluted protein peak was collected and injected into the spectrometer at a flow rate of 2 μl/min.

N-terminal sequence analyses of mutant IL-6, and fragments generated thereof by enzymic hydrolysis, were performed by automated Edman degradation using a model 477A pulsed-liquid phase sequencer with a model 120A on-line analyzer (Applied Biosystems) for the detection of phenylthiohydantoin-derivatives of amino acids. Digestions of mutant IL-6 (20 μg) were conducted with 0.4 μg tosylphenylalaninechloromethane-treated trypsin or with 0.4 μg endoproteinase Lys-C (Boehringer Mannheim) by overnight incubation at 37°C in 0.1 M Tris/HCl, pH 8, containing 1 M urea. A sample of mutant IL-6 (20 μg) was also digested overnight with 0.1 μg Glu-C endoproteinase from *Staphylococcus aureus* V8 (Boehringer) in 0.1 M NH_4HCO_3, pH 7.8, containing 1 M urea at 37°C. The proteolytic fragments were separated by reverse-phase HPLC on a Vydac C_{18} column using water/0.1% trifluoroacetic acid (phase A) and 95% acetonitrile/0.07% trifluoroacetic acid (phase B) as mobile phases. The column was eluted with a linear gradient of 5−75% phase B in 60 min at a flow rate of 1 ml/min. Eluted protein fragments were collected and submitted to N-terminal sequence analysis.

Conformational analysis of mutant IL-6. Circular dichroic (CD) spectra were recorded on a Jasco (Tokyo, Japan) model J710 spectropolarimeter equipped with a thermostatted cell holder. Far-ultraviolet and near-ultraviolet CD spectra were recorded at 20−22°C at a protein concentration 15−50 μM in 50 mM sodium phosphate, 0.1 M NaCl, pH 7.5. Analysis of far-ultraviolet CD spectra for estimating the amount of protein secondary structure was carried out by a computer program provided by Jasco (Yang et al., 1986).

Fluorescence measurements were performed on a Perkin-Elmer (Norwalk) fluorescence spectrophotometer model LS-50. Spectra were recorded at 25°C at a protein concentration of 2 μM in 50 mM potassium phosphate, 0.1 M NaCl, pH 7.5.

Second-derivative ultraviolet absorption spectra of HPLC-purified mutant IL-6 (100−150 μg) in 300 μl 50 mM potassium phosphate, 0.1 M NaCl, pH 7.5, were obtained on a Perkin-Elmer model Lambda-2 double-beam spectrophotometer at 25°C. Second-derivative spectra of both mutant and wild-type interleukin-6 were also measured for the fully unfolded species, as obtained utilizing protein solutions in potassium phosphate containing 6 M Gdn · HCl. Solvent accessibility of tyrosine residues was calculated according to Ragone et al. (1984).

Protein concentration was determined by ultraviolet spectroscopy at 280 nm utilizing an absorption coefficient at 280 nm (0.1%, by mass) for non-glycosylated human recombinant IL-6 expressed in *E. coli* (Barthelemy et al., 1993) and mutant IL-6 of 0.47 $mg^{-1} \cdot cm^2$ and 0.51 $mg^{-1} \cdot cm^2$, respectively, calculated according to Gill and Von Hippel (1989).

Urea-induced unfolding of mutant IL-6. Urea-mediated denaturation curves were determined by measuring the CD signal at 222 nm of mutant IL-6 thermostatted at 25°C. Solutions at different urea concentrations were prepared by diluting a 10 M urea stock in 50 mM sodium phosphate, 0.1 M NaCl, pH 7.5, with the same buffer. Samples of 100 μl protein (0.35 mg/ml) were diluted with 900 μl urea at the appropriate concentration and incubated at room temperature for 2 h before CD measurements. The reversibility of the protein unfolding process was at least 90%, as checked by diluting samples of the unfolded protein in 9 M urea to 0.9 M urea and measuring the recovery of the ellipticity at 222 nm. The free energy of unfolding, ΔG_d, from the curve of $[\theta]_{222}$ versus urea concentration was calculated by using the following equation:

$$\Delta G_d = -RT\ln K = -RT\ln[y_n - y)/(y - y_d)],$$

where K is the equilibrium constant, y is the ellipticity value at 222 nm, and y_n and y_d are the values of the ellipticity at 222 nm characteristic of the native and denatured state of the protein, respectively. The data for ΔG_d thus obtained were fitted to the equation $\Delta G_d = \Delta G_d^{H_2O} - m[\text{urea}]$, where $\Delta G_d^{H_2O}$ is the value of ΔG_d in the absence of urea and m is a measure of the dependence of ΔG_d on urea concentration (Pace, 1986).

Biological assays. *In vitro* bioactivity of both mutant and wild-type IL-6 was evaluated by the hybridoma proliferation assay using the IL-6-dependent mouse-mouse hybrid 7TD1 cell line (Sironi et al., 1989). Cells for the proliferation assay were washed twice in IL-6-free Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and resuspended at 20000 cells/ml. The cell suspension (100 μl) was added to an equivalent volume of medium containing scalar concentrations of the sample to be tested. After incubating cell cultures for 3 days at 37°C, 7% CO_2 in air at a relative humidity of 95%, the number of viable cells was determined by a colorimetric assay using the dye 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide (Sigma) (Mosman, 1983). Each sample was tested in quadruplicate in three separate experiments and the growth/response curves were analyzed using the Inplot program (Graphpad Software). The mitogenic activity of mutant IL-6 and the activity of non-glycosylated recombinant human IL-6 expressed in *E. coli* (Barthelemy et al., 1993) were compared to the proliferative response elicited by an interleukin-6 reference preparation (lot 88/514) kindly provided by the National Institute for Biological Standards and Controls (Hertfodshire, England). *In vivo* biological activity of the interleukin-6 species was also studied in a primate animal model. Five male and five female adult *Cynomolgus* monkeys (*Macaca fasciculata*) weighing 2.7−3.9 kg were bred individually in temperature-conditioned rooms. One animal of each sex was injected subcutaneously with either 0, 10 or 50 μg/day of the two proteins. The dose was administered daily, in two equal parts in the morning and in the afternoon, for 11 consecutive days. Blood samples for the determination of platelet counts were removed aseptically from the femoral veins on days 0, 5, 8 and 11 of the study. Platelet counts were determined using an automatic analyzer ELF-8 (Ortho Diagnostics).

RESULTS

Expression, purification and chemical characterization of mutant IL-6. Fermentation of a recombinant *E. coli* strain, host-

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1                                   20
S E R I D K Q I R Y I L D G I S A L R K

21                                  40
E T S N K S N M S E S S K E A L A E N N

41                                  60
L N L P K M A E K D G C F Q S G F N E E
                  |_____|
61                                  80
T C L V K I I T G L L E F E V Y L E Y L

81                                  100
Q N R F E S S E E Q A R A V Q M S T K V

101                                 120
L I Q F L Q K K A K N L D A I T T P D P

121                                 140
T T N A S L L T K L Q A Q N Q W L Q D M

141                                 160
T T H L I L R S F K E F L Q S S L R A L

161
R Q M
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Fig. 1. Amino acid sequence of the mutant human IL-6. With respect to the 185-residue chain of wild-type human interleukin-6 (Van Damme et al., 1987), the mutant has a deletion of 22 residues (APVPPGEDSKD-VAAPHRQPLTS) at its N-terminus and a Cys→Ser exchange at positions 45 and 51 of the polypeptide chain (numbering of wild-type IL-6). The location of the single disulfide bond between Cys52 and Cys62 is shown.

ing a plasmid coding for mutant IL-6, resulted in a biomass of approximately 60 g wet mass/l of culture. The biosynthesis of the mutant protein occurred as insoluble aggregates or inclusion bodies, which were solubilized in 6 M Gdn · HCl. The denaturated mutant IL-6 thus obtained was refolded by a tenfold dilution in renaturing buffer (see Materials and Methods). The protein recovery/refolding protocol was optimized by comparing the results obtained after solubilization of inclusion bodies in 6 M Gdn · HCl or in 7 M urea. Higher yields of mutant IL-6 were obtained by dissolving inclusion bodies with Gdn · HCl than with urea (60 mg versus 18 mg mutant IL-6/l fermentation solution), in analogy with previous reports also showing that Gdn · HCl is not exchangeable for urea in the preparation from inclusion bodies of IL-6 produced as a fused protein to growth hormone (Asagoe et al., 1988) and human IL-4 (Van Kimmenade et al., 1988). Moreover, the mutant protein obtained from the Gdn · HCl-solubilized pellet showed *in vitro* higher specific activity ($EC_{50} = 8.0 \pm 1.0$ pg/ml; see Materials and Methods) than the protein obtained from urea-solubilized pellet ($EC_{50} = 46.0 \pm 5.0$ pg/ml). The beneficial effect of Gdn · HCl in the recovery of biologically active cytokine is not understood, but it appears to act during the solubilization step of inclusion bodies. When a denaturation/renaturation treatment using either Gdn · HCl or urea was conducted on purified mutant IL-6, the yields of refolding, in terms of protein amount and specific activity, were comparable and independent from the denaturing agent used (data not shown).

The presence of reducing agents during the solubilization/dilution cycle did not influence the final yield of recombinant protein, indicating the spontaneous formation of the single disulfide bond in mutant IL-6 within the bacterial cell, without significant formation of protein intermolecular aggregates. Reverse-phase HPLC analysis of an aliquot of the solution containing the refolded protein (data not shown) established the presence of

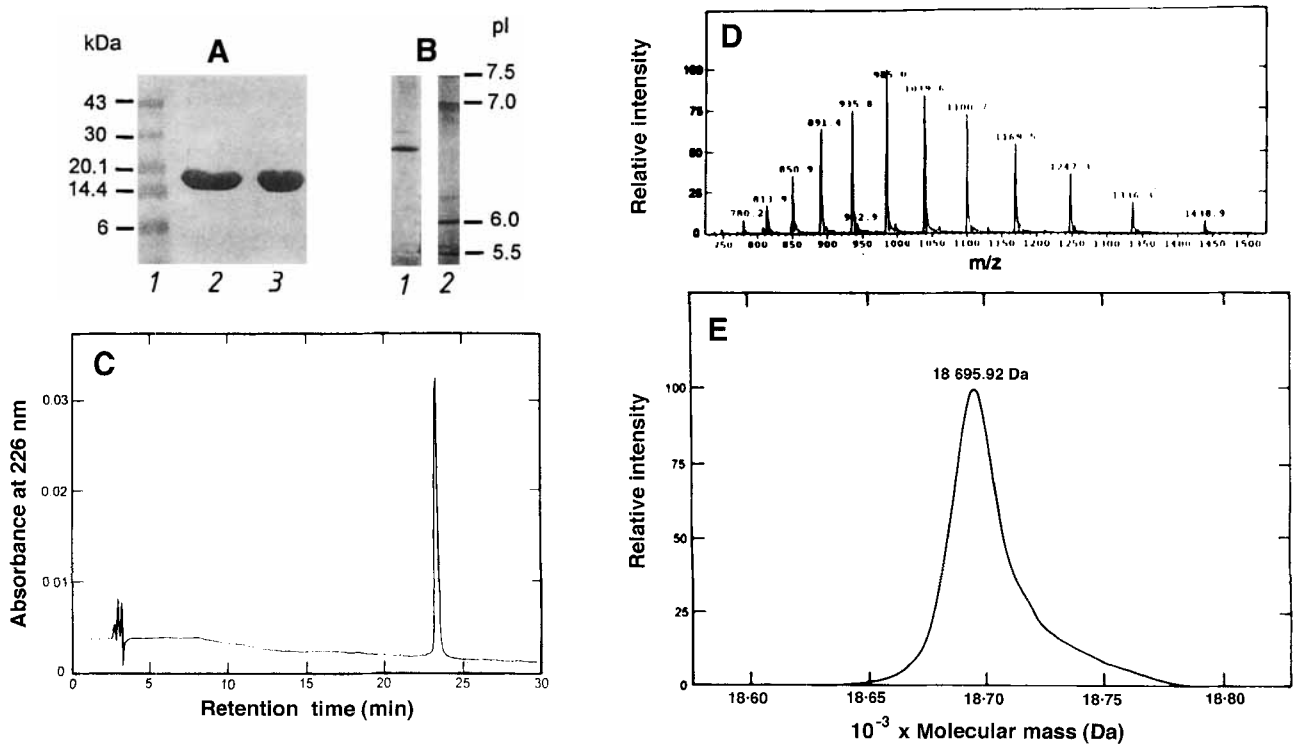

Fig. 2. Analytical characterization of mutant IL-6. Gel electrophoresis and isoelectrofocusing of purified mutant IL-6. (A) SDS/PAGE was conducted utilizing a 15% polyacrylamide gel under reducing (lane 2) and non-reducing (lane 3) conditions. Molecular mass standards (lane 1) were ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.4 kDa) and bovine trypsin inhibitor (6 kDa). (B) IEF of the purified protein (lane 1) was performed on an immobilized pH gradient system (Righetti et al., 1990). Reference pI markers are shown in lane 2. (C) Reverse-phase HPLC analysis of purified mutant IL-6. Analysis was conducted utilizing a Vydac C_4 column eluted with a gradient of 40–100% acetonitrile in water containing 0.1% trifluoroacetic acid. (D, E). Characterization of the covalent structure of mutant IL-6 by electrospray mass spectrometry. The ions observed (D) correspond to different charged states of a single molecular species of 18695 ± 2.1 Da (calculated value 18695.98 Da, average isotope composition). A deconvolution procedure was employed to mathematically reduce the raw data (D) to a single molecular mass (E).

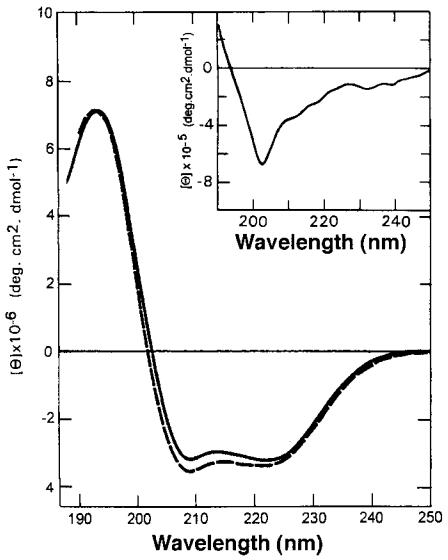

Fig. 3. Far-ultraviolet CD spectra of mutant and recombinant wild-type human IL-6. Spectra were taken at 22°C in 50 mM potassium phosphate, 0.1 M NaCl, pH 7.5, at a protein concentration of 0.49 mg/ml and 0.27 mg/ml for mutant IL-6 and recombinant IL6, respectively. Mutant IL-6 (——); recombinant wild-type human IL-6 (– – – –). The insert shows the difference spectrum obtained after subtraction of the mutant IL-6 spectrum from that of the full-length cytokine.

two major peaks of protein material corresponding to mutant IL-6 (30%) and to a contaminating bacterial protein (70%), which was precipitated from solution by adding ammonium sulfate at 1.5 M final concentration. An additional chromatographic step of the supernatant, obtained utilizing a hydrophobic chromatography column (phenyl-Sepharose), allowed us to obtain a preparation of the mutant protein that was at least 95% pure (see below). Further buffer exchange of the protein with 20 mM sodium phosphate, 50 mM NaCl, pH 7.4 and a final chromatographic step on an anion-exchange column yielded a preparation of mutant IL-6 with an endotoxin level less than 0.2 EU/mg protein. The final yield of purified and correctly folded cytokine was approximately 60 mg/l fermentation solution.

The homogeneity of purified mutant IL-6 was established by SDS/PAGE analyses carried out in both reducing and non-reducing conditions. Isoelectrofocusing (IEF) of the purified cytokine on an immobilized pH gradient showed a single protein band with a pI approximately 6.5, and reverse-phase HPLC analysis gave a single symmetric chromatographic peak indicating that the recombinant protein was at least 95% pure (Fig. 2.).

N-terminal sequence analyses of the first 28 amino acid residues of mutant IL-6 by automatic Edman degradation established that the recombinant protein had the expected N-terminal sequence (Fig. 1) and that the initiating methionine residue was completely removed during biosynthesis. Sequence analysis of internal portions of mutant IL-6 were determined after protein fragmentation with trypsin (cleavages at lysine and arginine), endoproteinase Lys-C (cleavages at lysine) and with the Glu-specific protease from *S. aureus* V8. Sequencing of several protein fragments obtained in this manner, and purified to homogeneity by reverse-phase HPLC, allowed the analysis of the 163-residue chain of mutant IL-6 (Fig. 1), with the only exception of the chain segment at positions 108–110 (data not shown).

The clearest indication of the identity and homogeneity of the mutant IL-6 preparation was obtained by electrospray mass spectrometry analysis (Fig. 2). The protein gave a spectrum with a bell-shaped distribution of multiply charged ions, from which an experimental value of molecular mass of a single protein component of 18695 ± 2.1 Da was derived, in excellent agreement with the value of 18695.98 Da (average isotope composition) calculated for mutant IL-6.

Conformational analysis. The far-ultraviolet CD spectra of both mutant and wild-type interleukin-6 (Fig. 3) were similar, with two minima at 222 nm and 209 nm and a maximum at 193 nm, typical of a polypeptide chain mainly consisting of a α-helical secondary structure (Adler et al., 1973; Yang et al., 1986). On the basis and limitations of the computing method employed (Yang et al., 1986), the amount of α-helical content of both cytokines was similar (67–70%). Both the shape of the CD spectrum and the ellipticity values of the full-length human interleukin-6 were in good agreement with those previously reported for human (Krüttgen et al., 1990; Nishimura et al., 1990; Li et al., 1993; Rock et al., 1992, 1994) and murine (Ward et al., 1993a,b) interleukin-6. The difference spectrum, obtained by subtracting the CD spectrum of mutant IL-6 from that of wild-type recombinant species, shows negative ellipticity at 250–200 nm and a minimum at approximately 200 nm, thus resembling the spectrum of a polypeptide in a random coil conformation (Adler et al., 1973; Yang et al., 1986; Fig. 3, insert). These CD data suggest that the 22 N-terminal amino acid residues of full-length IL-6 are in a random-coil conformation.

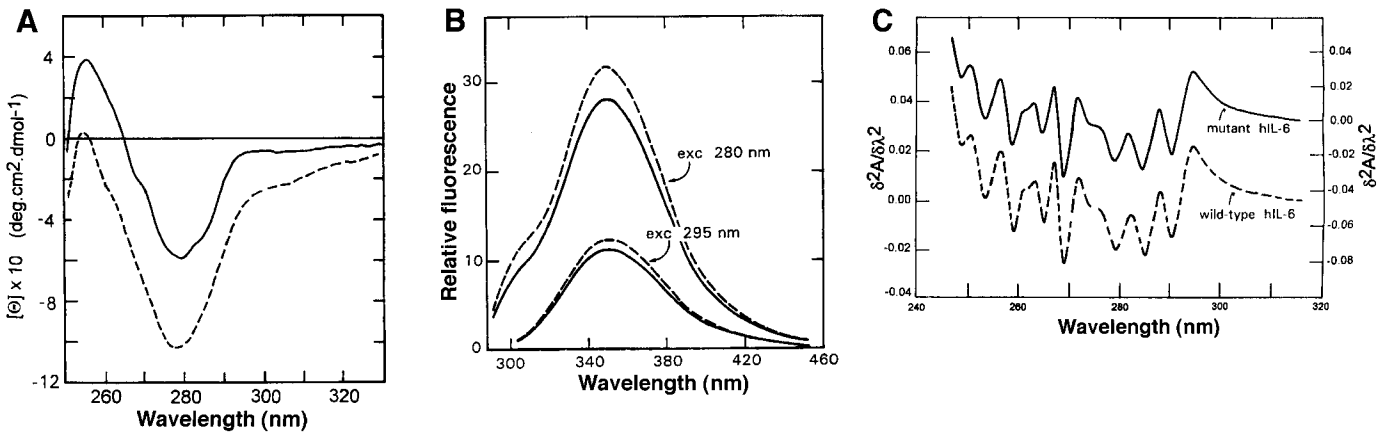

Fig. 4. Analysis of tertiary structure of mutant and wild-type IL-6. (A) Near-ultraviolet CD, (B) emission fluorescence and (C) second-derivative ultraviolet absorption spectra of mutant (——) and wild-type human (– – – –) IL-6. All spectra are the results of three separate measurements in 50 mM potassium phosphate, 0.1 M NaCl, pH 7.5 (see Materials and Methods section).

The near-ultraviolet CD spectrum of mutant IL-6 (Fig. 4A) shows spectral characteristics close to those reported for recombinant human (Krüttgen et al., 1990) and murine interleukin-6 (Ward et al., 1993a,b) with similarities in the shape of the spectrum, but reduced positive ellipticity at 250–260 nm. Absorption bands corresponding to the seven phenylalanine residues appear as two negative shoulders at 268 nm and 262 nm, superimposed onto the positive background disulfide contribution in this region (Strickland, 1974; Khan, 1979). There is no evidence of fine structure contribution for the single Trp136 in the region 287–293 nm, suggesting that the indolyl side chain has no fixed conformation and is thus exposed to the solvent (Strickland, 1974). However, appreciable differences between mutant and full-length interleukin-6 exist in terms of signal intensity and shape of the spectrum in the 250–260-nm region, where disulfide bonds contribute to the CD spectrum (Strickland, 1974; Khan, 1979; Fig. 4A). These differences can be explained by the lack of one disulfide bond in mutant IL-6 with respect to the full-length cytokine.

Fluorescence emission spectra for both mutant and wild-type interleukin-6 were obtained by excitation at 280 nm and 295 nm to observe the emission spectrum of tyrosine- and tryptophan-residues or the single tryptophan residue, respectively, of the two cytokines (Lakowicz, 1986). The fluorescence spectra of both proteins are essentially identical either after excitation at 280 nm or 295 nm (Fig. 4B). Moreover, the emission spectrum measured by excitation at 295 nm shows a maximum at approximately 350 nm, which is taken as an indication that the single Trp136 residue is fully exposed to the solvent medium in both cytokine species (Burnstein et al., 1973). In the 280-nm spectra, tyrosine fluorescence appears as a shoulder at approximately 303 nm, indicating that in both proteins there is a poor energy transfer between the three tyrosine and single tryptophan residues of each protein (Lakowicz, 1986).

Second-derivative absorbtion spectroscopy of proteins can be used as a finger-printing method for a given protein structure in order to compare aromatic side chain topology in mutant proteins with respect to the wild-type species (Craig et al., 1989). The superimposability of the second-derivative absorption profile for mutant and full-length interleukin-6 (Fig. 4C) strongly indicates that the aromatic amino acid side chains are located in a similar environment in the two proteins, thus providing direct evidence for strong similarities in their tertiary structures. Moreover, taking advantage of the variation of ultraviolet absorbance for a tyrosine/tryptophan-containing protein upon changing the polarity of the medium, second-derivative absorption spectroscopy can be used to calculate the degree of exposure (α) of tyrosine residues, defined as the fraction of the average solvent accessibility for all tyrosine residues in a given protein (Ragone et al., 1984). The degree of tyrosine exposure for the three tyrosine residues for mutant IL-6 ($\alpha = 0.336$) and wild-type recombinant interleukin-6 ($\alpha = 0.342$) was essentially identical, suggesting that the identity of the microenvironment of Tyr10, Tyr76 and Tyr79 in mutant IL-6 (Fig. 1) was similar to that of the corresponding tyrosine residues in the full-length recombinant interleukin-6. The one-third average exposure of the three tyrosine residues in both cytokines is also consistent with the results of photochemically induced dynamic nuclear polarization studies conducted on human interleukin-6, showing that only Tyr32 of this species is exposed to solvent (Nishimura et al., 1990).

Urea-mediated denaturation. The urea-mediated unfolding process of mutant IL-6 was monitored at 25 °C following the decrease of ellipticity at 222 nm after increasing the urea concentration. As shown in Fig. 5, the process is highly cooperative,

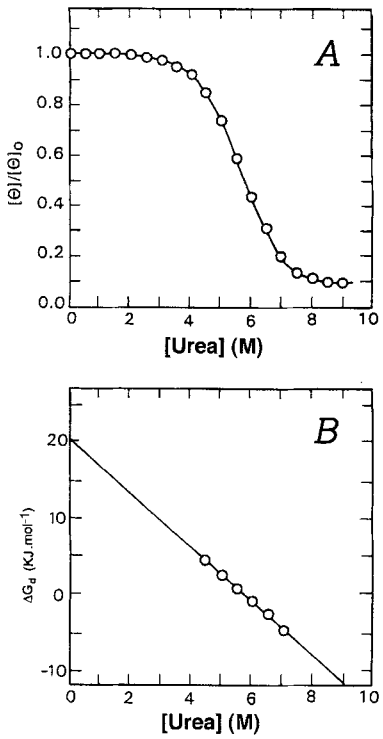

Fig. 5. Urea-induced unfolding transition of mutant IL-6. (A) The conformational transition of the protein was followed by monitoring the decrease of the CD signal at 222 nm while increasing the urea concentration. Measurements were carried out at 25 °C in 50 mM potassium phosphate, 0.1 M NaCl, pH 7.5, at a protein concentration of 0.35 mg/ml. Protein solutions were kept at room temperature for 2 h before performing measurements. Ellipticity data versus urea concentration are given as $[\theta]/[\theta]_o$, where $[\theta]_o$ is the ellipticity value measured in the absence of urea. (B) Linear extrapolation of the Gibbs free energy to determine the stability of mutant IL-6 in the absence of urea. The fraction of unfolded protein is derived from the experimental data by assuming a two-state transition of unfolding. The values for the Gibbs free energy in the presence of urea (ΔG_d) were calculated from the corresponding equilibrium constant (K_d) in the transition region.

with a midpoint denaturation at 5.5 M urea. The value of Gibbs free energy variation associated with the unfolding reaction in the absence of denaturant ($\Delta G_d^{H_2O}$), estimated by linear extrapolation of ΔG_d values in the transition region (Pace, 1986, Pace et al., 1988), was 20.9 ± 0.4 kJ · mol^{-1} (Fig. 5). The high cooperativity of the urea-mediated unfolding transition of mutant IL-6 can be taken as an indirect proof of the presence of a properly folded globular protein. Both the denaturant concentration at the midpoint denaturation, and the value for the free energy of unfolding compare favorably with corresponding values obtained for human (Rock et al., 1994) or murine (Zhang et al., 1992; Ward et al., 1993a,b) interleukin-6.

Biological activity. *In vitro* biological activity of mutant IL-6 was assayed as the proliferative response of 7TD1 cells, an IL-6-dependent hybridoma cell line. Dose/response curves were comparable for slope and maximal response for mutant and full-length interleukin-6 and the international standard preparation of human interleukin-6 included in the study (Fig. 6). In this assay, mutant IL-6 was the most potent among the cytokines tested with a concentration inducing half-maximal growth stimulation (EC_{50}) of 9.6 ± 0.4 pg/ml, whereas the EC_{50} of the reference standard IL-6 preparation and the full-length recombinant interleukin-6 were 26.0 ± 0.5 pg/ml and 37.0 ± 1.1 pg/ml, respectively.

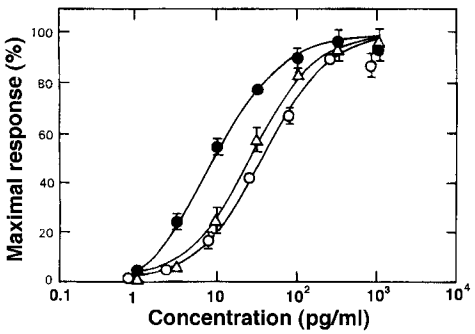

Fig. 6. *In vitro* biological activity of IL-6. Growth curves of 7TD1 cells incubated for 3 days with different concentrations of mutant IL-6 (●), international standard IL-6 (△) or recombinant human IL-6 (○). The data shown are the results of experiments conducted in triplicate.

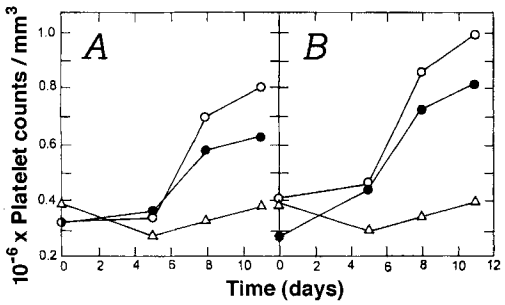

Fig. 7. Platelet counts in monkeys treated with mutant and recombinant wild-type human IL-6. One animal of each sex was subcutaneously injected with 0 (△), 10 (●) and 50 (○) μg · kg^{-1} recombinant human IL-6 (A) or mutant IL-6 (B) each day for 11 consecutive days. The data shown are mean values of two treatments.

The activity of mutant and full-length interleukin-6 was further established *in vivo* by measuring thrombopoiesis in primates, a well-documented pharmacological response induced by IL-6. An increase in platelet counts was dose related and measurable by day 8, and the reponse continued throughout the treatment period. A 2–3-fold increase of the basal values was reached on day 11, when treatment was ended. Animals treated with mutant IL-6 resulted in slightly higher platelet counts as compared to monkeys treated with the wild-type recombinant interleukin-6 (Fig. 7).

DISCUSSION

This study reports a detailed structural and functional characterization of a simplified form of human interleukin-6 bearing a deletion of 22-residues at its N-terminus, possessing only one of the two disulfide bonds and, moreover, devoid of post-translational modifications such as glycosylation and phosphorylation. Previous protein engineering experiments have shown that individually these three types of chain modification do not appear to impair the biological function of the cytokine (see Introduction). The 163-residue chain of mutant IL-6 containing a single disulfide bridge between Cys45 and Cys51 was produced as insoluble inclusion bodies by fermentation of a recombinant *E. coli* strain, from which the cytokine was redissolved utilizing concentrated Gdn · HCl, refolded and isolated to homogeneity in high yields, i.e. 60 mg correctly folded and biologically active protein/l fermentation solution. Even if in previous studies emphasis is given to the oxidation/renaturation step in the recovery from inclusion bodies of disulfide cross-linked proteins (Fischer et al., 1993), it is shown in this study that the solubilization step can be very critical for an efficient recovery of a recombinant, fully active protein. Special attention was devoted in this study to firmly establish the covalent structure of the polypeptide chain of mutant IL-6, since in other studies N-terminally and C-terminally truncated (Parekh et al., 1992; Zhang et al., 1992; Ward et al., 1993a) or even elongated forms (Danley et al., 1991) of recombinant interleukin-6 were described. Furthermore, the preparation of mutant IL-6 was of clinical grade, since bacterial endotoxin contamination was negligible and within the limits (less than 1 EU/dose) required for *in vivo* administration.

Several spectroscopic analyses allowed us to conclude that the overall three-dimensional structure of mutant IL-6 is very similar to that of the recombinant full-length human interleukin-6. In particular, fluorescence emission and second-derivative absorption spectra clearly indicated that the microenvironment of the aromatic residues is identical in both mutant and wild-type interleukin-6, thus indicating that deletion of the N-terminal 22 residues and removal of the first disulfide bridge by a Cys→Ser exchange, do not impair the overall folding of the cytokine molecule. This is an additional experimental observation of the fact that globular proteins appear to possess a core that is critical in dictating structure, stability and function, while some parts of the polypeptide chain can be dispensable or amenable to structural mutation. Indeed, from the results of a number of protein engineering experiments (Matthews, 1993) or analyses of mutant proteins existing in nature (Fontana, 1991a,b), it appears that proteins tolerate extensive structural variation, especially at their flexible or loose parts exposed to solvent. On this basis, the fact that mutant IL-6 maintains the conformational stability and functional properties of the full-length IL-6 allows us to propose that the mutations occur at flexible sites of the entire protein.

The three-dimensional structure of human IL-6 is not yet known and, consequently, the actual structural characteristics and location in the protein fold of both the N-terminal 22-residue segment and the first disulfide bridge between Cys45 and Cys51 are not known. Nevertheless, it has been proposed (Bazan, 1990), and generally accepted (Savino et al., 1993, 1994; Rock et al., 1994), that the interleukin-6 molecule forms a bundle of four antiparallel helices connected by flexible loops. Starting from the N-terminus of the full-length IL-6 chain, the first amino acid in α-helical configuration was predicted to be at position 18 (Bazan, 1990), 25 (Parry et al., 1991) or 26 (Savino et al., 1993). This is consistent with the results of CD measurements of this study, showing that the four-helix bundle is maintained in mutant IL-6 and that the 22-residue segment of full-length IL-6 is in a random coil conformation, as revealed by the analysis of the difference spectrum obtained by subtracting the far-ultraviolet CD spectrum of mutant IL-6 from that of the full-length cytokine (Fig. 3, insert).

From the models of IL-6 developed so far, the two disulfide bridges appear to be located in loop regions (Savino et al., 1993, 1994). Moreover, since the first disulfide bridge, Cys45 Cys51, is cleaved by excess thiol much more easily (10 s) than the second disulfide bridge (30 min; Rock et al., 1994), it can be proposed that the first disulfide moiety is exposed to solvent and is thus likely located in a hydrophilic environment, considering that exposure, hydrophilicity and loop flexibility are surface properties of globular proteins and usually strongly correlated (Ringe and Petsko, 1985), even if energy strain effects in disulfide bond reactivity cannot be excluded (Katz and Kossiakoff, 1986). Indeed, a calculated flexibility profile (Karplus and Schultz, 1985) of the polypeptide chain of mutant IL-6 reveals that the chain segment where the first disulfide bridge of the cytokine is located (positions 20–37, Fig. 1), shows enhanced

flexibility with respect to the rest of the chain (Polverino de Laureto et al., 1994). These observations allow us to propose that the two structural alterations of the 185-residue chain of human interleukin-6, leading to mutant IL-6, are located in exposed, flexible sites of the protein. In agreement with the concept of buried residues dictating protein structure/function (Matthews, 1993), the cleavage of the exposed first disulfide bridge does not impair structure/function of the cytokine, whereas cleavage of the buried second disulfide bridge leads to major alterations of protein structure, stability and function (Snouwaert et al., 1991b; Jean et al., 1993; Rock et al., 1994). These results can be compared to the analogous results obtained with ribonuclease T_1, in which cleavage of the fully exposed Cys2 Cys10 disulfide bridge decreases the protein thermal stability (t_m) by 6°C, while 86% of the functional activity is retained; the additional cleavage of the buried Cys6 Cys103 bond dramatically reduces t_m by approximately 43°C and leads to 65% reduction in activity (Pace et al., 1988). Nevertheless, disulfide bridges are known to stabilize globular proteins mainly by decreasing the entropy of the unfolded state and this stabilizing effect increases with the size of the loop (Pace et al., 1988). Therefore, the marginal contribution of the first exposed disulfide bridge to the net conformational stability of mutant IL-6 with respect to that of the wild-type protein (Rock et al., 1994) can be reasonably interpreted by also considering the short-sized loop connecting Cys45 to Cys51.

An interesting aspect of this study is that mutant IL-6 retains full, or even higher, biological activity of the native or recombinant human interleukin-6 both in the hybridoma proliferation assay *in vitro* and, more importantly, in the *in vivo* experiment in *Cynomolgus* monkeys. This is in agreement with the results of previous studies of Snouwaert et al. (1991b) and Jean et al. (1993) utilizing Cys→Ser substitutions, but is different from the results of Rock et al. (1994), which instead provided evidence that a Cys→Ala or a Cys→*S*-alkylated-Cys replacement at the level of the first disulfide bridge in human interleukin-6 produces 41% (alanine exchange) or 75% (*S*-alkylated-Cys exchange) reduction in biological potency of the cytokine. Thus, it seems that the actual modification of cysteine residue(s) determines different conformational/functional states of the cytokine. These different findings can probably be interpreted by assuming that the hydrophilic and approximately isosteric Cys→Ser exchange is more appropriate for the first disulfide bridge located at an exposed and flexible loop, whereas the Cys→Ala exchange leads to the introduction at this site of an unfavored amino acid residue due to the hydrophobic character of alanine and/or to the creation of a potentially destabilizing cavity within the protein structure by introducing a less bulky side chain ($-CH_2-SH \rightarrow -CH_3$; Inaka et al., 1991). Perhaps, the even more detrimental effects in the interleukin-6 potency of the Cys→*S*-alkylated-Cys exchange (see above) are due to steric or electrostatic effects. For example, reduction of the single disulfide bond in the C_2 fragment of immunoglobulin light chain retains a native-like conformation, unless the -SH groups are alkylated (Goto and Hamaguchi, 1979).

To summarize, in this study we have described a useful procedure for the large-scale production and the detailed chemical, physicochemical and functional characterization of a recombinant mutant IL-6. Sufficient amounts of a clinical-grade cytokine are now available for future structure/function studies and for investigating its therapeutic use, specifically in platelet recovery after radiotherapy, chemotherapy (Kimura et al., 1990; Carrington et al., 1992), and bone marrow transplantation (Herodin et al., 1992).

Part of this study was presented at the 6th Symposium of the Protein Society, San Diego, California, July 24–28, 1993 (Polverino de Laureto, P., De Filippis, V., Fontana, A. and Bertolero, F., communication 160-Y).

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