Periplasmic expression of human interferon- α 2c in *Escherichia coli* results in a correctly folded molecule

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Human interferon- α 2c (IFN- α 2c) was produced in *Escherichia coli* under the control of the alkaline phosphatase promoter using a periplasmic expression system. Compared with other leader sequences, the heat-stable enterotoxin II leader of *E. coli* (*STII*) resulted in the highest rate of correct processing as judged by Western-blot analysis. The fermentation was designed as a batch-fed process in order to obtain a high yield of biomass. The processing rate of IFN- α 2c could be increased from 25% to more than 50% by shifting the fermentation pH from 7.0 to 6.7.

IFN- α 2c extracted from the periplasm was purified by a new four-step chromatographic procedure. Whereas cytoplasmically produced IFN- α 2c does not have its full native structure, IFN- α 2c extracted from the periplasm was found to be correctly folded, as shown by c.d. spectroscopy. Peptide-map analysis in combination with m.s. revealed the correct formation of disulphide bridges. N-terminal sequence analysis showed complete removal of the leader sequence, creating the authentic N-terminus starting with cysteine.

INTRODUCTION

Human interferon- α 2 (IFN- α 2) was among the first proteins to be produced by recombinant DNA technology (Goeddel et al., 1980; Dworkin-Rastl et al., 1983; Thatcher and Panayotatos, 1986). The recombinant host was *Escherichia coli* and the protein was produced by cytoplasmic expression (Bodo and Maurer-Fogy, 1986).

There are, however, several serious drawbacks to the cytoplasmic expression of recombinant proteins in *E. coli*. The intracellularly synthesized protein remains in the reduced state and may either form insoluble inclusion bodies or is found in the soluble fraction after rupture of the bacterial cell wall. This protein does not have the correct conformation, and disulphide bridges are not formed. In order to obtain a native protein, the molecule has to be oxidized and refolded to achieve the correct conformation. This step, however, is not very efficient and in addition generates a variety of unwanted molecular forms that can be detected in such preparations: residual-reduced and partially reduced forms, oligomers formed by intermolecular disulphide bridges and molecular forms with unnatural intramolecular disulphide bridges ('scrambled' forms). The occurrence of these forms has been described for recombinant IFN- α 2c (Bodo and Maurer-Fogy, 1986).

The removal of the N-terminal methionine residue which marks the beginning of translation presents another problem of cytoplasmic expression. The cleavage efficiency of the aminopeptidase depends on the subsequent residues. IFN- α 2c with an N-terminal cysteine is a rather poor substrate. In addition, the cleavage efficiency is significantly reduced at the high expression rates obtained later in the fermentation process. Therefore fermentation has to be stopped at an early time point, at low cell density and with low IFN- α 2c yields, as the methionine-containing protein cannot be separated from the form without methionine. Most of these unwanted molecular forms can be minimized during the purification of the protein by preparative

separation techniques, albeit at the price of rather low yields of the correct form of IFN- α 2c.

In the present paper we describe an alternative approach to the production of human IFN- α 2c in *E. coli*, using the signal peptide from heat-stable enterotoxin II of *E. coli* (*STII*) (Lee et al., 1983; Picken et al., 1983) which leads to the secretion of IFN- α 2c into the periplasm. In contrast with cytoplasmic expression, periplasmic secretion has been shown to yield a correctly folded native molecule with correct N-terminal processing and formation of disulphide bridges.

MATERIALS AND METHODS

Construction of the expression vector

All cloning procedures were performed essentially following standard protocols (Sambrook et al., 1989). Construction of the expression cassette containing the phosphatase promoter, the *STII* leader and the human IFN- α 2c was performed by splicing by overlap extension (SOE)-PCR (Ho et al., 1989). The IFN- α 2c sequence was PCR-amplified from the *Hind*III-linearized bacterial expression construct pER21/1 (Dworkin-Rastl et al., 1983) using the 5' primer (ATGCCTATGCATGTGATCTGCCTCAACCCACAGC) and the 3' primer (GACTTCAGAAGCTTCTGCAGTTACGATCGTTATCATTCCTTACTTCTAAAC-TTTC, *Hind*III site underlined). The phosphatase promoter (Chang et al., 1986; Shuttleworth et al., 1986) and the *STII* leader (Lee et al., 1983; Picken et al., 1983) were amplified from the *Pvu*I-linearized pCF2 (expression vector for human IFN- ω 1 spliced to the *STII* leader sequence; R. Hauptmann, unpublished work) using the 5' (CGTCTTCAAGAAATTCGAGATTATCG, *Eco*RI site underlined) and 3' (GGCAGATCACATGCATAGGCATTGTAGCAATAG) primers. The purified PCR products were combined and amplified using the 3' primer of the first and the 5' primer of the second PCR reaction. The *Eco*RI-*Hind*III-cut PCR product was cloned into the corresponding

Abbreviations used: IFN- α , interferon- α ; *STII*, heat-stable enterotoxin II leader of *Escherichia coli*; SOE, splicing by overlap extension; RP-h.p.i.c., reversed-phase h.p.i.c.

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Figure 1 (a) Map of the plasmid pDH13 and (b) sequence of the *STII* leader-IFN- $\alpha 2c$ fusion construct (*EcoRI*-*PstI* fragment)

(a) *phoA*, alkaline phosphatase promoter; *Tet*, tetracycline-resistance gene. (b) The *STII* leader sequence is shown in small letters. The underlined sequences show the position of the respective restriction enzyme-cleavage sites.

sites of Bluescribe M13⁺; the nature of the insert was verified by sequencing.

The final expression plasmid pDH13 was obtained by inserting the expression cassette (*EcoRI* cut, filled in with Klenow, recut with *PstI* fragment from the Bluescribe intermediate) into the

large *SspI*-*PstI* fragment of pAT153 (Twigg and Sherratt, 1980). The correct assembly of the resulting expression plasmid pDH13 (Figure 1a) was verified by restriction enzyme analysis. The sequence of the *STII* leader and IFN- $\alpha 2c$ is shown in Figure 1(b).

Construction of expression vectors containing various leader sequences

In order to combine the reading frame for IFN- $\alpha 2c$ with the coding sequences of other bacterial and phage leader sequences (Table 1) oligonucleotide pairs with *EcoRI* and *HindIII* compatible ends were synthesized (Figure 2) and subcloned into Bluescribe M13⁺ cleaved with these two enzymes. The inserts were verified by sequencing. These constructs and pDH13 were used as templates for SOE-PCR for splicing the individual leader sequences on to the coding sequence of IFN- $\alpha 2c$. The resulting PCR products were cut with *EcoRI* and *HindIII* and subcloned into Bluescribe M13⁺. After sequence verification, the *XhoI*-*PstI* fragments were isolated and ligated into the large *XhoI*-*PstI* fragment of pDH13. The resulting plasmids were used to transform *E. coli* W3110 for the expression of the recombinant proteins.

Fermentation

Preculture

Autoclaved Luria-Bertani medium (700 ml) plus 5 mg/l tetracycline (sterile-filtered) in a 2-litre glass vessel were inoculated from a stock culture to an A_{546} of 0.01. The culture was incubated for 12 h at 37 °C with vigorous stirring (800 rev./min) and aeration (5 fermenter vol./min).

Main culture

Salts [10 mM (NH₄)₂HPO₄, 30 mM (NH₄)₂SO₄, 40 mM K₂HPO₄, 10 mM NaCl, 4.7 mM NH₄Cl, 3.4 mM trisodium citrate] were heat-sterilized in the fermenter. Trace elements, MgSO₄, glucose, thiamine, L-tryptophan, L-leucine, L-methionine and tetracycline, all sterile-filtered presterilized, were added aseptically after cooling, resulting in a starting volume of about 7 litres. Then 600 ml of the preculture was automatically inoculated into the fermenter. Fermentation conditions were: agitation 1000 rev./min, aeration 1 fermenter vol./min, back pressure 0.3×10^5 Pa, temperature 37 ± 0.1 °C; the pH was maintained at 6.7 ± 0.1 with NH₃ and H₂SO₄. The concentration of dissolved oxygen was kept above 15% air saturation (at 0.3×10^5 Pa back pressure) by aeration with oxygen-enriched air as necessary. After the initially available glucose had been consumed, a feed procedure was started, automatically triggered by the oxygen concentration, which contained glucose, thiamine, MgSO₄, L-tryptophan, L-leucine and L-methionine. The feeding rate started at 2.5 g of glucose/l per h and was continuously increased for 24 h to 5.0 g/l per h and then kept constant until the end of the fermentation process.

The fermentation run was terminated when a total of 350 g/l glucose had been added. At this time a typical A_{546} of 220–250 was obtained.

The broth was then cooled to about 10 °C and simultaneously adjusted to pH 2.0 with H₂SO₄ for inactivation of the biomass. The biomass was harvested by centrifugation and stored frozen at -70 °C.

Extraction and purification

Acid-inactivated biomass (approx. 0.5 kg) was suspended in

Table 1 DNA and amino acid sequence of the different signal sequences used for secretion of IFN- α 2c into the periplasm of *E. coli*

M13, M13 precoat protein. The codon of the first residue of the *phoA* leader peptide was changed from GTG (valine) to ATG (methionine).

Gene bank accession no.	Leader	DNA and protein sequence
M13763	<i>STII</i>	ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAT GCC TAT GCA M K K N I A F L L A S M F V F S I A T N A Y A
	<i>phoA</i>	ATG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAA GCC M K Q A T I A L A L L P L L F T P V T K A
J01655	<i>ompF</i>	ATG ATG AAG CGC AAT ATT CGT GCA GTG ATC GTC CCT GCT CTG TTA GTA GCA GGT ACT GCA AAC GCT M M K R N I L A V I V P A L L V A G T A N A
M16643	<i>lamB</i>	ATG ATG ATT ACT CTG GCG AAA CTT CCT CTG GCG GTT GCC GTC GCA GCG GGC GTA ATG TCT GCT GAG GCA ATG GCT M M I T L R K L P L A V V V A A G V M S A Q A M A
V00303	<i>malE</i>	ATG AAA ATA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC M K I T G A R I L A L S A L T T M M F S A S A L A
V00307	<i>ompA</i>	ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC M K K T A I A I A V A L A G F A T V A Q A
J01645	<i>lpp</i>	ATG AAA GCT ACT AAA CTG GTA CTG GGC GCG GTA ATC CTG GGT TCT ACT CTG CTG GCA GGT M K A T K L V L G A V I L G S T L L A A
M11454	<i>M13</i>	ATG AAA AAG TCT TTA GTC CTC AAA GCC TCT GTA GCC GTT GCT ACC CTC GTT CCG ATG CGT TCT TTC GCT M K K S Y V L K A S V A V A T L V R M L S F A

Figure 2 Structure of the synthetic DNA cloned into Bluescribe M13⁺

■, Location of restriction sites; ▨, ribosome-binding site (RBS).

500 ml of 1 % acetic acid using a Polytron homogenizer, and stirred at 0 °C for 1 h. Poly(ethylenimine) (50 % stock solution; Serva, Heidelberg, Germany) was added to a final concentration of 0.25 % (w/v). The suspension was adjusted to a pH of 10.0 with 5 M NaOH and stirred for an additional 2 h at 0 °C. After adjustment of the pH to 7.5 with 5 M HCl, the bacteria were pelleted by centrifugation at 17000 *g* (Beckman J2-21 centrifuge). The supernatant was loaded on to a silica column (35 mg of protein/ml of column material) equilibrated in 20 mM Tris/HCl, pH 7.5. The column was washed with 30 column vol. of this buffer, followed by a wash step with 20 mM Tris/HCl, pH 7.5, containing 100 mM tetramethylammonium chloride. IFN- α 2c was eluted by increasing the tetramethylammonium chloride concentration to 800 mM.

The IFN- α 2c-containing fractions were pooled, adjusted to 20 % (NH₄)₂SO₄ by the addition of solid (NH₄)₂SO₄ and loaded on to a phenyl-Sepharose column (Phenyl Toyopearl 650 S; Tosohaas) equilibrated in 20 mM Tris/HCl, pH 7.5, containing 30 % (NH₄)₂SO₄ (loading conditions). IFN- α 2c was eluted from the column with a linear gradient from 100 % loading conditions to 100 % 20 mM Tris/HCl, pH 7.5, containing 30 % ethylene glycol (solvent B). The pooled IFN- α 2c-containing fractions were adjusted to 20 mM sodium succinate, pH 5.0, by extensive dialysis. The final pH was adjusted to 3.0 with HCl before loading on to sulphopropyl ion-exchange resin (Toyopearl TSK SP 5PW; Tosohaas) equilibrated in 20 mM sodium succinate, pH 5.0 (loading conditions). IFN- α 2c was eluted from the column with a linear gradient from 100 % loading conditions to 100 % solvent C (20 mM sodium succinate, 500 mM NaCl, 10 % ethylene glycol, pH 5.5). The pooled IFN- α 2c-containing frac-

Figure 3 Comparison of the processing efficiency of different leader sequences (Western blot)

IFN- α 2c was expressed with different leader sequences in the *E. coli* strain W3110. Lane 1, purified IFN- α 2c; lane 2, *STII*; lane 3, *ompF*; lane 4, *M13* precoat protein; lane 5, *malE*; lane 6, *lamB*; lane 7, *ompA*; lane 8, *lpp*; lane 9, *phoA*. m, the position of the processed IFN- α 2c; p, position of the unprocessed IFN- α 2c, carrying the *STII* leader.

tions were dialysed against 10 mM Bistris, pH 5.8, and loaded on to a DEAE-Sepharose (DEAE-Sepharose Fast-Flow; Pharmacia) column equilibrated in the same buffer. Elution of IFN- α 2c occurred by a linear gradient into 10 mM BisTris, pH 5.8, containing 500 mM NaCl and 0.1 % Tween 20 (solvent D).

Immunoaffinity purification

IFN- α 2c expressed either peri- or cyto-plasmically was extracted from the biomass with 1 % acetic acid as described above. This extract was neutralized, dialysed against 25 mM Tris/HCl, pH 7.5, and immunoaffinity-purified using the IFN- α 2c-specific monoclonal antibody EBI-1 (Adolf et al., 1982) coupled to CNBr-activated Sepharose (Pharmacia), equilibrated with 25 mM Tris/HCl, pH 7.5. IFN- α 2c was eluted with 0.1 M citric acid/30 % ethylene glycol and dialysed against PBS.

Western blots

Portions of biomass were dissolved in SDS/PAGE loading buffer containing 10 mM dithiothreitol (about 1 mg of total protein/ml) and boiled for 5 min. Samples (15 μ l) were separated by SDS/PAGE (15 % gels). Western blots were carried out

Figure 4 IFN- $\alpha 2$ Western blot of biomass obtained from fermentations at pH 7.0 and pH 6.7

Lane 1, typical fermentation at pH 7.0; lane 3, fermentation at pH 6.7; lanes 2 and 4, purified IFN- $\alpha 2$ c. p, unprocessed precursor; m, the mature processed IFN- $\alpha 2$ c.

following standard protocols using the IFN- $\alpha 2$ c-specific monoclonal antibody EBI-1 (Adolf et al., 1982).

Reversed-phase (RP-) h.p.l.c.

Intact IFN- $\alpha 2$ c was analysed on a BakerBond C18 column (4.5 mm $\times$ 250 mm, 5 μ m particles, 30 nm pore size) at 30 $^{\circ}$ C. The samples were chromatographed using solvents A (0.1% trifluoroacetic acid in water) and E (0.1% trifluoroacetic acid in acetonitrile) using gradient conditions as described in the legend to Figure 7.

Tryptic digest

IFN- $\alpha 2$ c was digested with 1% (w/w) trypsin (sequencing grade, Boehringer-Mannheim) in 1% NH_4HCO_3 . Reduction was performed by incubating the cleavage mixture in 3 M urea/50 mM dithiothreitol for 2 h at 25 $^{\circ}$ C. RP-h.p.l.c. of the peptides was performed on a DeltaPAK C18-column (Millipore-Waters; 3.9 mm $\times$ 150 mm, 5 μ m particles, 10 nm pore size) at 30 $^{\circ}$ C, using the solvents described above. Peptides were eluted using a linear gradient from 0 to 55% solvent E in 55 min at a flow rate of 1 ml/min.

^{252}Cf plasma-desorption m.s.

Mass spectra of tryptic peptides were obtained on a BIO-ION 20 time-of-flight mass spectrometer (BIO-ION; Nordic AB, Uppsala, Sweden). Samples were dissolved in 0.1% trifluoroacetic acid/water and applied to nitrocellulose-coated targets (BIO-ION). Spectra were obtained at an acceleration voltage of 17 kV.

C.d. spectroscopy

Spectra of IFN- $\alpha 2$ c were measured with a JASCO 600 spectrometer with 10 mm pathlength at 25 $^{\circ}$ C, and scanned at 0.2 nm intervals at 50 nm/min. IFN- $\alpha 2$ c was dissolved at a concentration of 40 μ g/ml in 20 mM phosphate buffer, pH 7.4. The con-

centration was determined by measuring A_{280} . The absorption coefficient was determined using an IFN- $\alpha 2$ c standard quantified by amino acid analysis (results not shown).

RESULTS

Selection of the optimal signal sequence

It was thought that the presence of cysteine as the N-terminal amino acid residue of IFN- $\alpha 2$ c might affect the efficiency of the enzymic removal of the signal sequence for periplasmic expression. Therefore several different leader sequences from various secreted proteins were tested in small-scale fermentation experiments (10 litres). These sequences are listed in Table 1. Western blots were performed to analyse the processing efficiency which is reflected in the amount of IFN- $\alpha 2$ c lacking the signal sequence (Figure 3, band marked m). The signal sequence of *STII* was the only one which revealed partial processing resulting in the expected N-terminus of IFN- $\alpha 2$ c (as shown by sequence analysis of the purified protein; results not shown). The processing rate varied between 10 and 20%. Some of the other sequences examined exhibited unexpectedly high mobilities (Figure 3, lanes 4 and 9), but none showed any reasonable processing.

The processing efficiency of the *STII* leader construct could not be improved by using other *E. coli* strains nor by reducing the fermentation temperature to 32 $^{\circ}$ C (results not shown).

On the basis of these experiments, an expression system using the *STII* signal sequence and the *E. coli* strain W3110 was chosen for the production of IFN- $\alpha 2$ c (Figure 1).

Fermentation

The fermentation process was designed as a batch-fed procedure in order to achieve high cell densities. An A_{546} of 220–250 was reached routinely, resulting in about 1.8 kg of biomass/10 l fermenter volume. The expression of IFN- $\alpha 2$ c driven by the promoter of the alkaline phosphatase (*phoA*) was induced by phosphate concentration limitation in the medium. The average yield was routinely about 250–300 mg/l IFN- $\alpha 2$ c. Feeding of leucine and methionine was found to be necessary in order to inhibit the incorporation of norleucine instead of methionine into IFN- $\alpha 2$ c.

About 30% of the IFN- $\alpha 2$ produced was correctly processed by the signal peptidase, as indicated by the ratio of the two IFN- $\alpha 2$ c bands in the Western blot (Figure 4). Lowering of the pH of the fermentation broth to 6.7 increased the processing grade to about 50–60% (Figure 4, lanes 3 and 4). The harvested biomass had an average content of about 1.5 mg of IFN- $\alpha 2$ c/g of biomass.

Purification

IFN- $\alpha 2$ c secreted into the periplasmic space was extended at low pH followed by a high-pH step. This second step was empirically shown to give more reproducible yields of IFN- $\alpha 2$ c (results not shown). The extraction yield of 30–50% of total IFN- $\alpha 2$ c found in the biomass (as determined by e.l.i.s.a.) reflects the amount of the correctly processed IFN- $\alpha 2$ c.

IFN- $\alpha 2$ c was purified through a series of four chromatographic steps including adsorption chromatography on silica, hydrophobic interaction chromatography, cation- and anion-exchange chromatography. Figure 5 shows a representative set of chromatograms, and Table 2 summarizes the yield of the individual steps. The total yield of the purification procedure is about 14%. Extraction of 1 kg of biomass with 1.5 mg/g IFN- $\alpha 2$ c finally results in about 190 mg of IFN- $\alpha 2$ c. Figure 6 shows a representative SDS/PAGE of the individual steps. The final product

Figure 5 Chromatographic purification of IFN- α 2c extracted from biomass

(a) Adsorption chromatography on silica. The arrow indicates the elution with 800 mM tetramethylammonium chloride. ———, Conductivity. (b) Hydrophobic interaction chromatography on phenyl-Sepharose. The elution was performed with a linear gradient from 0 to 100% solvent B as indicated (-----). (c) TSK sulphopropyl cation-exchange chromatography. The elution was performed with a gradient from 0 to 100% solvent C as indicated (-----). (d)

Table 2 Yields of the four-step purification of IFN- α 2c

The IFN- α 2c content is given as percentage of the total protein content. The recovery is expressed as a percentage of all IFN- α 2c loaded on to the respective column.

Step	IFN- α 2c content (%)	Recovery (%)
Extraction	4.5	29.3
Silica	16.7	83.4
Phenyl-Sepharose	71.2	93.2
Sulphopropyl resin	97.6	70.9
DEAE-Sepharose	100.0	86.9

Figure 6 SDS/PAGE of the purification-step products

Lane 1, molecular-mass standards; lane 2, acidic extract; lane 3, silica pool; lane 4, phenyl-Sepharose pool; lane 5, TSK pool; lane 6, DEAE-Sepharose pool. The gel was stained with Coomassie Blue.

(lane 6) is essentially pure. No contaminating bands are observed even on silver staining (results not shown).

Characterization of purified IFN- α 2c

In order to demonstrate the correct folding and disulphide-bridge formation of IFN- α 2c in the periplasm, the purified protein was analysed by RP-h.p.l.c., by tryptic peptide maps and by c.d. spectroscopy. For this purpose, IFN- α 2c was extracted as described and rapidly purified by immunoaffinity chromatography. Significant disulphide formation or protein folding is minimal under these conditions. For comparison, cytoplasmically expressed IFN- α 2c (expression system without any leader sequence) was extracted and purified similarly. These preparations have been shown previously to contain reduced and 'scrambled' forms (Bodo and Maurer-Fogy, 1986).

The periplasmically expressed IFN- α 2c exhibited a single sharp peak in the RP-h.p.l.c. profile (Figure 7, tracing 1). No scrambled

Anion-exchange chromatography on DEAE-Sepharose. The elution was performed with a gradient from 0 to 100% solvent D as indicated (-----). The bar beneath the major peaks in each chromatogram indicate the IFN- α 2c-containing pools which were collected and used for the following step.

Figure 7 RP-h.p.l.c. profiles of immunoaffinity-purified IFN- α 2c obtained from periplasmic secretion (1) compared with IFN- α 2c from cytoplasmic expression before (2) and after (3) oxidation

The arrows indicate various IFN- α 2c species with incorrectly assembled disulphide bonds obtained from cytoplasmic expression. Elution was performed with a linear gradient (flow rate 1 ml/min) from 45 to 53% solvent E.

Figure 8 Tryptic peptide map of IFN- α 2c before (upper tracing) and after (lower tracing) reduction with dithiothreitol

a, Disulphide-linked peptides 1–12 + 84–112; b, disulphide-linked peptides 1–12 + 71–112; c, disulphide-linked peptides 24–31 + 134–144; d, disulphide-linked peptides 24–31 + 135–144. 1, Peptide 1–12; 2, peptide 84–112; 3, peptide 71–112; 4, peptide 134/135–144; 5, peptide 24–31. The peptides marked a, c and d were isolated and subjected to m.s. (Table 3). The identity of peptides 1–5 was verified by N-terminal sequencing (results not shown).

Table 3 Plasma-desorption analysis of disulphide-linked tryptic peptides

The numbers indicate the positions of the peptides in the sequence. a, b, c and d refer to the positions of the respective peptides in the tryptic map (Figure 8).

Peptide	Calculated mass (Da)	Observed mass (Da)
1–12 + 84–112 (a)	4615.2	4618.3
1–12 + 71–112 (b)	6047.8	6049.0
24–31 + 134–144 (c)	2247.6	2246.4
24–31 + 135–144 (d)	2119.4	2119.9

Figure 9 Comparison of c.d. spectra of immunoaffinity-purified IFN- α 2c obtained from periplasmic secretion and cytoplasmic expression

—, Periplasmic secretion; ----, cytoplasmic expression before oxidation; - · -, cytoplasmic expression after oxidation. Molar ellipticity is expressed as $[\theta] \times 10^{-3}$ degree $\cdot$ cm² $\cdot$ dmol⁻¹ using a mean residual weight of 116.87.

forms were seen. These forms account for up to 50% of the cytoplasmically expressed IFN- α 2c (Figure 7, tracing 2, arrows). Reoxidation can significantly reduce the amount of these forms (Figure 7, tracing 3).

In addition, the correct alignment of the two disulphide bridges in the periplasmically expressed IFN- α 2c was demonstrated by analysis of tryptic peptide maps of the reduced and unreduced protein (Figure 8). The dithiothreitol-sensitive peaks were shown by plasma-desorption m.s. to contain the expected disulphide-linked peptides 1–12 + 71/84–112 (a, b) and 24–31 + 134/135–144 (c, d). The m.s. data of these four peptides which are generated by the alternative use of tryptic-cleavage sites are summarized in Table 3. The identity of the reduced peptides (1–5, Figure 8) was verified by N-terminal sequence analysis (results not shown).

The correct folding of secreted IFN- α 2c was demonstrated by c.d. spectroscopy and compared with cytoplasmically expressed IFN- α 2c (Figure 9). Periplasmic IFN- α 2c exhibited the typical appearance expected for a mostly α -helical type-I IFN (Senda et al., 1992), which is characterized by the pronounced negative peaks around 210 and 218 nm. The spectrum of cytoplasmically expressed IFN- α 2c (Figure 9) was noticeably different. The characteristic peaks around 215 nm were slightly shifted to longer wavelengths and were less intense. An additional positive peak was observed at 200 nm, and this altogether indicates a reduced amount of secondary-structure elements. The spectrum of reoxidized material (Figure 9) showed a greater resemblance to the spectrum of the periplasmically expressed material, although the intensity of the correctly located negative peaks was slightly reduced.

DISCUSSION

High-level expression of eukaryotic proteins in *E. coli* frequently results in the formation of insoluble aggregates, known as the inclusion bodies (Schein, 1989), or in incorrectly processed and misfolded proteins, sometimes with incorrect disulphide-bridge formation.

The data presented here demonstrate that such errors, which are observed in cytoplasmically expressed IFN- α 2c, are overcome by periplasmic secretion. A more detailed analysis showed that not every signal sequence tested resulted in the expected pro-

cessing. Furthermore, significant differences in the processing efficiency were observed between different *E. coli* strains (results not shown). The combination of the heat-stable enterotoxin signal sequence (*STII*) and strain W3110 gave the best results. However, the processing was never quantitative, leaving 50–80 % of the precursor unaffected. The yield of correctly processed IFN- α 2c could be improved to over 50 % by lowering the pH in the fermenter from 7.0 to 6.7. Fermentation at lower temperatures, which might enhance the solubility of recombinant proteins (Schein, 1989) and therefore their availability for secretion and processing, did not exhibit any beneficial effects (results not shown). The reasons for the incomplete processing remain unclear. Possibly, the high expression rate of the recombinant protein exceeds the capabilities of the secretory pathway. Attempts to overexpress either a cytoplasmic component of this pathway, the chaperone SecB, or the signal peptidase (R. Hauptmann, unpublished work) did not improve the processing rate.

The fermentation process itself was designed as a batch-fed procedure, thus resulting in a high yield of biomass. The expression of IFN- α 2c under the control of the alkaline phosphatase promoter was induced by phosphate limitation in the medium. Using this promoter, the onset of the expression of the recombinant protein can be easily controlled. Total yields of about 250–300 mg of IFN- α 2c/l with and without signal peptide were routinely obtained at the 10- and 100-litre fermenter scale.

Processing of the precursor yields some 30–50 % mature IFN- α 2c and this was virtually all recovered by the extraction procedure. The precursor carrying the signal peptide was never observed in the extracts. Purified IFN- α 2c contained the correct N-terminal sequence starting with cysteine.

IFN- α 2c was extracted rapidly and purified by immunoaffinity chromatography in order to reduce refolding and was shown to be correctly processed and folded. The c.d. spectra exhibited typical α -helical features with a pronounced minimum around 215 nm. The correct formation of the two disulphide bridges between Cys-1 and Cys-98, and Cys-29 and Cys-138, was shown by tryptic peptide mapping in combination with m.s. By contrast, the c.d. spectra of cytoplasmically expressed IFN- α 2c looked quite different: after rapid extraction and immunoaffinity purification which reduces refolding, RP-h.p.l.c. revealed that the material contained up to 50 % scrambled forms (Bodo and Maurer-Fogy, 1986) with incorrect or open disulphide bridges. The c.d. spectrum of this material was shifted to higher wavelengths, and the bands were less intense. The spectrum of refolded material containing fewer scrambled forms had the appearance of the correctly folded material, but the intensity of the bands was lower. Chromatographic removal of the scrambled forms

(Bodo and Maurer-Fogy, 1986) resulted in material that exhibited a c.d. spectrum identical with that of the periplasmically expressed IFN- α 2c (results not shown).

All these data indicate that periplasmically expressed IFN- α 2c is produced as a correctly folded molecule. This is further substantiated by the full biological activity of 2.2×10^8 units/mg in an antiviral assay (Adolf, 1987), which is identical with the values obtained for natural human IFN- α 2 (Adolf et al., 1991).

A new purification procedure was developed for IFN- α 2c secreted into the periplasmic space of *E. coli*. This purification protocol included four chromatographic steps, circumventing the problems associated with the use of immunoaffinity chromatography. The yields of the individual steps were usually above 70 %, thus resulting in a yield of 40 % for the chromatographic purification and 14 % for the overall procedure including the extraction step.

In conclusion, the periplasmic expression system described in this study combined with a new efficient purification procedure permits the production of recombinant human IFN- α 2c suitable for therapeutic purposes without encountering the problems observed with cytoplasmic expression, such as incomplete removal of the N-terminal methionine or misfolding and incorrect disulphide-bridge formation.

REFERENCES

- Adolf, G. R. (1987) *J. Gen. Virol.* **68**, 1669–1676
- Adolf, G. R., Bodo, G. and Swetly, P. (1982) *J. Cell. Physiol. Suppl.* **2**, 61–68
- Adolf, G. R., Kalsner, I., Ahorn, H., Maurer-Fogy, I. and Cantell, K. (1991) *Biochem. J.* **276**, 511–518
- Bodo, G. and Maurer-Fogy, I. (1986) in *The Biology of the Interferon System 1985* (Stewart, W. E., II and Schellekens, H., eds.), pp. 59–64, Elsevier Scientific Publishing Co., Amsterdam
- Chang, C. N., Kuang, W.-J. and Chen, E. Y. (1986) *Gene* **44**, 121–125
- Dworkin-Rastl, E., Swetly, P. and Dworkin, M. B. (1983) *Gene* **21**, 237–248
- Goeddel, D. V., Yelverton, E., Ullrich, A., Heynecker, H. L., Miozzari, G., Holmes, W., Seeburg, P. H., Dull, T., May, L., Stebbing, N. et al. (1980) *Nature (London)* **287**, 411–416
- Ho, S. N., Hunt, S. N., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) *Gene* **77**, 51–59
- Lee, C. H., Mosely, S. L., Moon, H. W., Whipp, S. C., Gyles, C. L. and So, M. (1983) *Infect. Immun.* **42**, 264–268
- Picken, R. N., Mazaitis, A. J., Maas, W. K., Rey, M. and Heynecker, H. (1983) *Infect. Immun.* **42**, 269–275
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning – A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Schein, C. H. (1989) *Bio/Technol.* **7**, 1141–1147
- Senda, T., Shimazu, T., Matsuda, S., Kawano, G., Shimizu, H., Nakamura, K. T. and Mitsui, Y. (1992) *EMBO J.* **11**, 3193–3201
- Shuttleworth, M., Taylor, J. and Minton, N. (1986) *Nucleic Acids Res.* **14**, 8689–8695
- Thatcher, D. R. and Panayotatos, N. (1986) *Methods Enzymol.* **119**, 166–177
- Twigg, A. J. and Sherratt, D. (1980) *Nature (London)* **283**, 216–218