Purification and renaturation of recombinant human interleukin-2

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Recombinant human interleukin-2 (IL-2) expressed as *Escherichia coli* was isolated as insoluble aggregates of protein (inclusion bodies) after cell breakage. IL-2 and contaminants were dissolved in 6 M-guanidinium chloride/10 mM-dithiothreitol, pH 8.5, and further purified in reduced and denatured form by gel-permeation chromatography in the same solvent. Renaturation was effected by dilution and autoxidation; IL-2 of native specific activity was isolated at over 95% purity by reversed-phase h.p.l.c.; an additional peak of reduced protein was also observed. Most losses of native IL-2 occurred on refolding, probably because of an aggregation process; concentrations around 1 µg/ml were necessary to achieve 30% recovery. It was essential to maintain the denatured protein in reduced form before renaturation and autoxidation, which was most efficient at pH 8.5 with 1.5 µM-CuSO₄. A procedure based on these observations has been used to prepare IL-2 on the 50 µg scale.

INTRODUCTION

Interleukin 2 (IL-2) or T-cell growth factor, is a 15500-M_r single-subunit protein produced by T-lymphocytes that promotes the proliferation of activated T-cells (Robb, 1985). In addition it modulates growth and differentiation of other lymphocyte subsets, for example causing maturation of cytotoxic T-cells (Ezard *et al.*, 1985), induction of γ-interferon (Farrar *et al.*, 1981) and promotion of natural killer- and lymphokine-activated-killer-cell activity (Hinney *et al.*, 1981; Mule *et al.*, 1985).

IL-2 has been purified from natural and recombinant sources by a variety of methods, usually involving RP-h.p.l.c. (Henderson *et al.*, 1983; Stern *et al.*, 1984) as the principal purification step. The human IL-2 gene has been cloned and expressed in *Escherichia coli* as insoluble 'inclusion bodies' (Devos *et al.*, 1983). Inclusion bodies are dense aggregates, largely of recombinant protein and 2–3 µm in diameter, that can be separated from soluble bacterial proteins by low-speed centrifugation after cell lysis (Schoner *et al.*, 1985). In most cases inclusion bodies need to be dissolved in denaturing solvent and the protein then refolded and purified (Marston *et al.*, 1984). Proteins vary considerably in their optimal conditions for renaturation; various factors such as pH, salt concentration and type, rate of removal of denaturant, concentration of the target protein and of contaminants may strongly affect the recovery of authentic protein (Ghelis & Yon, 1982). Since IL-2 has one disulphide bridge and one free cysteine residue, redox conditions must also be considered so as to provide oxidation without excessive formation of wrong disulphides. The purification of recombinant IL-2 has been described in outline and the protein thoroughly characterized (Kato *et al.*, 1985; Liang *et al.*, 1985; Yamada *et al.*, 1986).

In the present paper we describe in detail the solubilization, refolding and purification of recombinant human IL-2. The strategy employed has been to purify

IL-2 partially in denatured form and then, following preliminary refolding experiments aimed at identification of the correctly folded species, to improve the recovery of this species by systematic examination of the folding conditions and to develop a purification scheme based on these data.

MATERIALS AND METHODS

Molecular genetics

A synthetic gene coding for the mature 133-residue protein was constructed on the basis of the cDNA sequence determined by Taniguchi *et al.* (1983), cloned and expressed in *E. coli* RB791. The IL-2 expression vector pTIL2E/2TT is a derivative of pAT153 (Twigg & Sherrat, 1980) containing the hybrid tac promoter from pDR540 (Russell & Bennett, 1982) inserted between the restriction-endonuclease-*Hind*III and *Bam*HI sites, a synthetic IL-2 gene inserted between the *Bam*HI and *Sal*I sites and a synthetic Trp A transcription terminator (Christie *et al.*, 1981) between the *Sal*I and *Ava*I sites. The synthetic IL-2 gene was composed of single-stranded oligonucleotides which were synthesized by the solid-phase phosphotriester methodology using a Bioscience Synthesis Automation Machine (Applied Biosystems) and ligated as described by Sagaramella & Khorana (1972). Codon usage was chosen for optimal expression in *E. coli*. The gene sequence was checked and was found to be correct.

Growth and disruption of cells

A 50 ml sample of an *E. coli* RB791 culture containing the IL-2 plasmid was grown overnight to stationary phase and this culture was used to inoculate 5 litres of medium in shake flasks. After 1 h at 37 °C cells were induced to produce IL-2 with isopropyl β-thiogalactoside, grown for 3 h and chloroform-killed. Cells (15–20 g wet wt.) were harvested in a Beckman J2-21 centrifuge at 2800 g for 5 min and disrupted by

Abbreviations used: RP-h.p.l.c., reversed-phase h.p.l.c.; DTT, dithiothreitol; PAGE, polyacrylamide-gel electrophoresis; 2-ME, 2-mercaptoethanol; f.p.l.c., fast protein liquid chromatography; GdmCl, guanidinium chloride; TFA, trifluoroacetic acid; g.p.c., gel-permeation chromatography; TX-100, Triton X-100; BSA, bovine serum albumin.

sonication in 50 ml of 10 mM-phosphate buffer, pH 7.4, containing 0.15 M-NaCl/10 mM-EDTA/10 mM DTT, the IL-2 inclusion bodies pelleted by centrifugation at 10000 *g* for 10 min and the pellet resuspended in an equal volume of the above buffer for use in subsequent experiments.

Solubilization of inclusion bodies

Solubilization was monitored by SDS/PAGE. Samples (100 μ l) of pellet suspension were centrifuged at 10000 *g* for 5 min and pellets were resuspended in 100 μ l of solvent and left for 10 min at 20 °C. After re-centrifugation supernatants were dialysed against 50 mM-Tris/HCl, pH 6.8; 2-ME (5% v/v) and SDS (1%) were added and samples boiled for 5 min. Pellets were washed with 190 mM-Tris/HCl, pH 6.8, boiled in 100 μ l of sample buffer to extract the remaining proteins, and re-centrifuged to remove particulate matter. Samples (50 μ l) of supernatants and pellet extracts were loaded on to gels and, after electrophoresis, proteins were revealed by staining with Coomassie Blue.

G.p.c.

Analytical g.p.c. was performed in 6 M-GdmCl/50 mM-Tris/HCl (pH 8.5)/10 mM-DTT (running buffer) with a 1 cm $\times$ 30 cm Pharmacia Superose 12 f.p.l.c. column and a Pharmacia f.p.l.c. apparatus; the flow rate was 0.5 ml/min. Samples (200 μ l) of pellet suspension were centrifuged at 10000 *g* for 5 min and the pellet dissolved in 8 M-GdmCl/10 mM-DTT, pH 8.5, for 1 h at 37 °C to ensure complete reduction and solubilization before chromatography. Preparative g.p.c. was performed on a 5 cm $\times$ 90 cm Sepharose CL-6B column equilibrated in running buffer and run at a flow rate of 70 ml/h; 40 ml of pellet suspension was centrifuged in a Beckman J2-21 centrifuge (JA-17 rotor) at 7000 *g* for 10 min and the pellet resuspended in 15 ml of 8 M-GdmCl/10 mM-DTT to give a final volume of 20 ml. IL-2-containing fractions were identified by silver staining after SDS/PAGE, pooled and stored at -70 °C. Pools were respectively termed 'g.p.c.-f.p.l.c.' and 'g.p.c.-6B'. To study oxidation in the absence of DTT, g.p.c.-6B pools were desalted in 6 M-GdmCl/0.1% TFA, pH 2.2, using a Pharmacia PD-10 gel-filtration column; this material ('g.p.c.-DS') was used within a few hours, since autoxidation is still appreciable, even at this pH (Saxena & Wetlaufer, 1970).

Refolding

Renaturation was effected by dilution of 1-3 ml (per experiment) of the g.p.c. pool into 50 mM-Tris/HCl, pH 8.5. All renaturations were performed at 20 °C. In order to vary the protein concentration at a constant GdmCl concentration, samples were appropriately diluted with 6 M-GdmCl or 6 M-GdmCl/10 mM-DTT before the final dilution in renaturation buffer. Renatured samples were often slightly cloudy. TFA was added to give a pH of 2.5-2.8, and samples were analysed by RP-h.p.l.c. Yields of IL-2 species, peak X and peak Y, were measured from RP-h.p.l.c. peak areas (see below).

RP-h.p.l.c.

RP-h.p.l.c. was performed on a Varian 5020 liquid chromatograph fitted with a UV-100 detector. Acetonitrile (HPLC Grade S; Rathburn Chemicals) and TFA (Pierce Sequenal grade) in Milli-Q-purified water

(Millipore) were filtered on a 0.22 μ m-pore-size filter before use. Synchropak RP-P C₁₈ columns (0.41 cm $\times$ 25 cm; Synchrom, Linden, IN 47955, U.S.A.) were obtained from Anachem. Fractions were stored at -70 °C. The amount of IL-2 protein was determined from peak areas using an $A_{1\text{cm}}^{280}$ value of $9.58 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Kato *et al.*, 1985).

SDS/PAGE and gel scanning

SDS/PAGE was performed with 15%-(w/v)-polyacrylamide gels as described by Laemmli (1970). H.p.l.c. column fractions in acetonitrile were 3-fold diluted in sample buffer [190 mM-Tris/HCl (pH 6.8)/3% (w/v) SDS/30% (v/v) glycerol/0.75% (v/v) Bromophenol Blue/5% (v/v) 2-ME]. Gels were silver-stained (Wray *et al.*, 1981) or Coomassie Blue R-250-stained. Protein standards (Pharmacia) were phosphorylase *b* (molecular mass 94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa). For quantification of IL-2 in g.p.c. pools, silver-stained gels were scanned densitometrically with a Joyce-Loebl Chromoscan-3 densitometer. Loads (50-500 ng) of h.p.l.c.-purified IL-2, of known concentration (determined by RP-h.p.l.c. peak area), were used for calibration, and samples were loaded at such dilution that their IL-2 content fell within this range. Total protein was measured by the Bradford method (Pierce Protein Assay Reagent).

Bioassay

Amounts (in units) of IL-2 were estimated with an IL-2-dependent cell-proliferation assay, essentially as described by Gillis *et al.* (1978), the murine T-cytotoxic cell line CTL-L being used. One unit of IL-2/ml was defined as that concentration giving 50% maximal incorporation of [³H]thymidine, and units were standardized to JURKAT IL-2 obtained from Dr. G. Thurman, NCI-Frederick Cancer Research Facility, Frederick, MD 21701, U.S.A.

Chemicals

Isopropyl β -thiogalactoside was obtained from Gibco. Acrylamide and bisacrylamide were from Bio-Rad; tetramethylethylenediamine, SDS and ammo-

Table 1. Solubilization of IL-2 inclusion bodies

Solubilization was estimated from SDS/PAGE gels (see, e.g., Fig. 1). 50% acetonitrile/0.1% TFA, 1% TFA and 10% TFA also gave negligible extraction.

Solvent	Approximate proportion of IL-2 dissolved (%)
8 M-GdmCl/50 mM-Tris, pH 8.5	> 95
1% SDS/50 mM-Tris, pH 8.5	> 95
70% Formic acid	> 95
8 M-Urea/50 mM-Tris, pH 8.5	70-90
50% Acetic acid	30-50
5% Triethanolamine/HCl, pH 10.9	10-20
40% Propan-2-ol/0.1% TFA	< 5
1 M-NaCl/50 mM-Tris, pH 8.5	< 5
0.5 M-2-ME/50 mM-Tris, pH 8.5	< 5
1% TX-100/50 mM-Tris, pH 8.5	< 5

Fig. 1. Solubilization of IL-2 inclusion bodies: SDS/PAGE analysis

Samples of pellet suspension were centrifuged and redissolved in the indicated solvent plus 10 mM-DTT. Supernatants (SN) were dialysed before boiling in SDS/2-ME; pellets (P) were re-extracted in an equal volume of sample buffer. Equal volumes of SN and P were then loaded. Tracks 1 and 2, 50%-acetic acid extraction, SN and P; tracks 3 and 4, 8 M-GdmCl extraction, SN and P; tracks 5 and 6, 70%-formic acid extraction, SN and P; track 7, M_r standards. The cause of the slight difference in mobility between acetic acid-solubilized (track 1) and insoluble (track 2) IL-2 is unclear; however, it does not result from hydrolysis or derivatization, as no change in apparent M_r or pI was observed on extensive incubation of pure IL-2 in 50% acetic acid.

nium persulphate were AnalaR grade from BDH. All other chemicals were from Sigma; GdmCl was of grade 1 purity.

RESULTS

Solubilization of IL-2

Over 98% of IL-2 was present in the pellet fraction and thus had to be dissolved. Levels of solubilization, as judged by SDS/PAGE, are shown in Table 1 for the various solvents tried. DTT at 10 mM was sometimes included, but made no observable difference to solubilization. Fig. 1 shows an SDS/PAGE gel monitoring extraction by acetic acid, formic acid and GdmCl; acetic acid at concentrations greater than 50% (v/v) did not effect greater solubilization, and below this concentration there was a steady decrease in percentage solubilization until the latter was negligible at 10% (v/v) acetic acid. A similar gradation was observed with formic acid, 2–6 M-GdmHCl and 4–8 M-urea; the high density of the latter two solvents may mean that some aggregated or membrane-associated protein did not spin down, although g.p.c. showed that 6 M-GdmCl was an effective solvent (see below). Since increasing solvent volume and time of extraction had little effect, and also re-extraction

of pellet from 50% acetic acid solubilization did not further dissolve IL-2, it appeared the IL-2 is heterogeneous with respect to solubilization.

GdmCl was chosen as solvent because of the relative ease of renaturation by dilution and the low risk of covalent modification of the protein when compared with the situation with other solvents. This solvent was also employed by Kato *et al.* (1985).

G.p.c.

G.p.c. in 6 M-GdmCl/10 mM-DTT achieved a 2.5–3-fold purification of IL-2 before the renaturation step (Fig. 2). IL-2 was detected by SDS/PAGE and by bioassay of fractions and was eluted at a position corresponding to an M_r of about 15000, indicating that it was unfolded and monomeric under these conditions. The purification is evident in densitometer scans (Fig. 3), which were used to estimate IL-2 concentrations in g.p.c.-purified samples, a method which was found to be more reliable (possibly owing to the denatured state of the protein) than radioimmunoassay. Data for g.p.c.-f.p.l.c., g.p.c.-6B and g.p.c.-DS pools are summarized in Table 2.

Renaturation of g.p.c.-purified IL-2

For initial refolding experiments, g.p.c.-6B material was diluted 20-fold in 50 mM-Tris/HCl, pH 8.5, to initiate renaturation/autoxidation and two peaks (X and Y) of IL-2 were observed on RP-h.p.l.c. analysis (Fig. 4). These species were over 95% pure by SDS/PAGE, as is

Fig. 2. G.p.c. of pellet extracts

(a) G.p.c. of a 200 μ l (5.4 mg/ml) pellet suspension dissolved in 6 M-GdmCl/10 mM-DTT, pH 8.5, using a 1 cm $\times$ 30 cm Superose 12 f.p.l.c. column equilibrated in the same buffer; the flow rate was 0.5 ml/min and the fraction size 1 ml. M_r markers were denatured γ -globulin (158000), ovalbumin (44000) and myoglobin (17000). (b) Preparative g.p.c. of a 20 ml pellet extract (9.25 mg/ml) on a 5 cm $\times$ 90 cm Sepharose CL-6B column in 6 M-GdmCl/DTT. The flow rate was 70 ml/h and 9.5 ml fractions were collected.

Fig. 3. Densitometer scans of silver-stained SDS/PAGE gels

(a) SDS-extracted pellet; (b) g.p.c.-6B pool from the Sepharose CL-6B/GdmCl column (Fig. 2b). The IL-2 concentration in g.p.c. pools (Table 2) was estimated from the peak area in densitometer scans by reference to a calibration curve of pure IL-2 standards (of known concentration as determined by RP-h.p.l.c. peak area) loaded on to the same gel. The relationship of peak area to IL-2 load was linear between 50 and 500 ng, and sample loads were adjusted to fall within this range.

Table 2. Summary of g.p.c. pools used in refolding studies

Details of preparative procedures are given in the Materials and methods section. IL-2 was determined by gel scanning (Fig. 3) and total protein by the Bradford method. G.p.c.-f.p.l.c.b (fractions 10–14; Fig. 2a) was deliberately mispooled in order to examine the effect of contaminants on renaturation yield.

Pool	Matrix	[Protein] ($\mu\text{g/ml}$)	Approx. [IL-2] ($\mu\text{g/ml}$)	Purity (%)
G.p.c.-f.p.l.c.a	6 M-GdmCl/50 mM-Tris/HCl (pH 8.5)/10 mM-DTT	290	24	8.3
G.p.c.-f.p.l.c.b	6 M-GdmCl/50 mM-Tris/HCl (pH 8.5)/10 mM-DTT	330	14	4.4
G.p.c.-6B	6 M-GdmCl/50 mM-Tris/HCl (pH 8.5)/10 mM-DTT	530	60	11.3
G.p.c.-DS	6 M-GdmCl/0.1% TFA, pH 2.2	420	40	9.5

shown for peak X in Fig. 5; the apparent M_r was 15030 (peak Y was approximately the same), which is consistent with the theoretical value of 15513 and with the value of 15000 reported for recombinant *E. coli* IL-2 by Liang *et al.* (1985). Specific bioactivity, relative to the JURKAT native IL-2 standard (10×10^6 units/mg), was $15.5 \pm 6.5 \times 10^6$ units/mg for peak X (mean $\pm$ S.E.M., $n = 6$); the specific activity of peak Y was 2.4×10^6 units/mg. Peak X grew at the expense of peak Y as renaturation time increased and, furthermore, thiol titration with 5,5'-dithiobis-(2-nitrobenzoic acid) (Anderson & Wetlaufer, 1975) showed a 2.6-fold higher cysteine content in peak Y, strongly suggesting that peak Y was reduced IL-2 and was being oxidized to X. This assignment is confirmed by reported specific activities (Liang *et al.*, 1986) and RP-h.p.l.c. retention times (Kunitani *et al.*, 1986) for reduced and oxidized

recombinant IL-2. In conclusion, peak X has the basic characteristics of native IL-2. Refolding and oxidation were thus optimized on the basis of peak-X yield as determined by RP-h.p.l.c. peak area; this method of quantification was found to be accurate when checked by amino acid analysis. The peak-X yield was divided by the total IL-2 in the g.p.c.-pool starting material (determined densitometrically; see Fig. 3) to give the percentage renaturation recovery. The principal parameters investigated with respect to renaturation recovery were protein concentration, IL-2 concentration and redox conditions.

Protein and IL-2 concentration

In order to monitor the effect of these variables on renaturation recovery, oxidation was allowed to go to completion. A progressive increase in peak-X recovery

Fig. 4. RP-h.p.l.c. purification of IL-2

RP-h.p.l.c. of refolded IL-2 was performed on a 0.41 cm $\times$ 25 cm column of Synchropak RP-P C₁₈. Refolding conditions: 1.5 ml of g.p.c.-6B diluted 20-fold in Tris/HCl, pH 8.5; t = 60 min. H.p.l.c. conditions: solvent A, 0.1% TFA; B, 65% acetonitrile/0.1% TFA. Gradient: 0–82% B, 10 min; 82% B, 10 min; 82–100% B, 24 min; the flow rate was 0.5 ml/min. Two IL-2 peaks, X and Y, were identified by SDS/PAGE; they were eluted at 56.5% and 59.5% acetonitrile respectively. Peak X was oxidized IL-2 and Y was reduced IL-2.

was seen as both protein and IL-2 concentrations were decreased concomitantly (Table 3); recovery was increased 1.7-fold with a 10-fold decrease in concentration. Presumably aggregation or misdirected folding was responsible. Protein concentration alone had no significant effect on recovery, whereas the concentration of IL-2 did (Table 3), indicating that a specific process was responsible. However, recoveries after renaturation of unpurified GdmCl extracts were only about 5%, possibly owing to interference from high- M_r membrane fragments or protein aggregates.

The fate of aggregated or misfolded IL-2 on RP-h.p.l.c. is unclear; a weak IL-2 band comprising less than 5% of the total IL-2 was seen, on SDS/PAGE, at around 45% (v/v) acetonitrile (19 min) in the gradient (Fig. 4), but no other species were detected. Aggregates are most likely to be found in the unretained fractions, although no bioactivity was detected in these fractions. Sub-denaturing concentrations of chaotrope have been seen to inhibit aggregation (Orsini & Goldberg, 1978), but 2 M-GdmCl had no effect on peak-X recovery, nor did 20% (v/v) acetonitrile or 25% (v/v) ethylene glycol.

Fig. 5. SDS/PAGE analysis of purified IL-2

IL-2 was purified by g.p.c. and RP-h.p.l.c. Proteins were revealed by silver staining. Track 1, M_r standards; track 2, 500 ng of IL-2 peak X (see Fig. 4) from C₁₈ h.p.l.c. of refolded g.p.c.-f.p.l.c. protein (samples for tracks 1 and 2 were reduced with 2-ME); track 3, sample as track 2, but unreduced, indicating little dimer in this preparation (although the latter is sometimes observed). The apparent M_r of IL-2 (calculated from the standards) was 15030.

Table 3. Renaturation: variation of protein and IL-2 concentration

Other conditions were: t , 16 h; [GdmCl], 0.6 M; [DTT], 1 mM. Peak X (fully bioactive, oxidized IL-2) yield was determined from RP-h.p.l.c. peak area, and percentage renaturation recovery calculated from these data and from the total (denatured) IL-2 in the g.p.c. pool as determined by gel scanning (Fig. 3). All IL-2 was oxidized within 16 h.

Pool	[Protein] (μ g/ml)	[IL-2] (μ g/ml)	Recovery of peak X (%)
(a) G.p.c.-f.p.l.c.a	29	2.4	23
	8.7	0.7	30
	2.9	0.2	38
(b) G.p.c.-f.p.l.c.b	16.5	0.7	31
G.p.c.-f.p.l.c.a	8.7	0.7	30
G.p.c.-f.p.l.c.b	8.7	0.4	38

Oxidation

In the presence of 1 mM-DTT at pH 8.5, it was necessary to leave IL-2 solutions for 6–24 h to oxidize fully, hence the g.p.c.-DS pool in 0.1% TFA was used to study oxidation by O₂ (with and without added Cu²⁺) and by thiol/disulphide mixtures (Table 4). Clearly, Cu²⁺ speeds the rate of autoxidation, as has been observed for ribonuclease (Ahmed *et al.*, 1975) and lysozyme (Yutani *et al.*, 1968).

Yields of IL-2 were similar to those obtained in the presence of DTT; however, if g.p.c.-DS samples were left at 4 °C in air for 24 h, substantial decreases in peak-X yield were observed that were reversed by reduction

Table 4. Oxidation to peak X

Other conditions were: *t*, 1 h; [GdmCl], 0.6 M; [DTT], 0; protein concentration, 42 µg/ml; [IL-2], 4 µg/ml; pool, g.p.c.-DS. The percentage oxidation to peak X was determined from RP-h.p.l.c. peak areas, with (peak X + peak Y) = 100%.

Added catalyst/oxidant	Oxidation (%)
No addition	34
1.5 µM-CuSO ₄	100
1 mM-GSSG	61
5 mM-GSSG/0.5 mM-GSH	100
1 mM-GSSG/1 mM-GSH	100
0.5 mM-GSSG/5 mM-GSH	95

and reoxidation, indicating the formation of wrong disulphide isomer(s) in the unfolded polypeptide.

GSSG/GSH mixtures also led to efficient oxidation (Table 4). The improved rate in the presence of GSH compared with GSSG alone indicates that thiol-disulphide reactions were rate-limiting in glutathione oxidation of IL-2, with mixed disulphide accumulating in the absence of free thiol (Creighton, 1978). Although rates were good with GSSG/GSH, there was the possibility that the third thiol of IL-2 had become derivatized, hence Cu²⁺/O₂ was considered the better oxidant.

Renaturation and purification protocol

On the basis of observations that IL-2 must be kept reduced during purification in GdmCl but that its oxidation/renaturation is inhibited by high levels of DTT, f.p.l.c. gel permeation was performed in 6 M-GdmCl/1 mM-DTT/20 mM-acetate buffer, pH 5; IL-2 remained reduced under these conditions for several days. Renaturation and autoxidation was effected at pH 8.5 in the presence of Cu²⁺; oxidation was attempted at lower pH values in the hope of increasing the yield, but was found to be extremely slow below pH 6. With IL-2 concentrations in the region of 1–2 µg/ml, highly purified IL-2 could be prepared conveniently at the 50 µg level of analytical RP-h.p.l.c. (Table 5). Since IL-2 will remain reduced on the time scale of preparative g.p.c. and, furthermore, renaturation is possible in bulk

solutions, then this methodology is amenable to scaling up to at least the milligram level.

DISCUSSION

Many heterologous proteins are expressed in *E. coli* as aggregates (Marston *et al.*, 1984); given the harsh conditions required to solubilize totally such IL-2 aggregates, it seems likely that much of this protein is improperly folded. The causes of inclusion-body formation are unclear; it may be that the nascent polypeptide aggregates in a manner analogous to that seen here *in vitro*; precipitation of folding intermediates has been demonstrated *in vivo* for P22 tailspike endorhamnosidase (King, 1986). IL-2 is a secreted protein and, in the eukaryotic cell, has a 19-residue signal sequence (Taniguchi *et al.*, 1983) that is lacking in our construct and may prevent aggregation by binding to the signal-recognition particle/endoplasmic reticulum (Walter & Blobel, 1982) and immobilizing the chain during folding. The lack of glycosylation of recombinant IL-2 (Liang *et al.*, 1985) is unlikely to play a part in aggregation, since 20% of native human IL-2 from cell culture is also unglycosylated (Robb *et al.*, 1984).

The assignment of peak X as conformationally correct IL-2 was based on specific activity and on evidence of oxidation. The *M_r* determination by SDS/PAGE showed that the polypeptide was largely intact; *N*- or *C*-terminal proteolysis remains a possibility on this evidence, although none has been reported for recombinant IL-2 from *E. coli*, except for variable aminopeptidase removal of the initiator methionine (Kato *et al.*, 1985; Liang *et al.*, 1985; Yamada *et al.*, 1986).

Assignment of peak X as correctly oxidized protein, with the Cys-58–Cys-105 disulphide bridge formed, was confirmed by its retention time on RP-h.p.l.c.; the mispaired isomers Cys-58–Cys-125 and Cys-105–Cys-125 are eluted much earlier in the gradient (Browning *et al.*, 1986) and may be responsible for the minor IL-2 peak at 45% acetonitrile. Recovery of Cys-58–Cys-105 IL-2 was much diminished if the protein was allowed to oxidize when denatured, since the Cys-105–Cys-125 bridge will predominate under these conditions (Browning *et al.*, 1986) and, in the absence of added thiol, reshuffling to the correct isomer did not occur at a significant rate on refolding.

In summary, the recovery of IL-2 renatured from

Table 5. Purification and renaturation of IL-2 by g.p.c. and h.p.l.c.

Notes: ^a, equivalent to 80 ml of *E. coli* culture (*A*₆₀₀ = approx. 1) or 280 mg of cell paste; ^b, recovery of denatured IL-2 assumed to be 100% for these steps (owing to difficulties in accurately measuring IL-2 in crude samples); ^c, IL-2 renatured by dilution to give a final [IL-2] of 1.5 µg/ml. Other conditions were: protein concentration, 17 µg/ml; [GdmCl], 0.3 M; [DTT], 0.05 mM; buffer, 50 mM-Tris/HCl (pH 8.5)/1.5 µM-CuSO₄; *t*, 3 h; temperature, 20 °C. Since recoveries from the C₁₈ column were near 100%, most losses of IL-2 occurred during renaturation. Abbreviation: ND, not determined.

	Total protein (mg)	Total vol. (ml)	Total IL-2 (µg)	Purity (%)	Purification (fold)	Recovery (%)
Whole cells ^a	28	–	150	0.5	1	ND
GdmCl extracts ^b of cell pellet	4.4	0.8	150	3.4	6.4	ND
Superose 12 g.p.c. ^b	1.7	5.0	150	8.8	16.5	100
Renaturation ^c /Synchropak RP-P C ₁₈	0.033	1.5	33	95	180	22

semi-purified extracts of *E. coli* has been improved by examination of the folding and oxidation conditions; such a strategy should also be useful for the production of other recombinant proteins that need to be denaturant-extracted and do not refold easily in high yield.

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