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Characterization and bioassay of post-translationally modified interferon α-2b expressed in *Escherichia coli*

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ABSTRACT

Examples of N-terminal acetylation are rare in prokaryotic systems, but in this study, we report one such example in which N-terminal Cys residue of recombinant human interferon α-2b produced in *Escherichia coli* is a favourite site for N^{α}-acetylation. The recombinant protein following Q-sepharose chromatography gave a single band on PAGE analysis. However, on reverse phase HPLC the material separated into three peaks. These were characterized by mass spectrometric techniques as: (a) the direct translation product of the gene retaining the N-terminal methionine, (b) a species from which the methionyl residue had been removed by *E. coli* methionyl aminopeptidase to give the native interferon α-2b and (c) in which the N-terminal Cys residue of the latter contained an acetyl group. Tryptic digestion of interferon α-2b gave fragments linking Cys^{1} to Cys^{98} and Cys^{29} to Cys^{138}, while that of N^{α}-acetyl-interferon α-2b gave the Cys^{1}–Cys^{98} fragment with an additional mass of 42 attributed to an acetylated N-terminal. Bioassay of the derivatives showed that N^{α}-acetyl-interferon α-2b had 10% of the activity of interferon α-2b. The results suggest that the lower activity derivative seen here in *E. coli* may also be produced when the protein is produced in yeast.

1. Introduction

Interferon α-2b is a member of a large group of proteins which has a range of biological activities, notable amongst which is the antiviral action originally described in the pioneering work of Isaacs and Lindenmann (1957), (Isaacs et al., 1957; Lindenmann et al., 1957; Burke and Isaacs, 1958; Isaacs et al., 1958; Isaacs and Burke, 1958). Historically, along with proinsulin, interferon was one of the first proteins to be produced using the methods of recombinant technology by the groups of Pestka and Weissmann (Maeda et al., 1980; Goeddel et al., 1980; Weissmann et al., 1982; for a review see, Pestka, 1986). Subsequently, various members of the interferon family have been cloned and expressed in *Escherichia coli*, yeast or mammalian cells, and various forms of interferon are now available for clinical use (Pestka, 1981).

The expression of a protein in *E. coli*, by necessity requires the presence in the gene of the protein-initiation codon for methionine (Met). The primary translation product, of the recombinant protein thus contains N-terminal methionyl residue. *In vivo* processing of which by the host methionine aminopeptidase depends on the nature of the residue following the N-terminal Met (Hirel et al., 1989). The mature form of interferon α-2b has as its N-terminal a cysteine (Cys) residue linked to Cys^{98} by a disulphide bond (Fig. S1) (Pestka, 1986), requiring the removal of the starter metionine from the primary translation product. In this paper, we describe the isolation of the species expressed by the human interferon α-2b gene in *E. coli* and the elucidation of their key structural features using mass spectrometry.

2. Materials and methods

2.1. Isolation and cloning of DNA encoding human interferon α-2b

The isolation of DNA, expression of the encoded protein and its purification is described by Mahmood (2009). Briefly, human genomic DNA was isolated from the blood of a healthy student using the method of (Blin and Stafford, 1976) and served as template for further manipulations carried out using the protocols described by (Maniatis et al., 1978). In the first PCR reaction, 1F

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and 1R (Table S1) were used as forward and reverse primers to give a 705 bp product. The latter then served as a template for the second PCR using 2F and 2R as forward and reverse primers. The resulting 625 bp DNA contained the coding sequence for interferon α-2b as well as codons for N-terminal methionine, stop codon and downstream sequence of 119 nucleotides. The DNA was transferred *via* pTZ57R/T (Fermentas®) into the expression plasmid pET-21a(+) (Novagen®) to give pET-21a-*Ifn.* The latter plasmid was used to transform *E. coli* BL21-CodonPlus (DE3)-RIL cells.

2.2. Cell biomass production and solubilization of inclusion bodies

Batches of bacterial cultures, from the transformed colonies above, in 2–10 l were grown in orbital shake flask incubators at 37 °C and 100 rpm. LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) was used as the growth medium. The primary inoculum was grown overnight (for 12–16 h) in 10 ml of LB broth and transferred to 100 ml of LB broth for further growth for 3 h. This secondary culture was then used as inoculum to grow bacteria on large scale, with inoculum:medium ratio of 1:99. When bacterial culture optical density at 600 nm (OD_{600}) reached 0.4–0.6, lactose (0.8 mM) was added, as expression inducer, and incubation was continued for further 5 h so that maximum expression of the desired protein could be achieved.

The cells were harvested by centrifugation at 5000 × *g* for 5 min and the pellet suspended in 15 ml of sonication buffer (50 mM Tris–Cl pH 8.0, 1.5% Triton X-100, 200 mM NaCl, 10 mM EDTA, 0.1% sodium azide and 2 mM DTT) per 3.0 g of cell pellet. The suspension was then subjected to sonication for a total of 45 min in 9 cycles. One cycle consisted of 5 bursts of sonication, each of 60 s, followed by cooling in ice for 120–180 s. After every cycle the cell lysate was subjected to centrifugation at 5000 × *g*, 4 °C for 20 min. The supernatant was discarded while to the pellet again sonication buffer was added and the above operation repeated for further 8 cycles. The entire time including centrifugation took from 8 to 10 h, when an aliquot of final pellet, following solubilization in SDS (sodium dodecyl sulphate), as described below, showed a 260 nm:280 nm ratio of around 0.64:1.

For refolding, inclusion bodies were suspended in a small volume of solubilization buffer (8 M urea, 50 mM Tris–Cl (pH 8.0), 50 mM glycine and 4 mM DTT; glycine was included in the solubilization buffer to help avoid carbamylation of protein) and sonicated on ice for 5 cycles of 30 s pulse and a gap of 2 min. Final volume was adjusted with the solubilization buffer to give protein concentration of 5-7 mg/ml and the mixture incubated at 37 °C for 30 min to allow complete reduction of disulfide bridges. A clear solution, obtained after centrifugation of solubilized protein at 5000 × *g*, 4 °C for 20 min, was further processed for refolding.

2.3. Quantification of proteins

For the quantification of protein in final suspension, the inclusion bodies in 100 μl of representative sample was centrifuged and the pellet solubilized in 5% SDS solution. An appropriate dilution of the protein solution in 5% SDS was used for recording the spectrum from which absorbance at 260 nm and 280 nm was measured. Protein quantification, from absorbance at 280 nm, sometimes leads to erroneous over estimation of protein concentration due to the presence of nucleic acids. In the present work most of the contaminating nucleic acids are removed by repeated sonication and centrifugation cycles to give a 260 nm:280 nm ratio of around 0.64:1. If the E_{260}:E_{280} ratio is ≥0.64 then the estimate is corrected for actual protein quantity using the following formula, given in equation below, which is an approximation derived from the original data of (Warburg and Christian, 1941) and further elaborated by (Layne, 1957).

$$\text{Corrected protein concentration} = 1.55 \times (280\,\text{nm absorbance}) - 0.76 \times (260\,\text{nm absorbance})$$

2.4. Refolding and protein purification by Q-sepharose column chromatography

Following is illustrative of the refolding procedure used in the present study. To the refolding buffer (1250 ml; 100 mM Tris–Cl pH 8, 2 mM EDTA, 0.5 mM cystine, 5 mM cysteine, 0.1 mM PMSF) was added the protein (610 OD_{280} units, 100 ml; 6.1 OD_{280} units/ml) in 10 pulses containing 60 OD_{280} units of protein in each pulse, every 4 h. The reaction was gently stirred and maintained at 4 °C. The final concentration of the protein in the refolding sink was about 0.5 OD_{280} units/ml. The reaction mixture was dialysed against 20 mM Tris–Cl pH 8, with 4–5 changes of the buffer, or subjected to diafiltration to reduce the volume of the folding mixture. In both cases the reaction mixture was clarified by centrifugation at 5000 × *g* for 20 min, at 4 °C.

A suspension of Q-sepharose (50 ml) was poured into a column 2.5 cm × 20 cm (diameter × length) which was washed with autoclaved distilled water (200 ml) to remove any preservative. The equilibration of column was carried out with 500 ml of 50 mM Tris–Cl (pH 8.0). After equilibration, the refolded protein in refolding buffer was loaded on to the column manually. The flow rate of column was adjusted to 1 ml/min so that proper binding of protein to the resin could take place. The column was washed again with the same buffer (50 mM Tris–Cl pH 8.0) until the OD at 280 nm of the flow-through was zero. The protein was then eluted with different concentrations of NaCl (0.1 M to 1.0 M) in 50 mM Tris–Cl pH 8.0. The volume of each concentration of NaCl applied to the column was 15 ml. The protein profile in the collected fractions was monitored by taking OD at 280 nm and the quality of purification was judged by 15% SDS-PAGE.

The protein fractions, judged pure by SDS-PAGE analysis, were mixed together and dialyzed against 0.1% TFA till concentration of salt became approximately 0.001 M. The weight of the lyophilized powder was determined and the amount of protein per 1 mg of the powder measured by taking OD at 280 nm. For this estimation the extinction coefficient of human interferon α-2b as determined from online ExPASy Proteomics Server, ProtParam tool (ExPASy, Switzerland) was used to give 1 mg of the protein in 1.037 OD_{280} units/ml.

2.5. Protein purification by reverse phase liquid chromatography

For reverse phase high pressure liquid chromatography (RP-HPLC) separation, preparative columns from Thermo Scientific® (Thermo Scientific; BioBasic C-18; particle size: 5 μm; length 250 mm × internal diameter 10 mm) were used and gradient was generated with 0.1% TFA (trifluoroacetic acid) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). About 0.5–1.0 mg of the above solid was solubilized in solvent A and loaded on column with 0% solvent B for 5 min and then eluted with 40%–80% gradient of solvent B at 0.5% ramp/min. Different fractions of protein were eluted between 59% and 61% solvent B. Individual fractions were collected in separate containers and freeze dried for further mass spectroscopic analysis of intact proteins as well as their tryptic fragments.