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Low temperatures stabilize interferon α-2 against proteolysis in *Methylophilus methylotrophus* and *Escherichia coli*

Julian A. Chesshyre and Alan R. Hipkiss

Department of Biochemistry, King's College London, University of London, Strand, London WC2R 2LS, United Kingdom

Summary. The accumulation of interferon (IFN) α-2 in transformed strains of *Escherichia coli* and *Methylophilus methylotrophus* was greater at 25° C than at 37° C. Interferon α-2 catabolism was followed by measuring the change in IFN titre (measured immunoreactively) with time at temperatures between 25° C and 37° C in chloramphenicol-treated cells. The IFN α-2 titre remained constant at 29° C and below, while at higher temperatures the titres declined. The $t_{1/2}$ values for IFN α-2 decreased with increasing incubation temperature. Pulse-chase studies using [^{35}S]methionine, sodium dodecyl sulphate-gel electrophoresis and autoradiography demonstrated that IFN α-2 was subjected to degradation at 37° C while at 25° C it was stable. It is proposed that the susceptibility of IFN α-2 to degradation in both *E. coli* and *M. methylotrophus* is affected by incubation temperature and 30° C may be a transition temperature above which the conformation of the molecule is recognised by the bacterial proteases.

Introduction

The use of recombinant strains of microorganisms for the production of foreign gene products is now well established. Much attention has been paid to factors affecting the expression of the desired polypeptide. Efficient modes of transcription and translation and the stable maintenance of plasmid-encoded genes have been well explored in the development of high-yielding recombinant systems. However, expression of heterologous proteins has often brought attendant problems of product instability toward proteolytic degradation. Although few principles have yet emerged which describe the predisposition of a protein towards intracellular catabolism in bacteria, it is likely that the degradation of cloned gene products may be influenced by variations in protein conformation.

Many aberrant cellular proteins, e.g. protein fragments and those containing certain amino acid analogues, as well as foreign polypeptides, are rapidly degraded in *Escherichia coli* (Kemshead and Hipkiss 1974; Goff and Goldberg 1985). The proteolytic system responsible contains both constitutive and inducible enzymes (Hipkiss 1979), some of which are ATP-stimulated but most are energy-independent (Swamy and Goldberg 1981). Many approaches can be employed either to decrease catabolism of cell proteins in general or, specifically to stabilise the commercially-useful heterologous polypeptide (Carrier et al. 1983). These include the use of cells possessing mutations in either a protease gene (Boss et al. 1984), for example, *lon* mutants that are defective in the ATP-stimulated protease La, or regulation of protease gene expression, i.e. *htpR* mutants that do not induce further synthesis of proteases brought about via a variety of stimuli including synthesis of foreign gene products (Snow and Hipkiss 1987). Overproduction of the required protein product, the addition of zinc ions (Gross et al. 1985), increasing product chain length (Shen 1985) and secretion into the periplasmic space or into the growth medium (Shiroza et al. 1984), have also been employed.

Temperature is a parameter which affects protein folding and stability (Privalov 1979); Lin and Zabin (1972) clearly showed that changes in temperature can profoundly affect intracellular catabolism of β-galactosidase fragments. We therefore investigated the effects of temperature upon the

Offprint requests to: A. R. Hipkiss

accumulation of human interferon α-2 (IFN α-2), constitutively expressed, in two differing Gram-negative bacteria both of which contained the same plasmid possessing the IFN α-2 gene.

Materials and methods

Bacterial strains, plasmids and growth conditions. Methylophilus methylotrophus AS1 pIFS 1303 was grown at 25° C or 37° C as previously described (Chesshyre et al. 1987) and was a gift from Dr. M. Worsey, ICI Corporate Bioscience Division, Runcorn, Cheshire, UK. *Escherichia coli* strains JA221 pIFS 1303 and JA221 pIFS 1205, gifts from Dr. R. C. Hockney, ICI Pharmaceuticals Division, Alderly Park, Macclesfield, Cheshire, UK, were grown in M9 minimal medium (Miller 1972) at 25° C or 37° C.

Plasmid pIFS 1205 is an expression plasmid for ampicillin resistance and IFN-α2 (Windass et al. 1982) using a synthetic IFN-α2 gene (Edge et al. 1986). Plasmid pIFS 1303 has the synthetic IFN-α2 gene and a sulphathiazole-resistance gene.

Determination of IFN-α2 in M. methylotrophus and E. coli. Aliquots of cell taken for estimation of IFN-α2 were from cultures grown as previously described (Chesshyre et al. 1987) to mid-logarithmic phase ($OD_{650}=0.4$). Cultures were treated with chloramphenicol (100 µg/ml) and aliquots added to hydrofluoric acid (48% w/v, BDH, Poole, UK) for *M. methylotrophus*, or hydrochloric acid (36% w/v) for *E. coli*, to give a final concentration of 0.1 *M* and stored at −20° C. After thawing at 20° C the samples were sonicated for 15 s at 4° C. For aliquots assayed without subsequent dilution the pH was adjusted to pH 7.0 using NaOH (10 *M*). Titres of IFN-α2 were measured using a commercially-available assay kit according to the manufacturers' instructions (NK2 IRMA — Celltech, Slough, UK).

Pulse chase analysis of IFN-α2 by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Escherichia coli JA221 pIFS 1205 was grown at 37° C from overnight stationary-phase cultures. At $OD_{650}=0.37$–0.47, cultures were labelled (L-[^{35}S]methionine 1.0 µCi/ml) for 60 min at 25° C. The cells were pelleted by centrifugation (12,000*g* for 5 min), resuspended in growth medium supplemented with 1 mg unlabelled methionine/ml and re-incubated at either 25° C or 37° C. At intervals 1 ml samples were removed and centrifuged (12,000*g* for 1 min); the pellets were immediately frozen at −70° C. For analysis by SDS-PAGE each pellet was resuspended in sample buffer (SDS, 1%; glycerol, 10%; 2-mercaptoethanol, 5%; TRIS-HCl, 0.065 *M*; EDTA, 2 *mM*; pH 6.8). The sample was then heated at 95°–97° C for 10 min prior to storage at −20° C. Samples were subject to electrophoresis according to Laemmli (1970) using 17% gels. Protein staining was carried out using Coomassie G-250 in TCA according to Neuhoff et al. (1985). Coomassie Brilliant Blue (CBB) G-250 (Sigma Ltd., Poole, UK) was purified according to Neuhoff et al. (1985). Following drying, the gels were exposed to Fuji RX 100 film at −70° C after treatment with fluor ("Enthance", New England Nuclear, Boston, Mass, USA).

Results

Interferon titres and growth temperature

Methylophilus methylotrophus ASI pIFS 1303 and *E. coli* JA221 pIFS1303 were grown at 37° C and

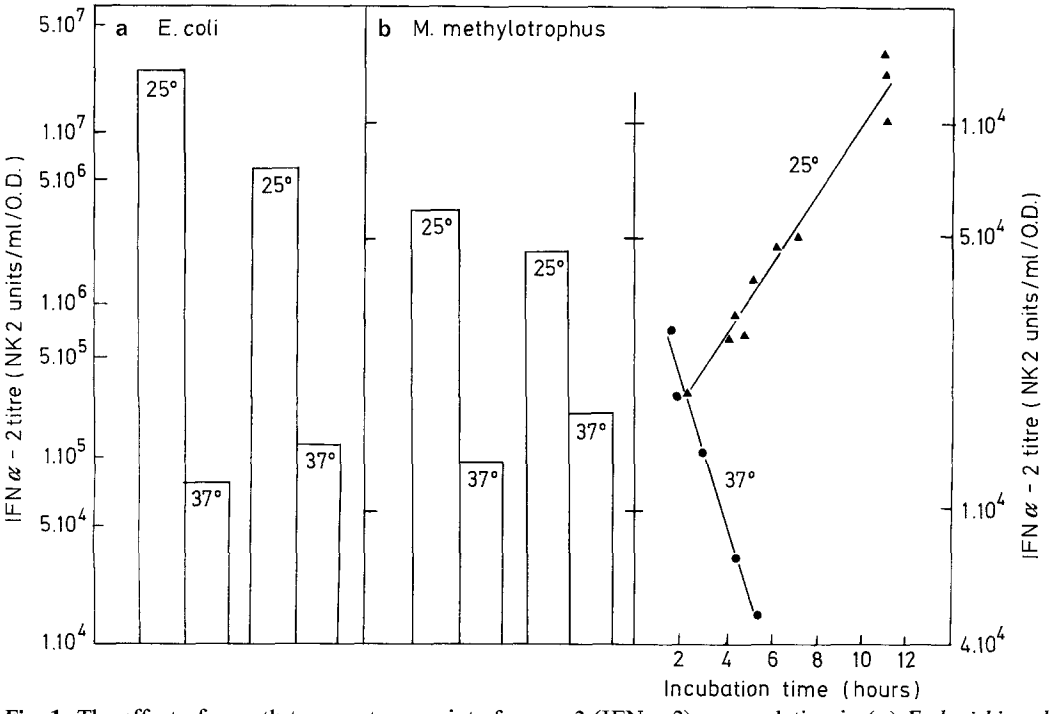

Fig. 1. The effect of growth temperature on interferon α-2 (IFN α-2) accumulation in (**a**) *Escherichia coli* and (**b**) *Methylophilus methylotrophus*. *E. coli* JA221 pIFS1303 and *M. methylotrophus* ASIpIFS1303 were grown at 25° C and 37° C and IFN α-2 titres were assayed at intervals; note scale difference for *E. coli*. In **b** incubation time represents the time subsequent to inoculation with stationary phase ("overnight") cultures into fresh medium

25° C and both organisms possessed greater IFN α-2 titres at the lower temperature (Fig. 1); up to a 250-fold difference for *E. coli*. Three other *E. coli* strains transformed with pIFS1303 were also found to accumulate more IFN α-2 at 25° C compared to 37° C (results not shown). We also found that at 25° C the IFN α-2 titres in *M. methylotrophus* increased logarithmically with lapsed time between inoculation from stationary phase overnight culture and the collection of cells in mid-logarithmic growth (Fig. 1b). However, the reverse relationship was observed when growth was conducted at 37° C (Fig. 1b).

Degradation of interferon-α2

Th accumulation of any protein is dependent on the rates of its synthesis and degradation. The results in Fig. 1 might be partly explained by differences in the rates of IFN α-2 degradation at the two temperatures. Therefore in order to detect IFN α-2 catabolism in the absence of further IFN α-2 synthesis, chloramphenicol (100 μg/ml) was added to cultures of *M. methylotrophus* ASI pIFS1303 and *E. coli* JA221 pIFS1303 following growth at 25° C. At 25° C the IFN α-2 titre remained constant (i.e. half-life ($t_{1/2}$) = ∞ and $1/t_{1/2}$ = 0) whereas an increase in temperature to above 29° C, following the addition of chloramphenicol to cells which had accumulated IFN α-2 at 25° C, gave rise to a decline in IFN α-2 titres (Fig. 2). Interferon α-2 synthesized at 37° C is similarly rapidly depleted in chloramphenicol-treated *M. methylotrophus* cells at 37° C (Chesshyre et al. 1987). In both organisms, transference from 37° C to 25° C resulted in stabilization of IFN α-2. Therefore we propose that the loss of immunogenic reactivity at temperatures above 29° C represents significant degradation of the IFN α-2 im-

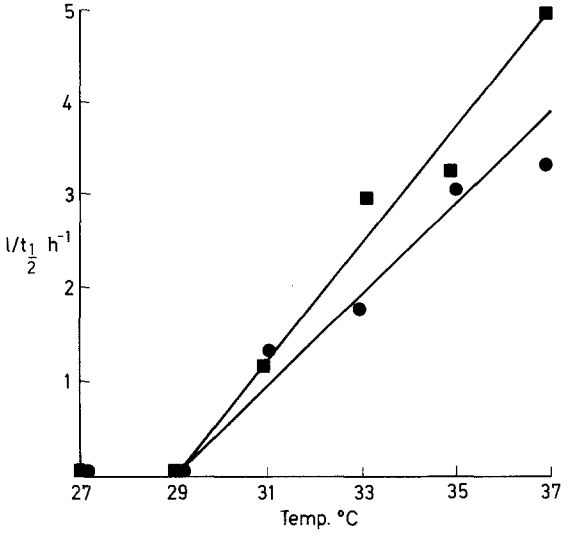

Fig. 2. The effect of temperature on the half-life ($t_{1/2}$) of interferon α-2 in *M. methylotrophus* ASIpIFS1303 and *E. coli* JA221 pIFS1303. Cells were grown logarithmically at 25° C to allow accumulation of IFN α-2, and then transferred to water baths at 27° C, 29° C, 31° C, 33° C, 35° C and 37° C with the simultaneous addition of chloramphenicol (100 μg/ml). The time taken for each IFN α-2 titre to fall to half of its initial value at each temperature was determined. The half-lives are corrected for expected kinetic effects as follows; at each temperature the half-life was adjusted by an appropriate factor to eliminate expected differences in IFN α-2 catabolism assuming $Q_{10}=2$, such that all corrected $t_{1/2}$ values should be the same (e.g. the observed half-life at 27° C should be twice that at 37° C if the differences in the rates of IFN α-2 catabolism were solely due to simple kinetic effects. Thus the $t_{1/2}$ value detected at 27° C was halved). Where $1/t_{1/2}=0$, no detectable decline in the IFN α-2 titre was observed in 120 min. At 37° C, IFN α-2 half lives of between 10 and 20 min were determined in *E. coli* JA221 pIFS1303 (■) and *M. methylotrophus* ASIpIFS1303 (●) respectively

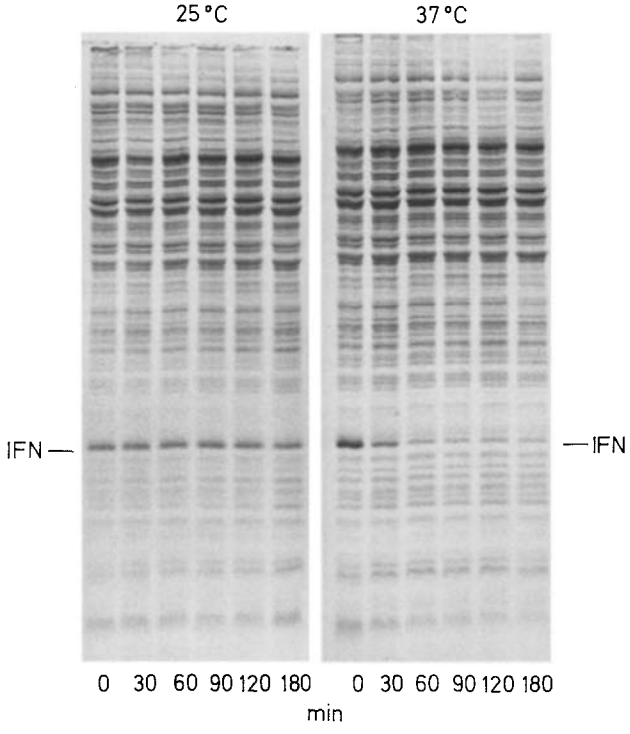

Fig. 3. Effect of incubation temperature on the degradation of IFN α-2 in *E. coli* JA221 pIFS1205. Cells grown at 25° C to mid-logarithmic phase were labelled at 25° C with 1 μCi/ml [^{35}S]methionine (specific activity 37 TBq/mmol) for 1 h. Following centrifugation, washing and resuspension in M9 medium supplemented with 1 mg unlabelled methionine/ml, the cells were re-incubated at either 25° C or 37° C. Samples were taken at the times indicated and the proteins therein separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and visualized by autoradiography

Table 1. Comparison of proteolysis of aberrant proteins in *Escherichia coli* and *Methylophilus methylotrophus* at 25° C and 37° C

	Rate of proteolysis (%/h) at	
	25° C	37° C
E. coli JA221 pIFS1303	31	50
M. methylotrophus AS1	10	16

Escherichia coli was treated with canavanine sulphate (100 μg/ml) for 35 min and pulse-labelled with 1.5 μCi/ml [^{3}H]leucine (183 Ci/mmol) for 10 min at 37° C; proteolysis was then assayed at 37° C or 25° C as described by Hipkiss (1979). *Methylophilus methylotrophus* was treated with puromycin hydrochloride (100 μg/ml) for 30 min and pulse-labelled with 0.3 μCi/ml [^{3}H]isoleucine (104 Ci/mmol) for 10 min; proteolysis was then assayed at 25° C or 37° C as described by Chesshyre et al. (1987)

munoreactivity at higher temperatures. The proposal for degradation of a significant proportion of the IFN α-2 molecule rather than a minor immunoreactive region is supported by the findings of Edge et al. (1986) who have shown that recognition of the NK2 epitope, which is the basis of the immunological IFN α-2 assay in this study, is still present in amino and carboxyl truncates of IFN α-2 analogues.

The catabolism of IFN α-2 in *E. coli* at 37° C was confirmed by autoradiography; a protein preferentially labelled with [^{35}S]methionine at 25° C and identified as IFN α-2 by SDS-PAGE/immunoblotting was rapidly degraded (Fig. 3b). Chloramphenicol had no effect upon the loss of radioactively labelled IFN α-2 at 37° C (results not shown). At 25° C no loss of ^{35}S-labelled IFN α-2 was detected (Fig. 3a). It should be noted that the proteolytic apparatus is still operative at 25° C in both organisms as evidenced by the degradation of aberrant cellular polypeptides (canavanyl proteins or puromycyl peptides) at more than half the 37° C rates (Table 1).

Discussion

The identical temperature of IFN-α2 stabilisation in both organisms suggests that a feature of the IFN α-2 molecule predisposes it towards proteolytic degradation. The use of chloramphenicol also served to prevent de novo synthesis of heat shock proteins, one of which is a known protease (Goff and Goldberg 1985), and therefore the catabolism of IFN α-2 was effected by proteases subject to the same physiological conditions under which IFN α-2 accumulated.

Interestingly, Schein and Noteborn (1988) have reported that in *E. coli* DS410 low culture temperature favours the formation of soluble forms of three different recombinant proteins, one of which was IFN α-2. It should be noted however that IFN α-2 is comparatively stable at 37° C in *E. coli* DS410, used by Schein and Noteborn (1988) ($t_{1/2}$ = 180 min) (Gow and Hipkiss 1989), whereas in *E. coli* JA221 (used in the present study) the recombinant gene product is labile ($t_{1/2}$ = 20 min, Fig. 2). The question of different solubility of IFN α-2 at various temperatures was not addressed in the present study. The accumulation of IFN α-2 in *M. methylotrophus* at any temperature is rather poor compared to many other recombinant systems and therefore it is unlikely to present problems of product precipitation in vivo. Even in *E. coli* the absence of degradation at temperatures greatly favouring solubilisation of IFN α-2 (i.e. 25° C) demonstrates that insolubility per se is unlikely to be the mechanism by which IFN α-2 *escapes* proteolysis. In fact we suggest the reverse, the factors which predispose the IFN α-2 to aggregate (e.g. exposure of hydrophobic surfaces) also promote its susceptibility towards degradation. Interferon α-2 is a hydrophobic molecule which has a tendency to form oligomers transiently in concentrated solution (> 1 mg/ml) in vitro (Thatcher and Panayotatos 1986), hence at high intracellular concentrations it is conceivable that precipitation might be due not only to intramolecular disulphide exchange but also to hydrophobic interaction.

As low temperatures are known to weaken hydrophobic interaction, solubilisation of IFN α-2 may be favoured. Mitraki et al. (1987) have reported that the aggregation of phosphoglycerate kinase is abolished at low temperatures in vitro. It has also been pointed out that intramolecular disulphide bonds preferentially form in IFN α-2 at 37° C (Wetzel et al. 1983) and that sulphydryl group reagents also cause temperature-dependent precipitation (Schein and Noteborn 1988). In the present study the acid lysis of cells used for IFN α-2 titre determination, would ensure that IFN α-2 would be fully reduced and presumably soluble (less than 5% of total IFN α-2 was associated with the particulate fraction of acid-lysed 25° C-grown *E. coli* (results not shown)). We also observed by immunoblotting preferential oligomer formation following SDS-PAGE under non-reducing conditions in cultures of *E. coli* grown at 37° C compared to 25° C. Therefore the disulphide bond status of IFN α-2 at low temperature may be related to its stability and solubility.

This report shows that the lower titres of IFN α-2 obtained at higher culture temperature ($>30°$ C) in two different organisms is due to protein catabolism. The determinant of heat-dependent labilization of this polypeptide has not been defined but it is tempting to hypothesize that alteration in the integrity of disulphide bonds and exposure of hydrophobic surfaces may be sufficiently disruptive to allow access to protease cleavage sites. However further investigations involving a combination of physico-chemical studies of purified IFN α-2 carried out at different temperatures coupled with the characterization of the protease which initiates degradation at the permissive temperature might reveal which temperature-dependent changes in the conformation of IFN α-2 are important with respect to stability.

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