Control of protein synthesis by amino acid supply

The effect of asparagine deprivation on the translation of messenger RNA in reticulocyte lysates

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The enzyme asparaginase, which hydrolyses asparagine to aspartic acid, inhibited cell-free protein synthesis by reticulocyte lysates. The inhibition was rapid and complete when sufficient enzyme was added but could be prevented or reversed by the addition of asparagine. The initial effect of asparaginase appears to be a block in polypeptide chain elongation due to asparagine deprivation, but there are some indications that prolonged incubation under these conditions may give rise to a secondary decrease in initiation of protein synthesis.

Asparaginase *Protein synthesis* *Asparagine deprivation* *mRNA translation*

1. INTRODUCTION

It is generally accepted that eukaryotic protein synthesis may be modulated at the level of translation by control of initiation involving either the availability of messenger RNA or the activity of initiation factors, particularly eIF-2 [1,2]. In addition, an adequate supply of all 20 genetically encoded amino acids is required and it has been known for some years that a number of tumour cells in culture respond to deprivation of an essential amino acid by inhibition of initiation, as shown by disaggregation of polyribosomes [3,4] and decreased formation of the 48 S pre-initiation complex [5]. In extracts from amino acid-starved cells the defect in the synthesis of the pre-initiation complex can be overcome by the addition of exogenous eukaryotic initiation factor eIF-2 [6] suggesting a mechanism similar to that involved in the haemin-controlled inhibition of protein synthesis [7]. It is, however, not clear how lack of an amino acid gives rise to such a block in protein synthesis initiation.

Investigations of the control of protein synthesis by amino acid supply are hampered by the difficulty of eliminating the availability of a particular amino acid rapidly and completely. Normal protein turnover replenishes the pool of endogenous amino acids by proteolysis in both intact cells and cell-free systems and to ensure complete depletion of an amino acid it is necessary to remove an amino acid efficiently and continuously as soon as it is released by protein breakdown. In this report we show that asparaginase can be used to deplete reticulocyte lysates of asparagine. The resultant inhibition of protein synthesis has been characterized and it appears that the primary effect is a block of polypeptide chain elongation rather than initiation.

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2. MATERIALS AND METHODS

2.1. *Preparation of reticulocyte lysates*

New Zealand white rabbits were made anaemic by subcutaneous injection of neutralized 2.5% phenylhydrazine hydrochloride in 1 mM mercaptoethanol (0.25 ml/kg body wt) on each of 5 successive days. After an interval of 2 days heparin (1 ml, 500 U) and nembutal (1.5 ml) were injected into the marginal ear vein and blood was collected by cardiac puncture. Reticulocytes were isolated at 4°C by sedimentation at 600 × *g* for 10 min, washed twice with 130 mM NaCl-5 mM KCl-7.5 mM $MgCl_2$ and lysed in an equal volume of water. Cell debris was removed by centrifugation at 28000 × *g* for 20 min and the supernatant was stored at −70°C in small aliquots.

The mRNA-dependent lysate was prepared [8] by incubation at 20°C for 20 min with *S. aureus* micrococcal nuclease (150 U/ml) in the presence of 1 mM $CaCl_2$, 25 μM haemin and 50 μg/ml creatine kinase. Nuclease activity was stopped by the addition of EGTA (2 mM final concentration). Calf liver tRNA (50 μg/ml) was added and the lysate was stored at −70°C.

Incubation mixtures (0.1 ml) were prepared by mixing lysate (40 μl), a cocktail (40 μl) containing creatine phosphate (37.5 mM), creatine kinase (75 μg/ml), all protein amino acids (0.1 mM) except methionine, Tris-HCl (50 mM, pH 7.6), dithiothreitol (2.5 mM), magnesium acetate (5 mM) and KCl (200 mM), 1 mM haemin (2 μl), [^{35}S]methionine (2 μl, 19.4 μCi) and water with other ingredients where stated (16 μl). Incubations were at 30°C. At appropriate times aliquots (5 μl) were removed and diluted with 1 ml of a solution containing 0.5 M NaOH, 2.5% (v/v) H_2O_2 and unlabelled methionine (1 mg). To precipitate the proteins 25% trichloroacetic acid (1 ml) was added and after 1 h at 4°C samples were filtered on to Whatman GF/C filters, washed four times with 5% trichloroacetic acid and dried. Radioactivity measurements were made in a Beckman LS-200C counter using a toluene/PPO/POPOP scintillation fluid.

2.3. *Analysis of labelled nascent polypeptides*

After incubation of lysates polyribosomes were sedimented by centrifugation at 100000 × *g* for 1 h. The pellets were resuspended in 1 N KOH (0.15 ml) and incubated at 37°C for 1 h to release nascent chains by cleavage of the peptidyl tRNA. The samples were buffered to pH 3.5–4.0 by the addition of 250 μl pyridine-acetic acid-water (1:6:25, by vol.). Debris was removed by centrifuging at 100000 × *g* for 2 h, aliquots of the supernatant (20 μl) were withdrawn for counting and the remaining solutions were chromatographed on a Sephadex G-15 column (10 × 0.5 cm diameter) using pyridine-acetic acid-water (1:6:25, by vol.) for elution. Fractions (100 μl) were collected and counted in a Beckman LS-100C spectrometer using pico fluor as scintillant.

2.4. *Materials*

[^{35}S]Methionine (1120 Ci/mmol) was purchased from New England Nuclear, [^{14}C]phenylalanine (504 mCi/mmol) from Amersham International, *E. coli* L-asparaginase (EC 3.5.1.1) from Sigma, calf liver tRNA from Boehringer, and poly(U) from Miles Laboratories. Poly(A)$^+$ globin mRNA was prepared from rabbit reticulocyte polysomes essentially as described by Krystosek et al. [9] using oligo(dT)-cellulose from P.L. Biochemicals for the affinity chromatography. Other chemicals were of Analar grade as supplied by BDH.

3. RESULTS

3.1. *Inhibition of protein synthesis by asparaginase*

The effect of different amounts of asparaginase on cell-free protein synthesis by the reticulocyte lysate is illustrated in fig.1. At or above 0.1 unit/0.1 ml incubation mixture the enzyme inhibited protein synthesis very rapidly, as shown by a decrease in the rate of incorporation of labelled methionine into protein to less than 10% of that of the control within 2 min. A lower concentration of asparaginase (0.01 unit/0.1 ml) had no observable effect for 4 or 5 min but thereafter protein synthesis was inhibited rapidly. This lag is probably due to the time required to hydrolyse the asparagine present in the lysate and in the amino acid mixture which was added to the cell-free system (see section 2.1).

3.2. *Reversal of asparaginase action by asparagine*

The inhibition of protein synthesis by asparaginase could be prevented or reversed by

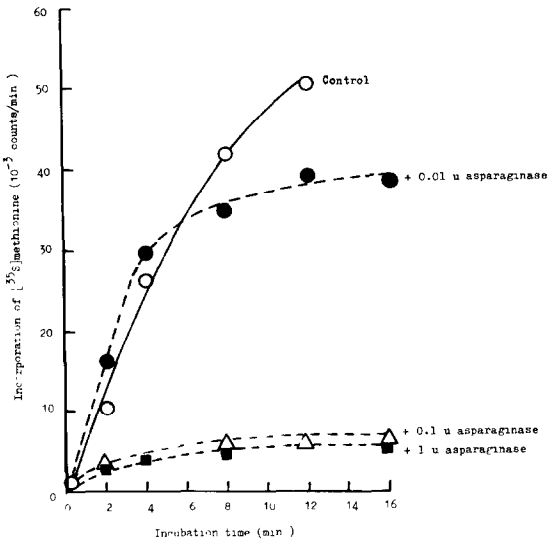

Fig.1. Inhibition of cell-free protein synthesis by asparaginase. Different amounts of asparaginase were added to the complete incubation mixture (0.1 ml) and the incorporation of [^{35}S]methionine into protein was assayed after incubation for up to 16 min.

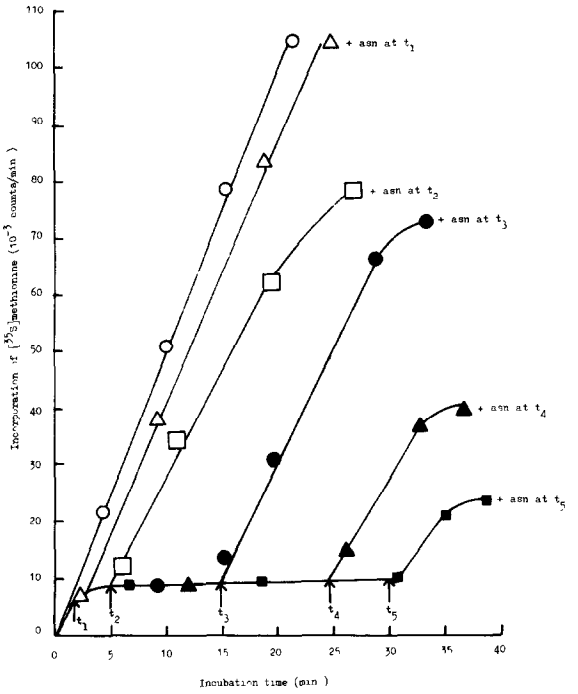

Fig.2. Reversal of the inhibitory action of asparaginase on protein synthesis. The complete cell-free system was incubated with asparaginase (0.1 unit/0.1 ml) and asparagine (10 μmol) was added at various times (t_1–t_5) as indicated. The incorporation of [^{35}S]methionine into protein after incubation for up to 40 min was assayed and compared with a control (○) to which no asparaginase or asparagine had been added.

adding asparagine (10 μmol) to the incubation mixture. A typical experiment involving addition of asparagine at various times up to 30 min after the start of the incubation with asparaginase is shown in fig.2. When asparagine was added protein synthesis resumed almost immediately at a rate which in all cases was almost identical with that of the control incubation mixture without enzyme.

3.3. *Specificity of asparagine in reversing the action of asparaginase*

The reversal of the inhibitory effect of asparaginase by asparagine is specific and the addition of lysine, which has two codons differing in the third base from the two asparagine codons AAU and AAC, or of glycine or glutamine does not counteract the inhibition significantly (table 1).

3.4. *Effect of asparaginase on protein synthesis by the messenger-dependent nuclease-treated reticulocyte lysate*

Asparaginase also inhibited protein synthesis by the mRNA-dependent lysate [8] although in this case the effect was observed after somewhat longer incubation with asparaginase, i.e. 10 min, than with the normal reticulocyte lysate (not shown).

Table 1

Effect of different amino acids on the inhibition of cell-free protein synthesis by asparaginase

Amino acid	Reversal of inhibition by asparaginase (%)
Asparagine	98.8 ± 1.5
Glutamine	5.1 ± 0.8
Glycine	1.2 ± 1.4
Lysine	2.3 ± 1.9

Complete incubation mixtures (0.1 ml) were incubated either with asparaginase (0.1 unit) or without enzyme (control). The amino acids (10 μmol) were added after 2–7 min and the incorporation of [^{35}S]methionine into protein was assayed after 15 min. The results are expressed as the % incorporation compared with the control (74000 cpm). The incorporation without amino acid addition was 9500 cpm. Figures are mean values of two, or in the case of asparagine three, experiments

After prolonged incubation periods (15–20 min) with asparaginase there was some decrease in the poly(U)-directed polymerization of phenylalanine and this effect was also prevented by supplementation of the incubation mixture with asparagine, even when added as late as 10 min after the start of the incubation with asparaginase (fig.3). When the nuclease-treated lysate was pre-incubated with asparaginase for 10 min to inhibit protein synthesis before addition of globin mRNA and labelled methionine, the inhibited lysate was still able to initiate protein synthesis as shown by the presence of short labelled nascent peptides on ribosomes in the absence of asparagine (fig.4). Addition of asparagine restored the ability of the lysate to synthesize nascent polypeptides of essentially the same size range as found in the control lysate which had not been treated with asparaginase.

4. DISCUSSION

The addition of asparaginase to reticulocyte lysates was found to give rise to a rapid and complete inhibition of cell-free protein synthesis which

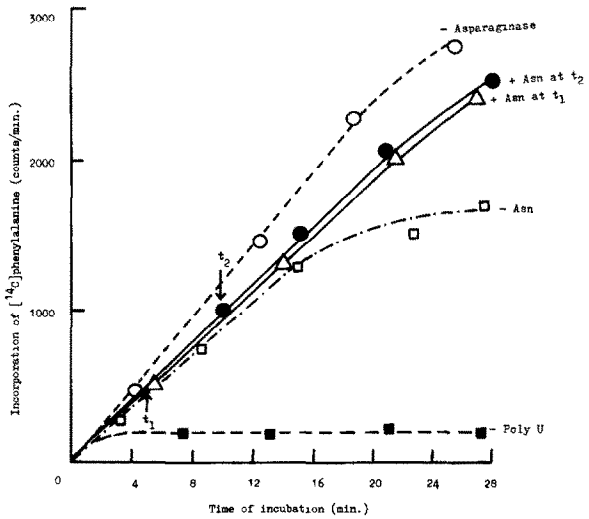

Fig.3. Poly(U)-directed polymerization of phenylalanine in the presence of asparaginase. A messenger-dependent nuclease-treated lysate was incubated with poly(U) and asparaginase (0.1 unit/0.1 ml) except that control incubations contained either no asparaginase (○) or no poly(U) (■). Where indicated, asparagine (10 μmol) was added to reverse the action of asparaginase.

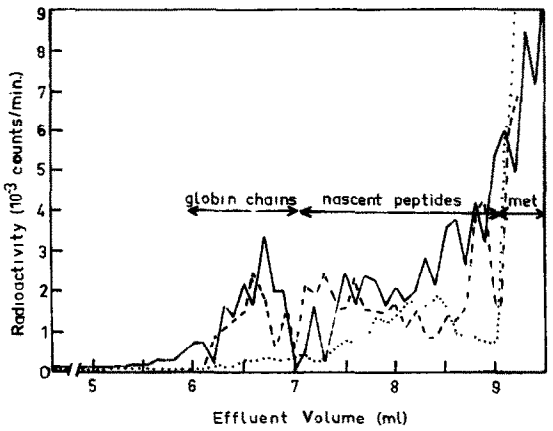

Fig.4. Synthesis of nascent polypeptide chains by the mRNA-dependent lysate in the presence of asparaginase. Complete incubation mixtures (0.2 ml) containing nuclease-treated lysate (0.16 ml) were preincubated with asparaginase (0.4 unit) for 10 min at 30°C. Poly(A)$^+$ globin mRNA (1 μg) and [^{35}S]methionine (95 μCi) were then added in water (50 μl) with or without asparagine (4 μmol) as indicated and the incubation was continued for a further 8 min. Polyribosomes were isolated and labelled nascent polypeptides analysed by Sephadex G-15 chromatography, as described in section 2.3. Radioactivity in nascent polypeptides from incubation in the presence of asparaginase (···), asparaginase plus asparagine (– – –), or no asparaginase control (——).

is evidently due to depletion of asparagine since supplementation with this amino acid prevents or reverses the inhibition. Neither lysine, whose codons differ from that for asparagine by one base, nor glycine or glutamine has any significant effect showing that the requirement for asparagine is specific. The inhibition is reversible by asparagine even after incubation with asparaginase for periods as long as 30 min and protein synthesis resumes with little or no lag.

Poly(U)-directed synthesis of polyphenylalanine is not immediately inhibited by asparagine depletion although after relatively long incubation periods there is some decrease, possibly by a secondary effect. The globin mRNA-dependent lysate was found to be able to synthesize short nascent peptides when the mRNA was added after asparagine depletion by asparaginase. The first asparagine residue in the rabbit α- and β-globin chains occurs in positions 9 and 19 respectively [10,11] and the synthesis of new but short nascent

peptides under these circumstances is in agreement with a block in elongation rather than initiation. The observation that polyphenylalanine synthesis continues for a significantly longer period of time after addition of asparaginase than does protein synthesis also suggests that the primary cause of the inhibition is the inability of the translation system to decode mRNA at asparagine codons in the absence of charged asparaginyl tRNA due to asparagine deprivation.

In Ehrlich ascites tumour cells or HeLa cells deprivation of essential amino acids, for example histidine, has been reported to give rise to inhibition of initiation [3,12]. It is not clear, however, whether the observed disaggregation of polyribosomes due to run-off was the result of prolonged incubation under conditions of incomplete amino acid depletion, which may inhibit initiation to a greater degree than polypeptide chain completion. In the present experiments, the only indication of a possible effect on initiation was the decrease in polyphenylalanine synthesis some 10–15 min after the general inhibition of protein synthesis. Although translation of poly(U) differs from that of messenger RNA in the mechanism of initiation, it is possible that prolonged incubation in conditions involving inhibition of elongation may eventually lead to a secondary block of initiation. In any case, however, the fact that the inhibition of protein synthesis by asparagine deprivation is readily reversible at least up to 30 min suggests that the mechanism of any such effect is likely to be different from the regulation of initiation by the haemin-controlled repressor, which gives rise to an irreversible inhibition after a comparatively short incubation period.

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