STIMULATION OF HAEMOGLOBIN SYNTHESIS IN RETICULOCYTE LYSATES BY INITIATION FACTORS

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1. Introduction

A lysate of reticulocytes [1] can synthesize haemoglobin at up to 25% of the rate of intact cells and, unlike fully fractionated systems, [2, 3] is active in polypeptide chain initiation. The high activity of the lysate is maintained for only 5–10 min at 37°; the rate of incorporation thereafter is very slow. This decline in activity, which is accompanied by polysome breakdown and accumulation of monomeric ribosomes, probably results from a failure of the system to bring about reattachment of ribosomes to endogenous messenger RNA for longer than a brief period of incubation.

A ribosomal extract, prepared by treating reticulocyte ribosomes with high concentrations of KCl has been shown to stimulate amino acid incorporation in various fractionated reticulocyte cell free systems [4–7]. The extract seems to contain at least three protein factors, all of which are required for *de novo* haemoglobin synthesis by washed reticulocyte ribosomes [5], and which may be similar to bacterial initiation factors [8, 9]. We show here that it is possible to sustain the activity of lysates for up to one hour by addition of these crude chain initiation factors [4, 5], and we discuss the possible significance of these results to the control of chain initiation in reticulocytes.

2. Methods

Reticulocyte-rich blood was obtained from rabbits injected with phenylhydrazine. The cells were sedimented and washed three times with medium RS [10] and lysis was effected by one of two methods. A concentrated lysate was prepared as described by Lamfrom and Knopf [1], and either used immediately for incorporation experiments, or stored at −70°. For all other procedures, cells were lysed by 3 volumes of RSB [11], followed 2 min later by 0.45 times the original cell volume of 2 M sucrose. Unlysed cells, cell debris and mitochondria were removed by centrifugation at 15,000 *g* for 10 min, and the ribosomes were pelleted by centrifuging at 165,000 *g* for 1 hr. The pellet was suspended in 0.1 M sucrose and adjusted to 0.6 M KCl [6]; the ribosomes were pelleted again at 204,000 *g* for 2½ hr, and the supernatant passed down a Sephadex G25 column, equilibrated in medium C (35 mM tris-Cl pH 7.6, 70 mM KCl, 3.5 mM $MgCl_2$, 6 mM β-mercaptoethanol). The haemoglobin-containing band, referred to as "factors", was collected and used immediately.

3. Results

The time-course of incorporation of a diluted lysate in the presence and absence of factors is shown in fig. 1. In the presence of factors, the initial rate of incorporation is slightly lower than in its absence but no drastic decline in activity occurs after 10 min of incubation. The system remains active for at least one hour if factors are present, although the rate of incorporation decreases progressively.

During incubation in the absence of factors, most of the polysomes are degraded to monomeric ribosomes within 10 min (fig. 2) releasing nascent peptide chains. The presence of factors partially prevents this degradation, so that more than 20% of the ribosomes

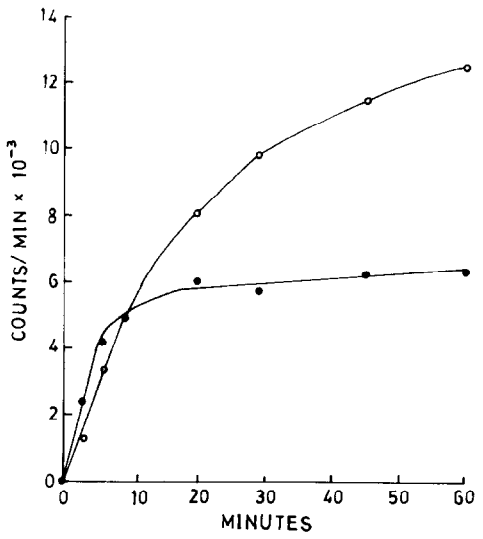

Fig. 1. Effect of factors on time course of incorporation of ^{14}C-phenylalanine. Each incubation finally contained, per ml: 1 μmole ATP, 0.2 μmole GTP, 20 μmoles tris-HCl pH 7.6, 110 μmoles KCl, 2 μmoles $MgCl_2$, 6 μmoles β-mercaptoethanol, 1.8 mg creatine phosphate, 0.2 mg creatine phosphokinase, 0.5 μCi ^{14}C-phenylalanine (specific activity 459 mCi/mmole), 20 mμmoles amino acids minus phenylalanine, 0.5 ml factors (in medium C) o—o, or 0.5 ml medium C (•—•), and 0.4 ml lysate. Tubes were incubated at 37°, and 0.1 ml samples were removed in duplicate at the times indicated. Each sample was precipitated with 0.5 M perchloric acid (containing unlabelled phenylalanine). The precipitate was spun down in a bench centrifuge, dissolved in 1 ml 1 M NaOH (containing unlabelled phenylalanine), and incubated at 37° for 1 hr. Protein was reprecipitated with 1 ml 2 M perchloric acid (containing unlabelled phenylalanine) and collected on glass fibre filters. The filters were stuck to the centre of cardborard discs with rubber solution, and counted in a Nuclear Chicago gas flow counter (efficiency 18%). The results are expressed as cpm/sample.

exist as polysomes after 30 min of incubation, and these are still actively synthesizing polypeptide chains.

We found that it was necessary to dilute standard lysates in order to obtain a response to the factors. Table 1 shows that the activity of a lysate is increased roughly 100% by factors if it is first diluted with at least three volumes of water. Such a dilution in itself, lowers the activity of a lysate quite substantially, but the addition of factors can restore the activity to the prediluted state. It is possible that the fall in activity results mainly from a dilution of the factors already present in the concentrated lysate.

Table 1
The response to factors as a function of lysate concentration.

Volumes of water added to 1 volume of concentrated lysate	Incorporation (cpm)		% stimulation
	+factors	−factors	
0	23,130	22,814	1
0.33	24,532	24,460	0
1.00	26,826	21,485	25
3.00	22,937	10,079	128
7.00	5,695	2,794	104

A concentrated lysate was diluted serially with water, and triplicate samples of each dilution were incubated, in the presence and absence of factors, under the conditions described in the legend to fig. 1, with the following minor differences: the volume of lysate (per ml incubation) was 0.25 ml, and the ^{14}C amino acid was leucine (specific activity 312 mCi/mmole). Incorporation was measured after 90 min of incubation at 34°, exactly as described in the legend to fig. 1. Results are expressed as cpm per incubation.

Sodium fluoride inhibits globin synthesis in cell free systems by interfering with peptide chain initiation but completion of peptides which were started in the intact cell is not affected by fluoride [12, 13]. The factors do not stimulate protein synthesis in diluted lysates if 10 mM NaF is present (table 2). It seems that the factors are increasing initiation of chains, just as they do in fractionated systems.

Table 2
Effect of factors in the presence of NaF.

	Lysate preparation 1	Lysate preparation 2
+factors	23,483	3,837
−factors	10,179	2,101
+factors, +NaF	3,435	575
−factors, +NaF	3,891	576

Two separate preparations of lysate (obtained from different animals) were diluted with 3 volumes of water, and incubated under the conditions described in the legend to fig. 1, with the following minor differences: the volume of lysate (per ml) was 0.2 ml, the volume of factors (per ml) was 0.4 ml, and the ^{14}C amino acid used was leucine (specific activity 312 mCi/mmole). Sodium fluoride was present at a final concentration of 10 mM and control tubes contained 10 mM sodium chloride instead. Incorporation was measured at 90 min of incubation at 34°, exactly as described in the legend to fig. 1. Results are expressed as cpm per incubation.

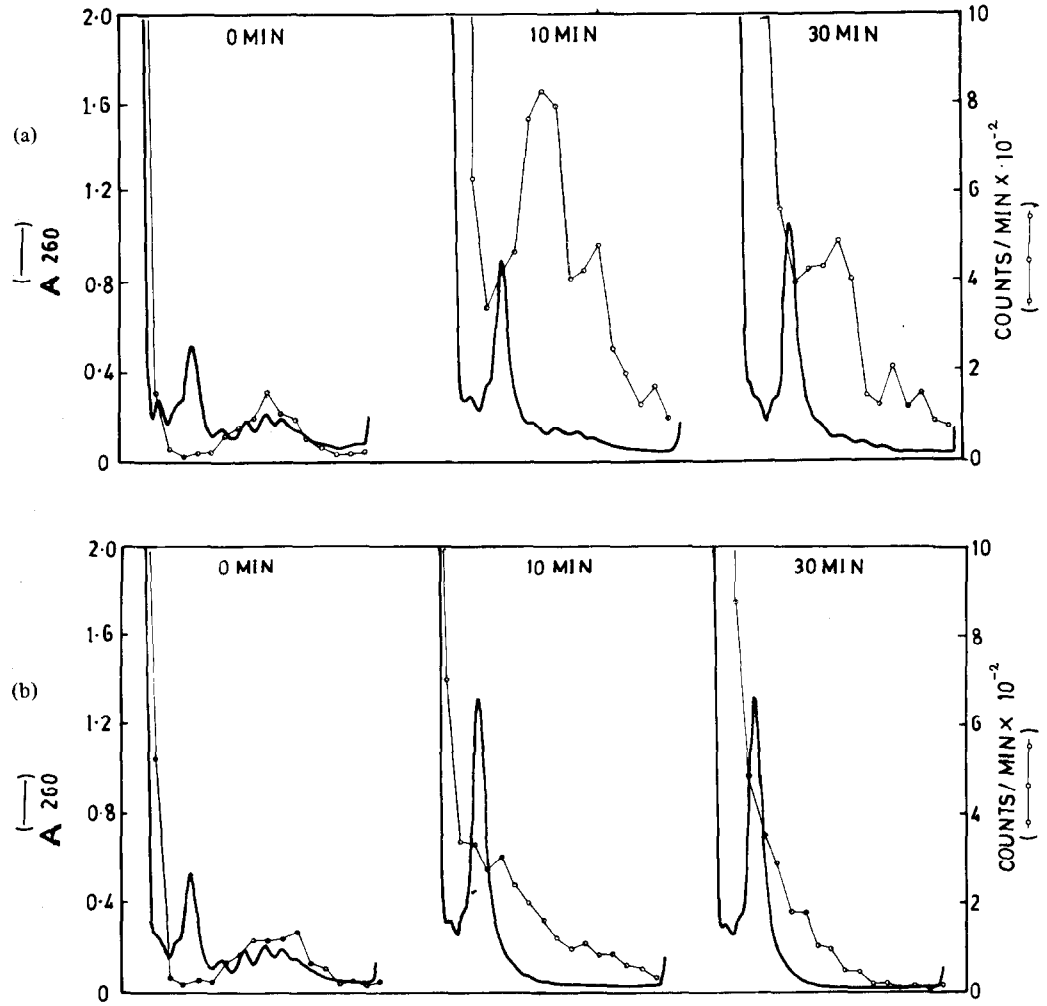

Fig. 2. Change in ribosome profile during incubation in presence and absence of factors. The lysate was incubated exactly as described in the legend to fig. 1, and samples were removed after 0 min, 10 min and 30 min of incubation. After chilling each sample to 0°, 1 ml of each was layered on a 12 ml 15–30% linear sucrose gradient (containing 20 mM tris-HCl pH 7.6, 110 mM KCl, 2 mM $MgCl_2$), and centrifuged for 80 min at 40,000 rpm in the SW40 rotor of the Beckman L2 65B preparative ultracentrifuge. After centrifugation, the bottom of the tube was punctured, and the gradient displaced upwards through an LKB flow cell by pumping 70% sucrose at a constant rate up through the tube. The absorbance at 260 nm of the gradient, as it passed through the flow cell, was monitored continously by a Unicam SP800 recording spectrophotometer. Gradient fractions were collected every 20 sec; 0.5 mg of carrier bovine serum albumin was added to each fraction, and precipitation with cold 0.5 M perchloric acid (containing unlabelled phenylalanine). The precipitates were collected on glass fibre filters and counted as described in the legend to fig. 1. (——) absorbance at 260 nm; (——o——o——) cpm. fig. 2a: + factors, fig. 2b: – factors.

4. Discussion

Fuhr et al. [14] found that a cell free system obtained by precipitating a lysate at pH 5.4 was also active in polypeptide chain initiation and that further chain initiation occurred when a soluble factor was added. The factors described in the present paper were obtained from ribosomes rather than from the soluble fraction, but it is possible that they may be similar to the factor of Fuhr et al. [14].

The stimulatory effects of ribosomal factors in unfractionated lysates described here are qualitatively very similar to the effects of haemin. Lysates incubated in the presence of exogenous haemin remain active for at least 30 min at 37°; polysomes are stabilised and chain initiation is increased [15, 16]. It is unlikely, however, that the active material present in our crude factors is merely haemin, since all activity is lost by heating to 80° for 2 min and the factors had been passed down a Sephadex G25 column. We cannot exclude the possibility that haemin is present and complexed with a protein.

Recently it has been suggested that haemin stimulates chain initiation indirectly by preventing formation of a soluble inhibitor [17]. The inhibitor accumulates if lysates are incubated in the absence of haemin, and is considered to be responsible for the cessation of protein synthesis which occurs, but it has not yet been possible to demonstrate this suggestion [18]. The simplest explanation of our results is that chain initiation factors stimulate initiation directly, presumably in a similar way to bacterial initiation factors. However, it is also possible that they interact in some way with soluble inhibitor. Further investigation is required to determine what role, if any, the soluble inhibitor plays in this system.

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