A translational inhibitor activated in rabbit reticulocyte lysates under high pO_2

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An inhibitor of protein synthesis was activated under high oxygen partial pressure (pO_2) in hemin-supplemented and glutathione disulfide-free lysates from rabbit reticulocytes. This inhibitor shared some common features with other translational inhibitors from rabbit reticulocytes; that is, hemin-controlled repressor, glutathione disulfide-activated inhibitor and high pressure-activated inhibitor. It caused biphasic kinetics of inhibition which could be potentiated by ATP. Its activation was prevented by cAMP or glucose 6-phosphate. The high pO_2-inhibitor could be partially purified from post-ribosomal supernatant containing ribosomal salt wash by precipitation between 0–50% $(NH_4)_2SO_4$-saturation, Sephadex G-100, and DEAE-cellulose chromatography.

Translational inhibitor *Reticulocyte lysates* *High pO_2* *Hemin-controlled repressor* *Glutathione disulfide* *Glucose 6-phosphate*

1. INTRODUCTION

Protein synthesis in rabbit reticulocytes and their lysates is inhibited by the deficiency of hemin, by low concentrations of glutathione disulfide (GSSG) or by double-stranded RNA (dsRNA) (for recent reviews see [1–3]). In all these cases the inhibition displays the following features: protein synthesis reveals biphasic kinetics, that is, it declines abruptly after having proceeded at the control rate for the first few minutes; a breakdown of polysomal structure with the conversion into 80 S monosomes occurs; inhibition is potentiated by ATP and reversed by cAMP, GTP and particularly by eukaryotic initiation factor 2 (eIF-2); the inhibition is dependent upon the activation of inhibitor(s) which, phosphorylate(s) specifically the same site(s) on 38 K subunit of the initiation factor eIF-2 [4].

Abbreviations: hpO_2, high pO_2; hpO_2I, high pO_2-induced inhibitor; HCR, hemin-controlled repressor; GSSG, glutathione disulfide; GSSGI, glutathione disulfide-activated inhibitor; dsRNA, double-stranded RNA; PAI, pressure-activated inhibitor; eIF-2, eukaryotic initiation factor 2; G6P, glucose 6-phosphate.

We asked whether the globin chain synthesis might be additionally under a control mechanism dependent on pO_2. Preliminary data suggested a reciprocal relationship between the activity in protein synthesis and pO_2 and, moreover, the activation of an inhibitor (hpO_2I) of protein synthesis in reticulocytes under high pO_2 [5]. As the further data to be reported below show, hpO_2I can be activated also in reticulocyte lysates and seems to share some common features with HCR or GSSGI. It causes biphasic kinetics of protein synthesis. Its effect is potentiated by ATP and prevented by cAMP or glucose-6-phosphate (g6P).

2. MATERIALS AND METHODS

Preparations of reticulocytes and reticulocyte lysates were as described [5]. For the preparation of ribosomal wash fraction, lysates were dialysed for 1 h against 5 mM $MgCl_2$ 25 mM KCl, 50 mM Tris–HCl (pH 7.4), 7 mM 2-mercaptoethanol (TKMM). The KCl-concentration was adjusted to 500 mM, the lysates were stirred for 30 min and centrifuged for 4 h at 120 000 × *g*. The upper two-thirds of ribosome-free supernatant which contained also ribosome salt wash were dialysed again

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for 1 h against TKMM, frozen and kept in liquid nitrogen. All steps were carried out at 4°C and all fractions were frozen and kept in liquid nitrogen. [^{3}H]Leucine (specific activity 52 Ci/mmol) was obtained from the Radiochemical Centre (Amersham), hemin chloride from Calbiochem. All other biochemicals were purchased from Boehringer (Mannheim).

The high pO_2-treatment of reticulocyte lysates was essentially as described for reticulocytes [5]. High pO_2-treatment was performed under normobaric conditions by blowing humidified O_2 (or, whenever indicated, 50% O_2 in N_2) for 15 min at 37°C through stoppered reaction tubes containing the lysates [5]. Samples were analysed in an ABL 1 Acid–Base Laboratory automatic gas analyzer (Copenhagen) for pO_2. Values over 25 kPa were regarded as high pO_2. Unless otherwise indicated, the lysate fractions, which before high pO_2-treatment were either dialysed against TKMM or gel-filtered on Sephadex G-25 with TKMM, contained 50 μM hemin chloride [6] and 1 mM ATP. The protein synthetic activity of the lysate fractions after exposure to high pO_2 was assayed under normal atmospheric air as described [5].

3. RESULTS

In agreement with the previous data obtained with reticulocytes [5], an inhibition of protein synthesis could be observed also in the lysates preincubated under high pO_2. In such high pO_2-lysates, the rate of protein synthesis was maintained during the first 3–5 min at the control level. There-

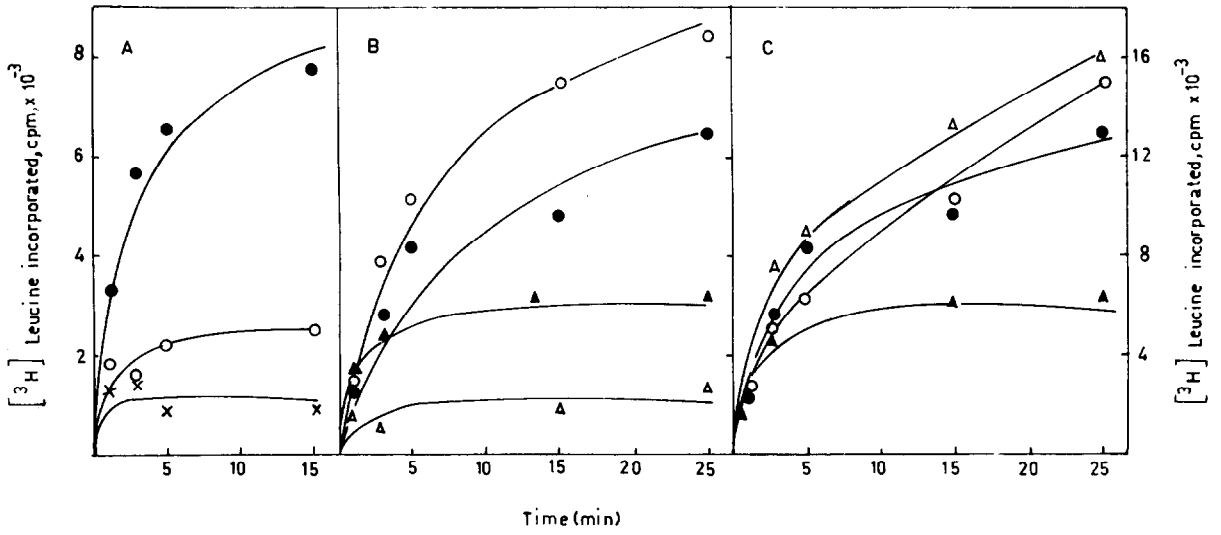

Fig.1. (A) Activation of hpO_2I in reticulocyte lysates under high pO_2. Experimental conditions were as described under Materials and Methods. The lysates in TKMM were preincubated for 15 min at 37°C in the presence of 1 mM ATP and 50 μM hemin chloride either under atmospheric air (•--•) or under 50% O_2 in N_2(○--○). These lysates (each 1 A_{260} unit) were assayed under atmospheric air in protein synthesis [5] either alone or mixed in 1:1 proportion (each 1 A_{260} unit) (×--×). [^{3}H]Leucine incorporated into hot CCl_3COOH-precipitable material in 15 μM aliquots was determined [5]. (B) Effect of ATP upon the activation of hpO_2I. The lysates in TKMM were incubated as under A under atmospheric air or high pO_2 either in the absence or presence of 1 mM ATP. Thereafter, 1 A_{260} unit lysate was assayed in protein synthesis as under (A). (○--○), lysate preincubated under atmospheric air with ATP; (•--•), lysate preincubated under atmospheric air without ATP; (△--△), lysate preincubated under high pO_2 with ATP; (▲--▲), lysate preincubated under high pO_2 without ATP. (C) Effect of cAMP upon the activation of hpO_2I. Lysates were preincubated under atmospheric air or under high pO_2 as above in the presence of 50 μM hemin chloride and 1 mM ATP with or without 2 mM cAMP. The lysates were, thereafter, assayed in protein synthesis as described under (A) and (B). (○--○), lysate preincubated under atmospheric air with cAMP; (•--•), lysate preincubated under atmospheric air without cAMP; (△--△), lysate preincubated under high pO_2 with cAMP; (▲--▲), lysate preincubated under high pO_2 without cAMP.

after, the synthesis stopped abruptly giving the typical biphasic kinetics (fig.1A). The addition of the lysate preincubated under high pO_2 depressed the control activity to the level of that of the high pO_2 lysate. As shown in fig.1B and C, the inhibition in the high pO_2-lysate could be potentiated by ATP and overcome by cAMP.

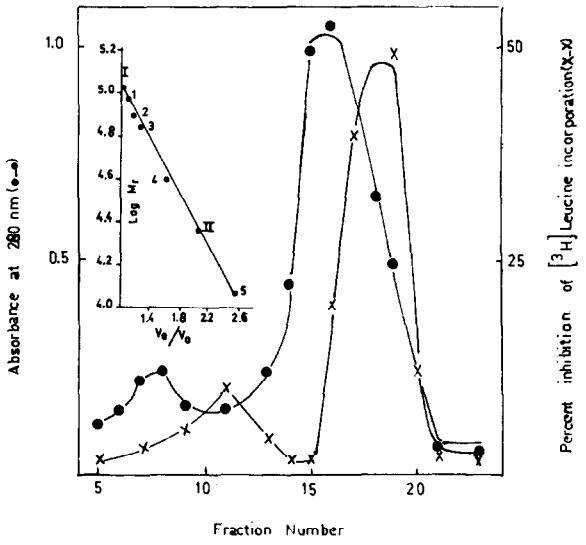

Fig.2. Chromatography of hpO_2I on Sephadex G-100. 6.2 mg protein enriched in hpO_2I activity by $(NH_4)_2SO_4$-precipitation between 0–50% saturation were applied to G-100 column (1×45 cm), equilibrated and eluted in TKMM buffer. 1 ml fractions were collected and 15 μl aliquots were assayed for hpO_2I activity in protein synthesis [5]. Control activity (0% inhibition) was 7500 cpm/20 μl reaction mixture. V_o (void volume) = 8.8 ml. The inset shows the repetition of the experiment on a second G-100 column (1×42 cm) calibrated with: (1) adenosine disphosphate ribosylated-elongation factor 2 (96 500) [7]; (2) creatine kinase (80 000); (3) bovine serum albumin (68 000); (4) ovalbumin (45 000); and (5) cytochrome *c* (12 500). V_e/V_o ratios, where V_e = elution volume corresponding to the peak concentration of each (standard) protein and V_o = void volume of the column (= 8.3 ml), were determined and plotted against log molecular weights. (I) corresponds to the hpO_2I activity eluting behind the void volume and (II) to the hpO_2I-activity eluting behind the main A_{280} peak. V_e used for hpO_2I (I) (= 8.5 ± 0.5 ml) and V_e for hpO_2I (II) (= 17.4 ± 1.5 ml) are means from four determinations. A third inhibitory activity of small molecular weight eluting with V_e = 29 ± 2 ml has not been investigated further and is not shown in the diagram.

As shown in table 1, the activation of hpO_2I could be also prevented by the addition of glucose-6-phosphate (G6P) ± glutathione (GSH) to the lysate prior to treatment with hpO_2. GSH alone seemed to render some protection against hpO_2I activation but, at the same time, to depress slightly the control activity.

hpO_2I-activity was found in the ribosomal (salt) wash fraction from the hpO_2-lysates, but not in S-100 or in the 2 M sucrose wash fraction (data not shown). hpO_2I-activity could be partially purified from the ribosomal salt wash fraction of the hpO_2-lysates. The inhibitory activity was first enriched by precipitation between 0–50% $(NH_4)_2SO_4$-saturation. When this fraction was chromatographed on Sephadex G-100, hpO_2I-activity was eluted slightly behind the main A_{280} (hemoglobin) peak (fig.2). A slight inhibitory activity eluting behind the void volume was also detected. When G-100 fractions containing hpO_2I-activity were subse-

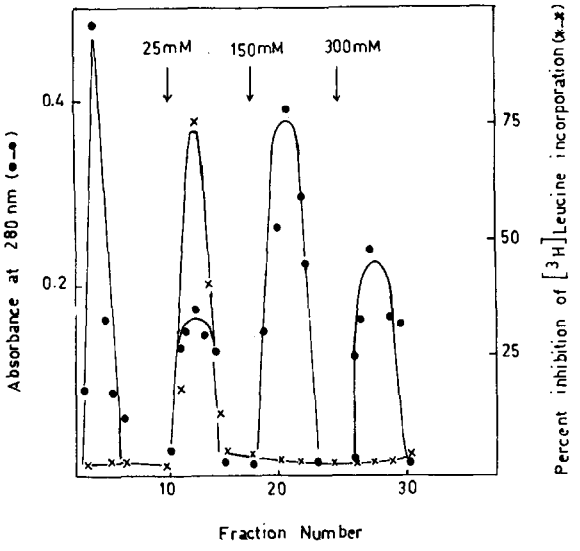

Fig.3. Chromatography of hpO_2I on DEAE-cellulose. The protein fraction (4.8 mg) containing the hpO_2I activity from G-100 column was applied to a column of Whatman DE32 (1×2 cm), equilibrated with 50 mM Tris–HCl (pH 7.4), 0.1 mM EDTA (pH 7.0), 7 mM 2-mercaptoethanol. Elution was carried out stepwise with 25 mM, 150 mM and 300 mM KCl in the same buffer. 15 μl aliquots from the indicated fractions (1 ml) were assayed for hpO_2I activity in protein synthesis [5]. Control activity (0% inhibition) was 12 200 cpm/20 μl reaction mixture.

Table 1

Effect of G6P and/or of GSH on the activation of hpO_2I

System	[^{3}H]Leucine incorporated (cpm)
(N)-lysate	944
(H)-lysate	97
(N)-lysate + (N)-lysate	1335
(N)-lysate + (H)-lysate	180
(N)-lysate + (N)-lysate + G6P	1283
(N)-lysate + (H)-lysate + G6P	1485
(N)-lysate + (N)-lysate + GSH	715
(N)-lysate + (H)-lysate + GSH	856
(N)-lysate + (N)-lysate + G6P + GSH	1297
(N)-lysate + (H)-lysate + G6P + GSH	920

Experimental conditions were as indicated under Materials and Methods. The dialysed lysates were incubated under normal atmospheric air (N) or high pO_2(H) in the presence of 50 μM hemin chloride and 1 mM ATP. The concentrations of G6P and of GSH, whenever they were present during preincubation, were 3 mM and 1 mM, respectively. Protein synthetic activity of the preincubated lysates alone or mixed in 1:1 proportion (each 0.2 A_{260} unit) were assayed for 10 min at 37°C as described [5].

quently chromatographed on DEAE-cellulose, the inhibitory activity was found in the 25 mM KCl-eluate (fig.3).

4. DISCUSSION

The findings above show that an inhibitor of protein synthesis is activated under high pO_2 in hemin-supplemented and GSSG-(GSH) free (i.e., gel filtrated) lysates. This inhibitor, hpO_2I, differs from HCR in molecular weight and chromatographic behaviour on DEAE-cellulose [7–11]. However, it shares also some common features with HCR, GSSGI and other translational inhibitors. It produces biphasic kinetics of inhibition which can be potentiated by ATP and overcome by cAMP. Moreover, the prevention of hpO_2I activation in the presence of G6P (± GSH) implicates a relationship between hpO_2I and HCR as well as GSSGI [12–17]. In view of some biochemical lesions which follow exposure to high pO_2 [18], it is likely that the activation of hpO_2I involves – possibly like that of the other translational inhibitors [12,19,20] – modification, i.e., oxidation, of some sulfhydryl groups. The relationship of hpO_2I to PAI [20,21] deserves also particular attention on account of similiarities in molecular weight (behaviour on G-100 chromatography [19]), activation procedures and apparent involvement of sulfhydryl groups during inhibitor activation. Future work will show whether all these repressor activities, such as cellular response to different environmental changes, are attributed to the same protein(s).

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