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Heat-stable translational inhibitor from rabbit reticulocyte lysates

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We have purified to apparent homogeneity a heat-stable (HS) factor from the postribosomal supernatant of rabbit reticulocyte lysates [(1988) FEBS Lett. 236, 479–483]. HS inhibits translation in hemin-supplemented lysates and induces phosphorylation of the α-subunit of the eukaryotic initiation factor 2 as does hemin deficiency. The translational inhibition produced by addition of HS to hemin-containing reticulocyte lysates and the accompanying phosphorylation of the eIF-2α subunit can be prevented or reversed by NADPH generators including glucose 6-phosphate, NADPH itself, and also by dithiols, e.g., dithiothreitol, but not by fructose 1,6-bisphosphate or by monothiols, e.g., 2-mercaptoethanol. When added to crude preparations of the proinhibitor form (proHCI) of the heme-controlled translational inhibitor (HCI), HS produces a pronounced increase of the HCI to proHCI ratio. It appeared possible that HS might be oxidized gluatathione (GSSG) but this is not the case, for HS is not a substrate for highly purified glutathione reductase from rabbit erytrocytes. The spectral analysis of highly purified HS is consistent with the idea that HS could be a nucleotide derivative.

Polypeptide chain initiation; Translational inhibition; eIF-2α kinase; HCI activation

1. INTRODUCTION

Protein synthesis in reticulocytes is regulated at the level of the eukaryotic polypeptide chain-initiation factor 2 (eIF-2) [1,2]. This factor forms a ternary complex with GTP and eukaryotic initiator methionyl tRNA (Met-tRNA$_i$), which then binds to a 40 S ribosomal subunit. Recycling of eIF-2 is catalyzed by GEF, the GDP-GTP exchange factor, and is inhibited when the eIF-2α subunit is phosphorylated by cAMP-independent protein kinases [3–5]. One of these kinases, the heme-controlled translational inhibitor (HCI), is activated in lysates incubated in the absence of added hemin or, with hemin present, upon addition of either oxidized glutathione (GSSG) [6] or Ca^{2+}-phospholipid [7]. In reticulocyte lysates HCI is present largely in the form of an inactive precursor, proHCI. Once activated, HCI inhibits translation in hemin-containing reticulocyte lysates, but the mechanism of activation remains obscure (for reviews see [1 and 2]).

We have previously reported that activation of proHCI in hemin-supplemented lysates can be elicited in a reversible fashion by Ca^{2+} and phospholipid (PL) [7]. Like the activation of HCI in lysates by GSSG or NADPH depletion [8], the activation by Ca^{+} and PL could be largely prevented or reversed by NADPH-generating systems or by dithiols [9].

Recently, we have described the purification of a novel heat-stable translational inhibitor from postribosomal supernatant of rabbit reticulocyte lysates (HS) [10]. HS factor inhibits translation in hemin-supplemented lysates by promoting phosphorylation of the α-subunit of eIF-2. According to the spectrum, the HS could be a nucleotide analogue and in this case, the concentration of HS required for inhibition of protein synthesis would be in the nanomolar range. Our results are consistent with the notion that HS activates the heme-stabilized proinhibitor form (proHCI) of HCI in reticulocyte lysates. Like the activation of HCI in lysates by GSSG or Ca^{2+}-PL [6,7], the activation by HS may largely be due to oxidation of labile SH groups which are involved in the activation of HCI.

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Abbreviations: eIF-2, eukaryotic polypeptide chain-initiation factor 2; Met-tRNA$_i$, eukaryotic initiator methionyl-tRNA; HCI, heme-controlled translational inhibitor (an eIF-2α kinase); proHCI, the proinhibitor (inactive) form of HCI; HS, heat-stable translational inhibitor from rabbit reticulocyte lysates; DTT, dithiothreitol; G6P, glucose 6-phosphate; FDP, fructose 1,6-bisphosphate; NEM, *N*-ethylmaleimide; GSSG, oxidized glutathione; GEF, guanine nucleotide exchange factor; PL, phospholipid; H^{+}-NMR, proton nuclear magnetic resonance

2. MATERIALS AND METHODS

2.1. *Materials*

G6P, FDP, GSSG and NEM were purchased from Sigma. All resins and the HPLC equipment were used earlier [10].

2.2. *Assays*

HS was assayed by its inhibitory effect on translation and its capacity for activating HCI in hemin-containing reticulocyte lysates. The effect is proportional to HS concentration up to about 70% inhibition. Translation and eIF-2α phosphorylation assays were as described [7,8]. Activation of proHCI was assayed by phosphorylation of the eIF-2α subunit as described in the legend to fig.3.

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Glutathione reductase was assayed spectrophotometrically by following the oxidation of NADPH by GSSG at 340 nm.

2.3. *Preparations*

Reticulocyte lysates were prepared from phenylhydrazine-treated rabbits by the method of Hunt et al. [11] with slight modifications. Initiation factor eIF-2 and HCI, both from rabbit reticulocyte lysates, were purified as described [12]. Labeled ATP was prepared as described by Schendel and Wells [13]. Crude proHCI was prepared by the Gross and Rabinovitz procedure [14] as described [15]. Hemin was added to the CM-Sephadex fraction (25 μM final concentration) and this was stored in small aliquots in liquid nitrogen. Highly purified glutathione reductase from rabbit erythrocytes was the gift of Dr C. Palomo of this laboratory.

3. RESULTS

3.1. *Translational inhibition by HS*

HS was purified from postribosomal supernatant of 400 ml of rabbit reticulocyte lysates as described [10]. Fig.1A confirms our previous work [10] in that the HS activity was eluted from the last HPLC size-exclusion column of a sharp, symmetric peak, with a maximum of absorbance at 256 nm (fig.1A, inset). The translation inhibitory activity also coincided precisely with the peak of absorbance (not shown) [10]. HS appears to be a potent inhibitor of translation in reticulocyte lysates. Inhibition is proportional to HS concentration up to about 70% (fig.1B). The amount of highly purified HS causing 50% inhibition of hemin-dependent translation was 0.7×10^{-3} A_{260} units (in 30 μl assay volume). Like the translational inhibition due to heme deficiency or HCI addition to hemin-containing lysates, HS produces increased phosphorylation of the eIF-2α subunit in hemin-supplemented lysates (fig.1C). At high HS concentrations, this phosphorylation is no greater than that caused by hemin deficiency (cf. lane 1 with 3 and 4).

3.2. *Translational inhibition by HS is prevented or reversed by NADPH generators and dithiothreitol*

Like the inhibition by Ca^{2+} and phospholipid [9], the inhibitory effect of HS on translation in hemin-containing lysates is largely prevented by glucose 6-phosphate, NADPH, or dithiothreitol but not by 2-mercaptoethanol or fructose 1,6-bisphosphate that can neither be converted to G6P nor metabolized via the hexosemonophosphate shunt. However, the inhibition caused by heme deficiency is not prevented or restored by NADPH generators of by dithiols (table 1).

Fig.2A suggests that translational inhibition produced by HS is oxidative in nature because the HS-promoted phosphorylation of the eIF-2α subunit in hemin-containing lysates (cf. lanes 2 and 3) is largely prevented by dithiothreitol (lane 6), NADPH (lane 7), or glucose 6-phosphate (lane 8) but not by fructose 1,6-bisphosphate (lane 4) or 2-mercaptoethanol (lane 5). Moreover, this phosphorylation is reversed by glucose 6-phosphate and dithiothreitol (fig.2B). Already at 5 min of incubation, the degree of phosphorylation of the eIF-2α subunit is greatly increased by HS (lane 3) as compared with control lysates (lane 2). Clearly, the phosphorylation of this band is significantly diminished if the lysates are supplemented with either G6P or DTT at 5 min of incubation (cf. lane 6 with 10 and 11). The activation of HCI by heme deficiency is not prevented by G6P (lane 5). As seen before (fig.2A) the effect of highly purified HS is largely prevented by G6P (lane 8) or DTT (lane 9).

3.3. *Activation of HCI by HS and GSSG*

A likely explanation for the inhibition of translation in hemin-containing lysates by HS is that it promotes the first step of the reaction proHCI → reversible HCI → irreversible HCI. To verify further this idea, we look-

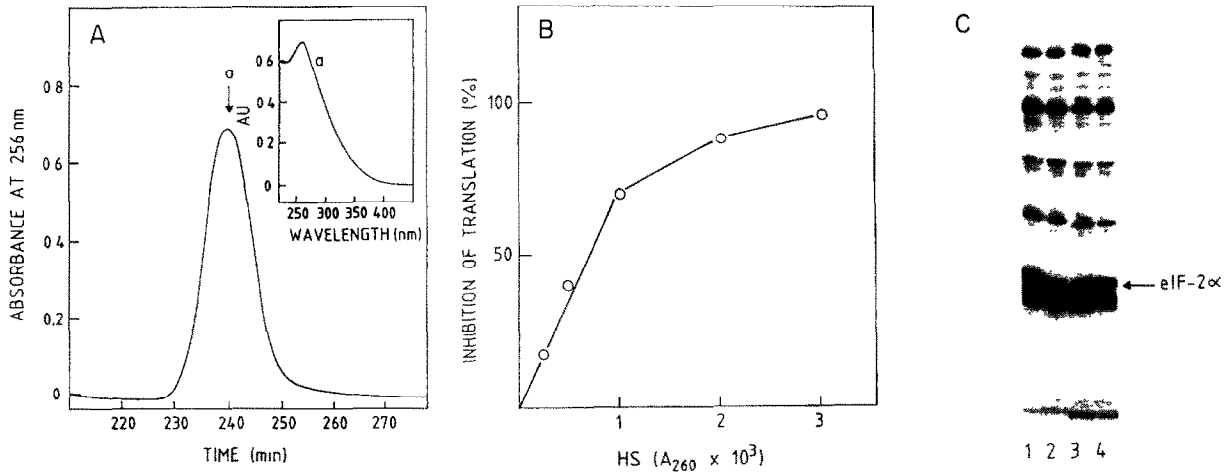

Fig.1. (A) Rechromatography of the HS activity in the size exclusion HPLC column [10]. (Inset). Automatic overlay of spectra acquired from peak maxima (a) from 220 nm to 450 nm. (B) Increasing amounts of purified HS were assayed for translational inhibition. (C) Effect of highly purified HS on phosphorylation of the eIF-2α subunit. The conditions were (lanes): (1) without hemin; (2) with 20 μM hemin; (3) with hemin and 2×10^{-3} A_{260} of HS; (4) with hemin and 4×10^{-3} A_{260} of HS.

Table 1

Effect of glucose 6-phosphate (G6P), NADPH, and dithiothreitol (DTT) on translational inhibition by HS in hemin-supplemented reticulocyte lysates

Additions	[^{14}C]Leucine incorporation (cpm × 10^{-3})		Relative hemin-dependent activity (%)
	Total	Due to hemin	
None	14.3		
G6P (1 mM)	14.8		
NADPH (1 mM)	14.5		
DTT (1 mM)	13.3		
2-mercaptoethanol (2-ME) (1 mM)	14.3		
Fructose 1,6-bisphosphate (FDP) (1 mM)	14.3		
Hemin (25 μM)	70.1	55.8	100
+ HS[a] (6×10^{-3} A_{260})	18.8	4.5	8
+ HS + G6P	60.3	45.5	81
+ HS + NADPH	51.2	36.7	66
+ HS + DTT	64.6	51.3	92
+ HS + 2-ME	17.6	3.3	6
+ HS + FDP	19.8	5.5	10

[a] HS from Sephadex G-15 [10] (peak I) was used

ed at the effect of HS on a crude preparation of proHCI (fig.3). Clearly, this preparation contains the inactive precursor form of HCI (lane 1) that is fully converted to HCI by NEM (lane 2) [1,2]. Incubation of crude pro-HCI with highly purified HS (lane 5) or with peak III of HS from Sephadex G-15 [10] (lane 3) results in HCI increase as judged by the increased phosphorylation of the eIF-2α subunit, but to produce this effect, one needs larger amounts of HS than are required with lysates. Curiously, while GSSG activates proHCI in lysates, it is less effective on partially purified proHCI (lane 4). An amount of GSSG that promotes HCI activation in hemin-supplemented lysates is not sufficient to activate crude proHCI (data not shown). This may

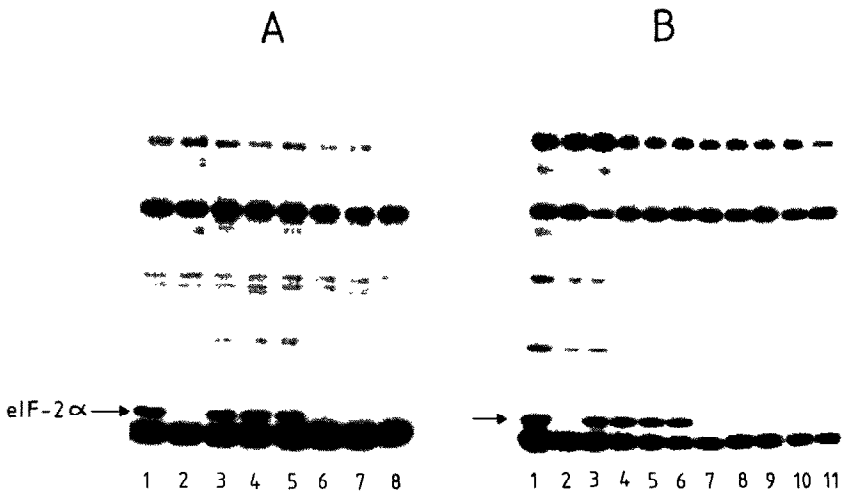

Fig.2. (A) HS-promoted phosphorylation of the 38 kDa α-subunit of eIF-2 and the effect of various compounds thereon. Samples were incubated for 60 min at 30°C and aliquots were assayed for protein synthesis and phosphorylation as described. The hemin-dependent incorporation of [^{14}C]leucine (in cpm × 10^{-3} per sample) is indicated in parentheses for each lane. Lanes: 1, without hemin; 2, with 20 μM hemin (32.0); 3, with hemin and 6×10^{-3} A_{260} of HS from Sephadex G-15 [10] (peak I) (3.4). Samples corresponding to lanes 4–8 all contained hemin and HS and, in addition, contained the following: 1 mM FDP (3.0) (lane 4); 1 mM 2-mercaptoethanol (2.6) (lane 5); 1 mM DTT (37.5) (lane 6); 1 mM NADPH (31.5) (lane 7); 1 mM G6P (30.1) (lane 8). (B) Phosphorylation of the 38 kDa α-subunit of eIF-2 promoted by highly purified HS is reversed by glucose 6-phosphate or dithiothreitol. Samples were incubated for 40 min at 26°C and aliquots were assayed for protein synthesis as described. Aliquots (3 μl) for the phosphorylation assay were taken as follows: lanes 1–3, at 5 min; lanes 4–11, at 40 min. The corresponding 40 min translation values are indicated in parentheses. Lanes: 1, without hemin; 2, with hemin; 3, with hemin and 2×10^{-3} A_{260} of highly purified HS; 4, without hemin (10.0); 5, with 1 mM G6P (10.3); 6, with hemin and HS (11.1); 7, with hemin (45.3). Samples corresponding to lanes 8–11 all contained hemin and HS from the start of incubation and, in addition, contained the following: 1 mM G6P (39.3) (lane 8) or 1 mM DTT (48.0) (lane 9) from the start and 1 mM G6P (34.1) (lane 10) or 1 mM DTT (42.7) (lane 11) added at 5 min.

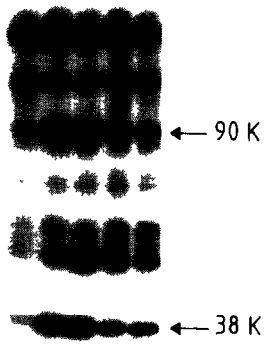

Fig.3. Effect of HS_r on activation of HCI. Samples (30 μl) containing 25 mM Tris-HCl, pH 7.6; 25 mM KCl, with proHCI (40 μg of protein) and when present, 2 mM MEM, 2 mM GSSG; 40×10^{-3} A_{260} of HS from Sephadex G-15 [10] (peak III) or 6×10^{-3} A_{260} of highly purified HS, were incubated for 15 min at 34°C. The excess NEM was neutralized with 4 mM DTT. Aliquots of 6 μl were used as a source of eIF-2α kinase source in a second incubation for 9 min at 30°C in a solution (20 μl final volume) containing 25 mM Tris-HCl, pH 7.6, 5 mM $Mg(OAc)_2$, and 0.1 mM [γ-^{32}P]ATP (30×10^6 cpm/sample). The 38 kDa bands of the autoradiograms were scanned at 626 nm with a Chromoscan 3 densitometer and the values are indicated in parentheses for each lane. Lanes: 1, proHCI (8 μg of protein) (3267); 2, proHCI and NEM (11 616); 3, proHCI and HS (peak III) (12 969); 4, proHCI and GSSG (8322); 5, proHCI and highly purified HS (9085). Arrows show the position of the 90 and 38 kDa bands determined in a parallel run in which a partially purified HCI was used (not shown).

suggest that an unknown component (limiting in crude proHCI preparations) may be involved in the activation of HCI by both HS and GSSG.

4. DISCUSSION

Our results provide further support for the view [10] that a homogeneous heat-stable factor (HS) from rabbit reticulocyte lysates is a potent translational inhibitor. Like GSSG, HS inhibits translation in hemin-supplemented reticulocyte lysates by activation of HCI, resulting in increased phosphorylation of the eIF-2α subunit, but it appears to be much more active. Unlike GSSG, HS is not a substrate for highly purified glutathione reductase from rabbit erythrocytes (table 2). Recently, we have reported that HS appears to be neither a peptide nor an oligonucleotide since HS activity was insensitive to proteolytic or nucleolytic digestion [10]. The spectral analysis of highly purified HS ([10] and fig.1A) is consistent with the idea that HS could be a nucleotide analogue. The molar extinction coefficients of nucleotides (260 nm) are about 1×10^4. Therefore, the amount of the highly purified HS required for 50% inhibition of protein synthesis (7×10^{-4} A_{260} unit)) is in the nanomolar range (70 nM). Thus, HS may be an extremely active inhibitor of protein synthesis. In attempting to identify the structure of HS, we have studied the effect of the nucleoside diphosphates (ADP, GDP, CDP and UDP) with compatible molecular masses and similar but not identical spectra (not shown). All of them inhibited protein synthesis. The concentration causing 50% inhibition in hemin-dependent translation was about 500 μM in either case. This inhibition, however, was not accompanied by increased phosphorylation of eIF-2α subunit (not shown). Furthermore, the analyses performed by the H^+-NMR technique were unable to identify the structure of HS, due to the large amount required. Therefore, we have begun with the purification of HS on a preparative scale. On the other hand, using fast atom bombardment (FAB) in mass spectrometry, the molecular weight of HS appears to be 429 (not shown).

Earlier [8] we emphasized that, unlike the activation of HCI in hemin-supplemented lysates by GSSG addition or NADPH depletion, the activation caused by heme deficiency is not prevented by NADPH generators or by dithiothreitol. It was therefore argued that there are at least two fully independent mechanisms for proHCI activation: (i) heme deficiency and (ii) oxidation processes that appear to lead to the conversion of certain SH groups to disulfides [1,16]. We have also shown [9] that proHCI can be reversibly activated via oxidation by agents other than GSSG, namely polyunsaturated fatty acids and phospholipids. HCI may be activated in a number of ways [2], but the mechanisms are unknown. The activation by GSSG is of interest because of its possible physiological significance. Reversibility of the oxidative activation of HCI is a requirement for physiological significance. Although the presence of enzymes capable of phosphorylating the eIF-2α subunit has been reported in non-erythroid cells [2], their mode of activation and physiological significance are unknown. Thus, although there is no doubt on the physiological relevance of the heme control of HCI activity in reticulocytes, the significance, if any, of the oxidative activation of proHCI remains a matter for speculation.

Table 2

HS is not a substrate for glutathione reductase

Incubation (min)	Δ OD_{340}		
	None	GSSG	HS
1	0.006	0.139	0.004
2	0.009	0.308	0.004
3	0.013	0.437	0.003
4	0.015	0.518	0.002
6	0.015	0.575	0.002

The assay was carried out with highly purified glutathione reductase from rabbit erythrocytes. The OD_{340} average value at 0 min was 0.602

The high activity of HS, its reversibility, and its presence in unheated lysates (data not shown) would be compatible with the notion that this factor may be involved in translational control.

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