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Purification of a novel heat-stable translational inhibitor from rabbit reticulocyte lysates

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We have purified to apparent homogeneity a novel heat-stable (HS) factor from postribosomal supernatants of rabbit reticulocyte lysates by heating for 10 min at 80°C, fractionation on Sephadex, anion-exchange chromatography on QMA Accell, and gel filtration HPLC. The apparent molecular mass of HS is 500–1000 Da on the basis of its behaviour on gel filtration. Like a factor from bovine heart [(1982) Proc. Natl. Acad. Sci. USA 79, 3134–3137], the reticulocyte HS inhibits translation in hemin-supplemented lysates with biphasic kinetics similar to hemin deficiency and promotes phosphorylation of the α-subunit of the eukaryotic initiation factor eIF-2. It is active at nanomolar concentrations. Reticulocyte HS appears to be neither a peptide nor an oligonucleotide since HS activity was insensitive to proteolytic or nucleolytic digestion.

Polypeptide chain initiation; Translational inhibition; Protein phosphorylation; HPLC fractionation

1. INTRODUCTION

Protein synthesis in reticulocytes is regulated at the level of the initiation factor 2 by phosphorylation of its α-subunit (for a review see [1,2]). There are at least two cAMP-independent eIF-2_α kinases in rabbit reticulocyte lysates. One, the heme-controlled inhibitor is activated from a precursor when lysates are incubated in the absence of added hemin or, with hemin present, upon addition of either oxidized glutathione (GSSG) [3] or Ca^{2+}-phospholipid [4]; the other, the double-stranded RNA (dsRNA)-activated inhibitor, is activated by low concentrations of dsRNA in the presence of ATP. Both HCI and DAI phosphorylate the α (38 kDa)-subunit of the eukaryotic chain initiation factor eIF-2, causing translational inhibition. Once activated, HCI and DAI inhibit translation in reticulocyte lysate in the presence of heme, but the mechanism of activation remains obscure [1,2].

Earlier [5], we purified from bovine heart a heat-stable protein that, like the heat-stable reticulocyte factor described by Henderson et al. [6], promoted increased phosphorylation of the eIF-2 α-subunit in reticulocyte lysates. In this paper we describe a novel heat-stable (HS) factor from postribosomal supernatant of reticulocyte lysates which, at nanomolar concentrations, inhibits translation in hemin-supplemented lysates by promoting phosphorylation of the α-subunit of eIF-2. The HS preparation maintains its activity upon treatment with either proteases (proteinase K, pronase) or nucleases (micrococcal endonuclease, phosphodiesterase). The molecular structure of HS is as yet unknown.

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Abbreviations: eIF-2, eukaryotic protein synthesis initiation factor 2; HCI, heme-controlled translational inhibitor; DAI, double-stranded RNA-activated translational inhibitor; pro-HCI, the proinhibitor (inactive) form of HCI

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2. MATERIALS AND METHODS

2.1. *Materials*

Sephadex (G-15, G-50) and protease from *Streptomyces griseus* (pronase E) were obtained from Sigma. Accell QMA anion-exchange media were from Waters Chromatography Division. Proteinase K was from Merck, micrococcal endonuclease (EC 3.1.31.1.) from Worthington and phosphodiesterase (EC 3.1.4.1.) from Boehringer Mannheim. All other chemicals were of reagent grade.

2.2. *HPLC equipment*

The chromatograph consisted of two Waters M6000A pumps, a Waters 680 automated gradient controller and a Waters 990 photodiode array detector with a dynamic range from ultraviolet to the visible (UV/VIS) region (190 nm to 600 nm), based on a NEC APC III personal computer. Sample injections were performed with a Waters U6K universal injector.

2.3. *Assays*

HS was assayed using its inhibitory effect on translation and its capacity for specifically activated eIF-2$_\alpha$ kinase, in hemin-containing reticulocyte lysates. Translation and eIF-2$_\alpha$ phosphorylation assays were as described [4,7]. Treatments of reticulocyte HS with proteases or nucleases was performed under optimal conditions as follows.

2.4. *Protease digestion*

Samples (20 μl final volume) containing 20 mM Tris-HCl (pH 7.6), 10 mM NaCl (for both proteases), 1 mM EDTA (only for proteinase K treatment) and, when present, 40×10^{-3} A_{260} of HS from Sephadex G-15 (pool I), and pronase or proteinase K (150 μg/ml) were incubated for 16 h at 37°C. Then, the protease was destroyed by heating for 5 min at 90°C. Aliquots (5 μl) were assayed for protein synthesis and phosphorylation of the eIF-2 α-subunit.

2.5. *Nuclease digestion*

2.5.1. Micrococcal nuclease

The complete preincubation mixture (7 μl) contained 20 mM Tris-HCl, pH 7.6, and 1 mM $CaCl_2$, and when present, 1 unit of micrococcal endonuclease (EC 3.1.31.1.) and 24×10^{-3} A_{260} of HS from Sephadex G-15 (pool I). After incubation for 15 min at 20°C, all samples received 2 mM EGTA (final volume 8 μl). Aliquots (2 and 4 μl) were assayed as described in table 1.

2.5.2. Phosphodiesterase

Samples (25 μl final volume) containing 20 mM Tris-HCl, pH 7.6, and when present 7.5×10^{-3} units of phosphodiesterase (EC 3.1.4.1.) and 20×10^{-3} A_{260} of HS from Sephadex G-15 (pool I) were incubated for 2 h at 37°C and the excess phosphodiesterase was destroyed by heating for 10 min at 80°C. Aliquots (6 and 9 μl) were assayed as described in table 1.

2.6. *Preparations*

Reticulocyte lysates were prepared from phenylhydrazine-treated rabbits by the method of Hunt et al. [8] with slight modifications. To obtain postribosomal supernatants, lysates were centrifugated at $160\,000 \times g$ for 2 h at 2°C. Initiation factor eIF-2 and HCI, both from rabbit reticulocytes, were partially purified as described [9]. Labeled ATP was prepared as described by Schendel and Wells [10].

2.7. *HPLC*

Size exclusion HPLC was performed on a TSK 3000 SW6 (21.5 × 300 mm) column (Toyo Soda, Tokyo, Japan) fitted with a TSK 3000 SW6 guard column, by isocratic elution with 0.1 M ammonium acetate buffer (pH 5.0). The column was operated at room temperature at a flow rate of 0.5 ml/min. Reversed-phase HPLC was performed with a Nova-Pak column (Waters) (3.9 × 150 mm) protected by a guard column packed with μ Bondapak C_{18}/Corasil (Waters). The column was eluted with acetonitrile gradients containing 0.1% trifluoroacetic acid. The column was operated at room temperature at a flow rate of 0.5 ml/min.

3. RESULTS

3.1. *Purification*

HS was purified from postribosomal supernatant of rabbit reticulocyte lysates. Supernatant from 200 ml of lysates was diluted with one volume of 1 mM Tris-HCl, pH 7.6, heated for 10 min at 80°C with continuous mechanical stirring, and the copious precipitate removed by centrifugation. The supernatant (HSS_{80}) was concentrated by lyophilization, applied to a column (1.6 × 90 cm) of Sephadex G-50 previously equilibrated with 1 mM Tris-HCl, pH 7.6, and eluted with the same buffer. HS activity was eluted as a single relatively broad peak. The active fractions were pooled and concentrated (HS-50). The solution was further fractionated by chromatography in a long column (2.5 × 190 cm) of Sephadex G-15 equilibrated and eluted with 10 mM $(NH_4)HCO_3$, pH 8.8. The HS-50 fraction was resolved in three major peaks, all of them with HS activity. The peak I was lyophilized and dissolved in 50 mM ammonium acetate buffer, pH 5.5, and applied to a self-packed Accell QMA anion-exchange column (0.9 × 12 cm) equilibrated and washed with the same buffer, until A_{260} was negligible. The column was then eluted stepwise with this buffer containing successively 15, 50 and 80% acetonitrile (CH_3CN).

The bulk of the A_{260} units was in the unretained material together with about 50% of the HS units. The retained HS units were eluted with 15% CH_3CN and the corresponding fractions pooled, lyophilized and dissolved in 100 mM ammonium acetate buffer, pH 5.0. This solution was loaded onto an HPLC size exclusion column and chromatographed as described in section 2. As

seen in fig.1 (A–C), the material was eluted from the column as a single, non-symmetrical peak. The retention time of the peak was 237 min. Automatic spectral analysis throughout the peak revealed at least three different components (fig.1D). Fractions with the highest specific activity (fig.1E) were pooled, lyophilized and rechromatographed in the same HPLC column. HS activity now eluted from the column as a sharp, symmetric peak at several wavelengths (fig.2A–C). Spectral analysis showed a single component (fig.2D) throughout the peak with a maximum of absorbance at 256 nm. The translation inhibitory activity coincided with the peak of absorbance (fig.2E).

3.2. *Comments on the purification procedure*

HS activity cannot be reliably determined in cruder cell fractions for they are contaminated with other translational inhibitors that do not affect eIF-2$_\alpha$ kinase activity. These contaminants were virtually removed after filtration on Sephadex G-15. In order to obtain homogeneous HS, only pooled fractions with the highest specific activity from each purification step were used for the subsequent chromatography. Thus, the yield was rather low. Moreover, Sephadex G-15 chromatography revealed the existence of several HS fractions with different apparent molecular masses and the relationship between them is as yet unknown. However, when a partially pure HS fraction was chromatographed on a reverse-phase HPLC column as described in section 2, HS activity eluted as a single peak with the washing buffer (not shown). Preliminary data are consistent with the idea that, only the HS factor of the peak I is present in unheated lysates (not shown). Peaks II and III could contain related molecular entities formed by partial degradation during purification.

3.3. *Properties*

The apparent molecular mass of HS from gel filtraton data is about 500–1000 Da. HS inhibits translation in hemin-containing lysates with biphasic kinetics (fig.3A) typical of hemin lack or HCl addition to hemin-containing lysates [1,2], and the inhibition is similarly relieved by large amounts of GTP (not shown). At high HS concentrations, eIF-2$_\alpha$ phosphorylation is not greater than that caused by hemin deficiency (figs.3B and 4A). At low HS concentrations, the dose-response

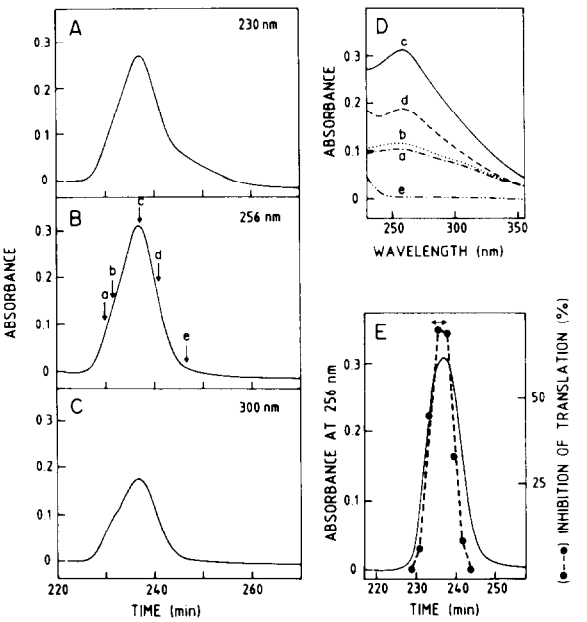

Fig.1. Size exclusion HPLC of the eluate of 15% CH_3CN from the QMA column. (A–C) Automatic multichromatogram analysis of the absorbance at 230 nm, 236 nm and 300 nm, respectively. (D) Automatic overlay of spectra acquired from right slope (a,b), peak maxima (c) and left slope (d,e) from 220 nm to 350 nm. (E) Localization of the HS activity in the peak. Fractions corresponding to the bar were pooled and lyophilized.

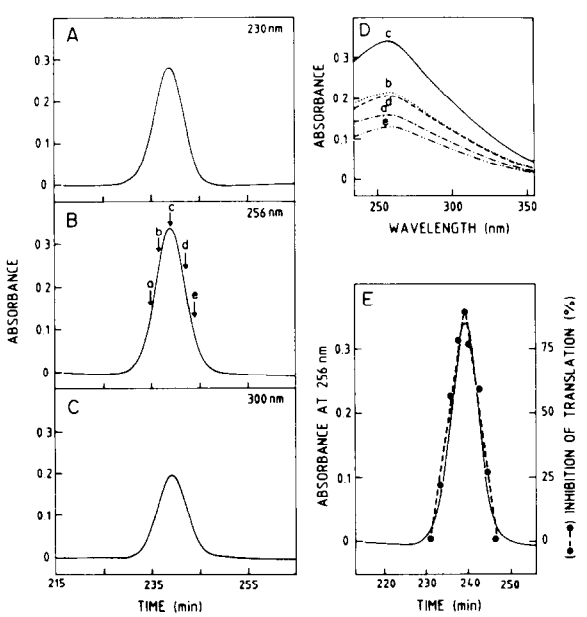

Fig.2. Rechromatography of the HS activity from fig.1 in the same size exclusion HPLC column. A–E are as described in fig.1.

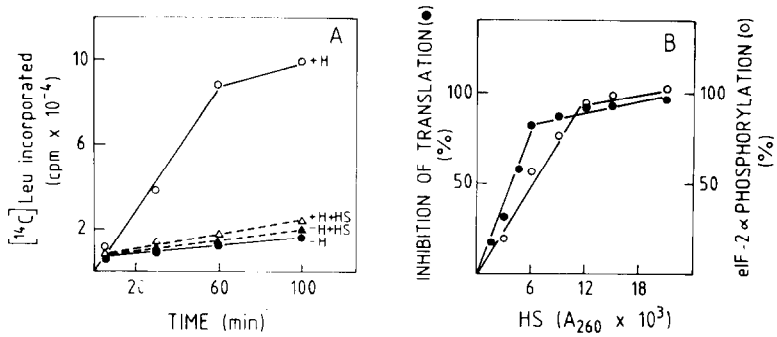

Fig.3. (A) Kinetics of translational inhibition by HS from Sephadex G-15 (pool I) without (●,▲) and with (○,△) hemin; and without (●,○) and with (▲,△) 8×10^{-3} A_{260} of HS. (B) Translational inhibition and eIF-2_α phosphorylation by HS from Sephadex G-15 (pool I). (●) Inhibition of translation, a function of the concentration of HS. (○) Phosphorylation of the 38-kDa α-subunit of eIF-2 promoted by HS. The 38 kDa bands of the autoradiograms (fig.4A) were scanned at 626 nm with a Chromoscan 3 densitometer.

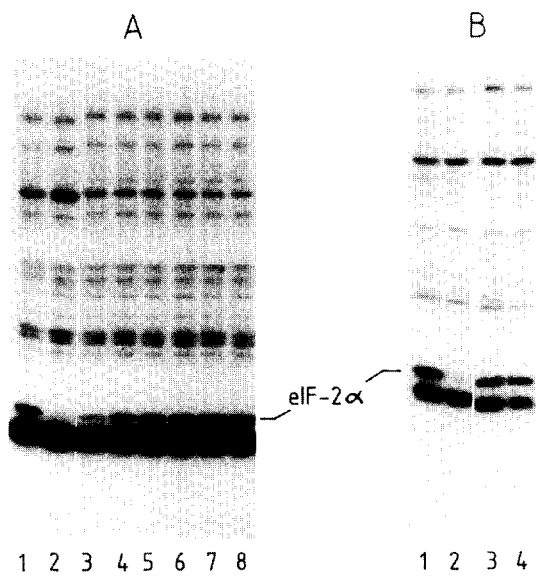

Fig.4. Phosphorylation of the 38 kDa α-subunit of eIF-2 promoted by HS. (A) Translation reaction samples were incubated for 60 min at 30°C when aliquots were taken for assay of [^{14}C]leucine incorporation (fig.3B) and eIF-2 phosphorylation as described. Lanes: 1, without hemin; 2, with hemin; 3–8, with hemin and increasing amounts of HS from Sephadex G-15 column (pool I): 3, 3×10^{-3} A_{260}; 4, 6×10^{-3} A_{260}; 5, 9×10^{-3} A_{260}; 6, 12×10^{-3} A_{260}; 7, 15×10^{-3} A_{260}; 8, 21×10^{-3} A_{260}. (B) Effect of highly pure HS on phosphorylation of eIF-2 α-subunit. The conditions were (lanes): without hemin (1); with 20 μM hemin (2); with hemin and 1×10^{-3} A_{260} of HS (3); with hemin and 2×10^{-3} A_{260} of HS (4). The corresponding 60 min translation values were 13917, 73745, 31923 and 19214 cpm.

Table 1

Nucleases have no effect on the heat-stable translational inhibitor

Preincubation mixture	Hemin-dependent [^{14}C]leucine incorporation (cpm $\times 10^{-3}$)	Inhibition (%)
A. None	33.7	
	34.6	
+ HS	9.0	73
	2.6	93
Micrococcal nuclease	34.0	
	34.2	
+ HS	10.0	71
	3.3	90
B. None	48.0	
	48.5	
+ HS	18.0	63
	9.2	81
Phosphodiesterase	50.0	
	52.9	
+ HS	18.1	64
	8.7	84

(For series A) Aliquots (2 and 4 μl) were assayed for their effect on translation in hemin-supplemented reticulocyte lysate. The incorporation of [^{14}C]leucine in a control with no added hemin was 15.9×10^3 cpm. The enzyme is fully active on globin mRNA degradation, thus, the incorporation of [^{14}C]leucine in a nuclease-treated lysate was 0.3×10^3 cpm. (For series B). Aliquots (6 and 9 μl) were assayed as above. The incorporation of [^{14}C]leucine in a control with no added hemin was 12.6×10^3 cpm. The enzyme was fully active on Ap_4A (not shown)

curves of translational inhibition and eIF-2_α phosphorylation are linear and closely related (fig.3B). These results suggest that the HS effect is eIF-2_α kinase-mediated and this suggestion is borne out by the fact that, whereas HS does not phosphorylate eIF-2 by itself (not shown), it produces increased phosphorylation of the eIF-2 α-subunit in lysates (fig.4). Homogeneous HS contained no amino acids and was not affected by protease treatments (not shown). Pretreatment of HS with micrococcal nuclease and phosphodiesterase had no effect on its activity (table 1). These results indicate that the HS is neither an oligopeptide nor an oligonucleotide.

4. DISCUSSION

A homogeneous heat-stable factor isolated from rabbit reticulocyte is an extremely potent inhibitor of translation in reticulocyte lysates. Like the Henderson factor [6], HS inhibits chain initiation by promoting phosphorylation of the α-subunit of the initiation factor eIF-2. However, the two factors are different for our HS factor is not a protein by nature and is apparently of much lower molecular mass. Reticulocyte HS could either activate HCI, DAI, or an unknown eIF-2_α kinase, or inhibit protein phosphatase(s) acting on eIF-2(αP). DAI activation and inhibition of eIF-2(αP) dephosphorylation can be ruled out, the former because high concentrations of dsRNA (which block DAI activation) are without effect on HS activity (not shown), the latter because eIF-2(α-^{32}P) is rapidly dephosphorylated in lysates whether HS is present or not (not shown). We have preliminary results suggesting that HS inhibits translation in hemin-supplemented reticulocyte lysates by increasing the HCI to proHCI ratio, resulting in increased phosphorylation of the eIF-2 α-subunit. In attempting to identify the structure of HS, we have studied a family of compounds (Ap_2A, Ap_3A, Ap_4A, Gp_3G and Gp_4G) with compatible molecular masses and similar but not identical spectra (not shown). Only the dinucleoside tetraphosphates (Ap_4A, Gp_4G) inhibited protein synthesis. The concentration causing 50% inhibition in hemin-dependent translation was about 1 mM in either case. This inhibition, however, was not accompanied by increased phosphorylation of the eIF-2 α-subunit (not shown). Whether HS has a physiological function is unknown.

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