Purification and Some Properties of an Oxydative Inhibitor in Rabbit Reticulocyte Lysates

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Protein synthesis in rabbit reticulocyte lysates in the presence of heme is inhibited by 50% by the addition of 4 mM GSSG (oxidized glutathione). The incubation of the rabbit reticulocyte lysate with 4 mM GSSG at 30 °C for 30 min will cause activation of an inhibitor of protein synthesis which could be purified from the lysates through a five-step procedure. The inhibitor results in a 70-80% inhibition after a 1 h incubation. The inhibitor consists of one polypeptide of 23 kDa apparent molecular weight and is 90% pure as judged by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. However, in the presence of cAMP (10 mM) or GEF (guanine nucleotide exchange factor) (0.3 μg), protein synthesis in the inhibited reticulocyte lysate will be already recovered.

Introduction

The phosphorylation of the eIF-2α kinases is a widely used mechanism of translational control under a variety of conditions of cellular stress, the binding of initiator Met-tRNA; to the 40S ribosomal subunit, catalyzed by initiation factor eIF-2 is the rate-limiting step and the phosphorylation of eIF-2α leads to an increased affinity of the initiation factor for GEF and thus increases the proportion of the latter that is trapped as an inactive complex with phosphorylated eIF-2 and GDP (Rowlands *et al.*, 1988a). Therefore, changes in the state of phosphorylation of eIF-2α in the cell thus lead to overall changes in protein synthesis.

The direct control of GEF activity can also regulate the initiation rate at the level of guanine nucleotide exchange on eIF-2 (Akkaraju *et al.*, 1991). Iron or heme (haemin) deficiency in erythroid cells leads to a general inhibition of protein synthesis. In general, increases in eIF-2α phosphorylation are observed in cells responding to various environmental stresses (Rowlands *et al.*, 1988b). HCR can be activated by heat shock or by conditions that mimic this stress in reticulocyte lysates (Matts *et al.*, 1991, 1993; Matts and Hurst, 1992). In addition to HSP 90, HCR can also bind to other proteins involved in cellular stress responses, such as members of the HSP 70 family, p56 (Matts and Hurst, 1992) and these may also regulate its activation during heat shock which causes increased phosphorylation of eIF-2α and inhibits eIF-2 activity in other cell types besides reticulocytes (Duncan and Hershey, 1984, 1989; Scorsone *et al.*, 1987, Hu *et al.*, 1993, Panniers, 1994, Sierra 1994) suggesting that activation of the same or related protein kinase(s) is involved in these systems as well. Cellular stresses such as heat shock, viral infection, nutrient deprivation and ischemia stimulate the phosphorylation of eIF-2α in various systems. The changes in eIF-2α phosphorylation are involved in the normal regulation of cell growth (Mundschau and Faller, 1991). Control of the phosphorylation state of eIF-2, or of the activity of eIF-2α-specific kinases occurs in response to stimulation of cell proliferation by mitogens and growth factors as

Abbreviations: cAMP, cyclic adenosine monophosphate; eIF, 2-eukaryotic initiation factor 2; GSSG, oxidized glutathione; GEF, guanine nucleotide exchange factor; HCR, Hemin control repressor; HSP, heat shock protein.

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well as the induction of cell differentiation (Petryshyn *et al.*, 1984, 1988). It is not always clear whether the alterations in the phosphorylation state of eIF-2α , and hence the activity of the eIF-2/GEF system, are causes or consequences of various responses that have been studied in a different system.

In heme-supplemented lysates, high pO2 or glutathione disulfide (GSSG) causes the activition of an inhibitor of protein synthesis which is heat-stable and has an apparent molecule weight of approx. 23 kDa (Ochoa, 1983). According to another reported finding a similar inhibitor seems to exist in spontaneously activated states in rabbit erythrocyte lysates (Erdogdu *et al.*, 1993).

Studies on some heat-shock proteins have shown that they take part in functions such as molecular chaperoning and translocation of proteins across subcellular membranes. Gaitero *et al.*, (1988, 1989) reported a heat-stable factor from the postribosomal supernatant of rabbit reticulocyte lysates which might be activated due to the oxidation of labile SH groups which are involved in the activation of HCR. It is suggested that the 23 kDa protein is predominantly induced in macrophages by oxidative stress and sulfhydryl-reactive agents (Sato *et al.*, 1993).

We have developed an improved procedure for the isolation of a 23 kDa inhibitor from reticulocyte lysates (derived from ten rabbits) which yields 2 mg of factor with about 90% purity. Furthermore, we also show that in the presence of cAMP [10 mM] or GEF [0.3] μg, protein synthesis in the inhibited reticulocyte lysate will be restored.

Materials and Methods

Reagents and chemicals

All reagents and chemicals used were of analytical grade and purchased from Fluka, Merck, and Sigma. Polyethylene glycol 8000 was obtained from Aldrich. Chemicals for PAGE were purchased from BioRad. All solvents were distilled before use.

Ion exchange chromatography

A Mono Q/P HR 5/5 (Pharmacia) anion exchange column was equilibrated with 20 mM Tris-HCl [Tris (hydroxymethyl) aminomethane] buffer, pH 7.4, and operated at a flow rate of 1 ml/min. Aliquots (5 ml) of solution were applied to the column.

The column was eluted with the same buffer using a KCl gradient (0-1 M KCl) and the absorbance was monitored at 280 nm, using a single path monitor UV-1 with optical and control unit (Pharmacia). Fractions containing inhibitor activity were collected.

Gel filtration

The gel filtration of inhibitor protein was loaded on a column of Superose (2.0 x 100 cm), previously equilibrated with 20 mM Tris-HCl (pH 7.4), 100 mM KCl, 2 mM DTT (dithiothreitol), 50 μM sodium-EDTA and 10% glycerol. The column was developed with the same buffer at a flow rate of 0.1 ml/min. Fractions of 1.0 ml were collected, and the activity of the inhibitor was assayed in the reticulocyte system.

Protein content

A modification of the procedure of Bradford (1976) was used to estimate protein contents. The absorbance of a mixture of the Coomassie solution and of the appropriate sample was measured at 595 and 465 nm. The ratio of the optical densities at 595 nm and 465 nm was determined. Bovine serum albumin was used as standard.

Gel electrophoresis

SDS/polyacrylamide gel electrophoresis was performed as described (Oldfield *et al.*, 1992) in gels prepared from 10% acrylamide and 0.1% N,N'-methylene-bis acrylamide. Gels were stained with Coomassie blue and dried.

Reticulocyte lysate assay system

The inhibitor effect on protein synthesis in rabbit reticulocyte lysates was assayed as described before (Erdogdu *et al.*, 1993).

Results and Discussion

The method used for the purification of GSSG-activated inhibitor allows the preparation to be carried out more convenient and yields inhibitor protein of great purity. The chromatography on DEAE-cellulose resolves inhibitor (eluted with

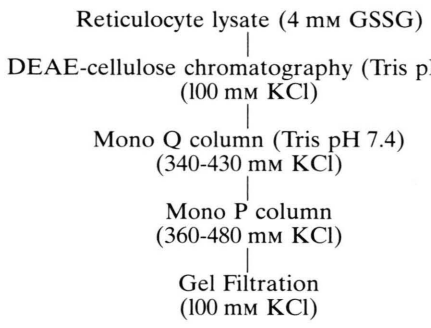

Fig. 1. Flow diagram illustrating the purification procedures for a 23 kDa inhibitor from rabbit reticulocyte lysates. Reticulocyte lysate was prepared from the blood of ten rabbits and fractionated as shown. Proteins were eluted with a step gradient of KCl.

100 mM KCl) from resin, although the pool containing the inhibitor has small purity. Further purification of protein was achieved by chromatography on Mono Q which was eluted at 340-430 mM KCl. Subsequent chromatography fractions containing protein on Mono P. The inhibitor was eluted from a Superose column at approximately 100 mM KCl and usually, the peak fractions were 80-90% pure (as judged by the staining intensity) and were not purified further (Fig. 1) (Table I). Subsequently, fractions containing inhibitor protein were identified by analysis on 10% SDS/polyacrylamide gels. The molecular weight of one polypeptide in fractions collected from the Superose column was determined at ≈ 23 kDa by SDS-PAGE. The activity of inhibitor protein did not change when it was heated at low protein concentration (Table II).

Table I. The inhibitory activity of purified fractions was determined in a 5 µl *in vitro* protein synthesis assay containing 0.5 A260 units of extract from rabbit reticulocyte lysates which were induced. One arbitrary unit (a.u.) of inhibitory activity was defined to represent the amount which leads to 50% inhibition of 35S- methionine incorporation in proteins in these assays. Control incorporation was equivalent to 3 pmol of methionine.

Fraction	Total activity (a.u.)	Specific activity (a.u./mg protein)	Purification (fold)
DEAE-cellulose	2150	3,5	1
Mono Q	1344	31,4	3.47
Mono P	850	126	4.2
Superose	34.7	520	180.4

Table II. Effect of heat on a GSSG-activated ≈ 23 kDa inhibitor protein (gel filtration fraction) in rabbit reticulocyte lysates.

Inhibitor protein	Preincubated Time	Preincubated Temperature °C	[14 C] leucine incorporated, cpm
None	–	–	14,124
23 kDa protein	0	–	1,953
23 kDa protein	15	100	1,848
23 kDa protein	60	100	1,964

Gel filtration-purified ≈ 23 kDa inhibitor protein was preincubated at a concentration of 0.2 µg/ml at the indicated temepratures and then subsequently assayed for inhibitory activity in the reticulocyte lysate system. Incubations at 100 °C were performed in a sealed tube to prevent evaporation of the solvent.

In order to gain some information pertaining to the mechanism, protein synthesis assays were performed as described in the Materials and Methods. Our results (Figs 2 and 3) showed that the activity

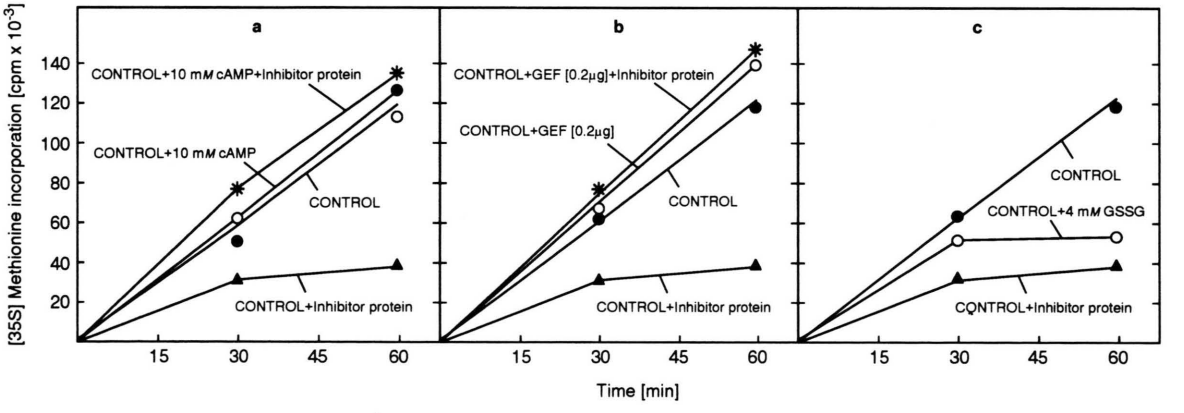

Fig. 2. Time course of the effect of a) cAMP (10 mM), GEF (0.2 µg) and c) GSSG (4 mM) before/after the addition of inhibitor protein (0.2 µg/ml) which was eluted from a Superose column in rabbit reticulocyte.

of protein synthesis was inhibited 70-80% by the addition of inhibitor after 1h incubation and in the presence of cAMP [10 mM] or GEF [0.3] μg/ml, protein synthesis in the inhibited lysate was recovered.

We have developed a simple purification scheme that allows the preparation of an inhibitor protein of 23 kDa apparent molecular weight of high purity. (Sato *et al.*, 1993) suggested that in the freshly prepared macrophages, the synthesis of a 23 kDa protein was very weak, however, in the cells incubated for 11h with glucose oxidase which produces hydrogen peroxide in the culture medium, the synthesis of a 23-kDa protein was enhanced more prominently. The 23-kDa protein which seems to be induced by the oxidative stress as GSSG plays a role in protective mechanism directed against oxydative stress and the function of this protein still remains to be investigated.

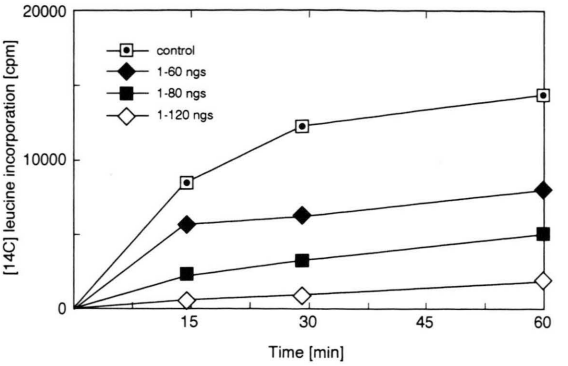

Fig. 3. The effect of inhibitor on rabbit reticulocyte lysate in vitro protein synthesis. Protein synthesis in reticulocyte lysate was performed as described under Methods. 50 μl reaction mixture contained 60, 80, 120 ngs inhibitor which is eluted form a Superose column. Incorporation of [^{14}C] leucine was given as counts per min [$\times 10^{-3}$] per 5 μl mixture.

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