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Some Properties of a Partially Purified Inhibitor of Protein Synthesis isolated from Bovine Cornea

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Summary. Bovine cornea extracted with 0.154 M NaCl yielded a protein fraction which (i) inhibited protein synthesis in rabbit reticulocyte lysates, and (ii) reduced the incorporation of formyl-methionine from f[^{35}S]Met-tRNA$_f$ into polypeptides. The inhibition was reversed by millimolar concentrations of glucose 6-phosphate or cAMP and partially reversed by the addition of initiation factor eIF-2. Thus, the corneal inhibitor may act by directly interfering with the activity of eIF-2.

Recent advances in our knowledge of eukaryotic protein synthesis have mainly come from studies using rabbit reticulocyte lysates (1-5). A number of laboratories have independently established the reaction sequence leading to the 80S formation. Such an assembly requires numerous initiation factors, mRNA, ATP and GTP, initiator tRNA, 40S and 60S ribosomal subunits (1-5). With regard to eukaryotic translational control, there is good evidence pointing to chain initiation as the rate limiting step. Complexity of regulation of peptide initiation is supported by the findings that polyamines, ATP deficiency, hemin, oxidized glutathione, sugar phosphates, ethanol, NAD^+, elevated temperature, high pressure, double-stranded RNA have all been shown to affect the process of initiation (6-15). Evidence has also been presented showing that a proteases(s) is involved in arresting peptide initiation in rabbit reticulocyte lysates (16-22). Although it is not clear whether a common mechanism exists for all of the inhibition observed, it is commonly accepted that the covalent modification of initiation

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factors, especially phosphorylation of eIF-2, is a key component associated with the underlying molecular dysfunction.

Skoza et al.(23) isolated a trypsin inhibitor from bovine cornea. Its mechanism of action at the molecular level is unknown. This report shows that the corneal protease inhibitor (PI) strongly inhibits proteins synthesis, primarily at the initiation step, in rabbit reticulocyte lysates.

Materials and Methods

[U-^{14}C]Leucine, f[^{35}S]met-tRNA$_f$ (14 μCi/mg), [^{35}S]met-tRNA$_f$ (9.7 μCi/mg) were from New England Nuclear. Rabbit reticulocyte lysates were purchased from Clinical Convenience Products (Madison, WI). Highly purified eIF-2 (85%) was provided by Dr. William Merrick. Partially purified eIF-2 was prepared from the 0.5 M KCl ribosome wash by 50% ammonium sulfate precipitation and phosphocellulose column chromatography (24,25). The bovine cornea protease inhibitor was obtained by extraction of cornea with 0.154 M NaCl followed by column chromatography on Sephadex G-75, using water as the eluant (23). Active fractions in the excluded volume were pooled, and used for the present studies without further purification. Cell-free protein synthesis assays were done with the addition of creatine phosphate and creatine phosphokinase (10, 11). Ternary complex formation was assayed by Millipore filtration except that bovine serum albumin (10 μg) was included in each assay (10, 24). The input of [^{35}S]met-tRNA$_f$ was 22,000 cpm. Protein synthesis assays with f[^{35}S]Met-tRNA$_f$ also contained nonradioactive methionine (30 μM) and 2.64x10^5 cpm of f[^{35}S]Met-tRNA$_f$ in a total volume of 30 μl.

Results and Discussion

Extraction of bovine cornea with 0.154 M NaCl and subsequent chromatography of the extract on Sephadex G-75 identified fractions with trypsin inhibitory activity. Aliquots of active fractions inhibited protein synthesis in rabbit reticulocyte lysates (Figure 1). Fractions containing the most inhibitory activity (herein referred to as PI) were pooled and further characterized. Figure 2A shows the effect of varying concentrations of PI on the kinetics of leucine incorporation into peptides. PI had no effect on the initial rate of peptide synthesis (0-15 min), but significantly reduced the rate thereafter. To further define the action of PI,

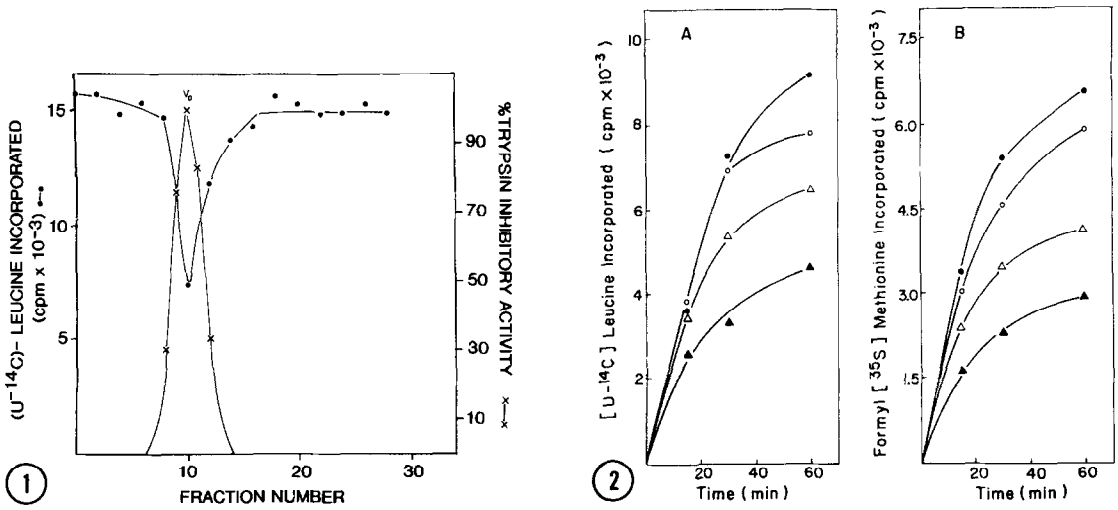

Figure 1. Correlation between leucine incorporation (•) and trypsin inhibitory activity (○) of 0.154 M NaCl corneal extract. A 23 mg lyophilized sample, dissolved in 1.5 ml of water, was applied to a Sephadex G-75 column (0.9 x 60 cm) and eluted with water. Two milliliter fractions were collected. V_o refers to void volume. Trypsin inhibitory activity was assayed as described (23).

Figure 2. Effects of PI on the kinetics of leucine and formylmethionine incorporation into polypeptides in rabbit reticulocyte lysates. **A.** Effects on leucine incorporation. Incubations were at 30°C and 5 μl aliquots were removed at the indicated times. •-•, control, 1.02 μg of PI; ▵-▵, 2.04 μg of PI; ▲-▲, 3.67 μg of PI. **B.** Effects on formylmethionine incorporation. Symbols are identical to those in Figure 2A.

the incorporation of formylmethionine from f[^{35}S]Met-tRNA$_f$ into the NH_2-terminus of globin, which directly measured peptide initiation (25, 26), was examined. Results in Figure 2B shows that PI reduced the incorporation of formylmethionine with a two-phase kinetics. Such an inhibitory mode has previously been shown to be associated with the activation of a eIF-2α-specific protein kinase (1-4). The possibility that a similar mechanism may be involved in the action of PI was tested as follows. First, lysates were incubated with varying concentrations of PI for 10 min at 30°C, then master mix (containing amino acids, buffer, ATP, GTP, the energy regenerating system, and hemin) was added to start the reaction. Results show that such a procedure significantly increased the translational inhibitory activity of PI (Figure 3A,B). Second, the effects of G6P or cAMP on the inhibition of protein synthesis by PI were studied.

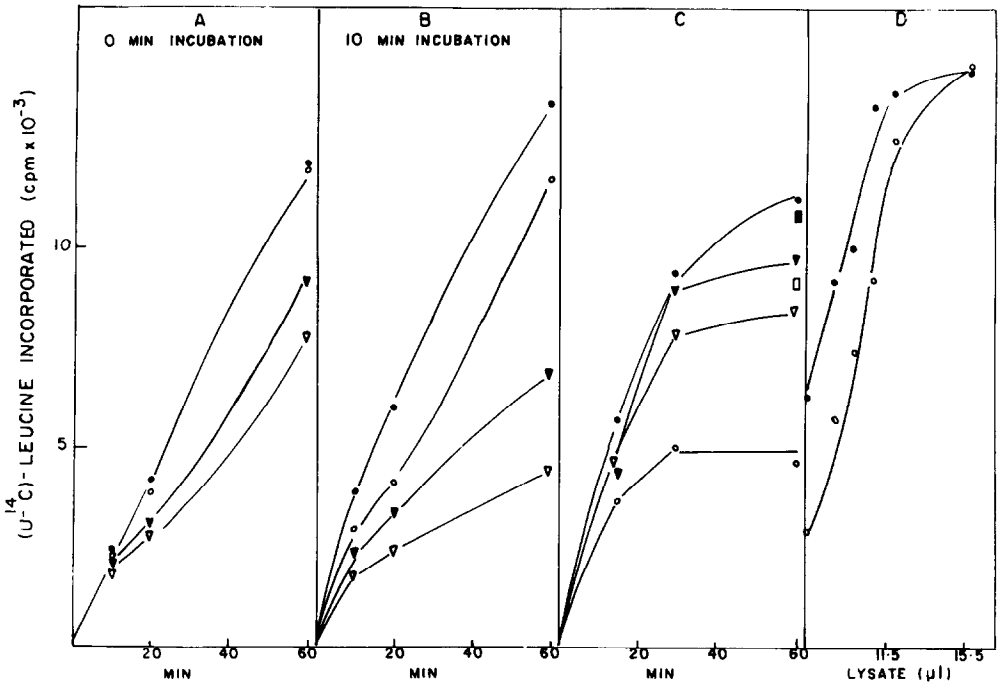

Figure 3. Properties of PI. **A.** Effects of PI on leucine incorporation. A different lysate than the one in Figure 1 was used. Incubations were at 30°C and 5 µl aliquots were removed at the indicated times. •-•, control, no inhibitor; o-o,1.02 µg of PI; ▼-▼, 2.04 µg of PI; ▽-▽, 3.67 µg PI. **B.** Preincubation with PI. Lysates (16 µl) were preincubated with PI for 10 min before the addition of the master mix. Symbols are identical to those in Figure 2A. **C.** Effects of G6P and cAMP on the inhibition of protein synthesis by PI. The lysate in Figure 1 was used for these studies. •-•, control; ■, control and G6P (0.51 mM); □, control and cAMP (4.67 mM); 0-0, 2.7 µg PI; ▼-▼, 2.7 µg PI and G6P; ▽-▽, 2.7 µg PI and cAMP. **D.** Effects of varying concentrations of lysate on inhibition by PI. Lysates were preincubated with 3.67 µg PI for 10 min, 30°C, master mix was then added and the reaction mixture incubated for an additional 60 min. •-•, minus PI; o-o,plus PI.

As shown in Figure 3C, the inhibitory effect of PI was completely reversed by the addition of G6P and cAMP, and partially reversed with the addition of highly purified eIF-2 (Table 1). The results of these experiments suggest at least two possible mechanisms of PI. First, PI may activate an endogenous translational inhibitor which is similar or identical to the previously reported heme- or double-stranded RNA-regulated eIF-2α kinase (1-4). Second, PI may directly interact with a factor required for protein synthesis. The first possibility was eliminated by incubating pro-eIF-2α kinase

Table 1
Effect of Highly Purified eIF-2 on the Inhibition of Protein Synthesis by PI

Addition	Polypeptide Synthesis[a] (cpm)		
	15 min	30 min	60 min
None	1,960	2,630	4,640
+eIF-2[b]	1,890	2,360	3,740
+PI[b]	1,670	1,610	1,870
+eIF-2 and PI	1,730	1,960	2,840

[a]7.5 μl of lysate was used per 30 μl reaction mixture.
[b]eIF-2 concentration was 1.9 μg and PI concentration was 3.5 μg.

with PI and showing that the latter lacked ability to activate the former. To test the second possibility, lysates were increased in the reaction mixture and the inhibition by a fixed concentration of PI studied. Figure 3D shows that as the lysate concentration was increased from 25% to 53%, the inhibition by PI was effectively eliminated. These results raised the possibility that PI acts by interacting quantitatively with an endogenous factor which is required for protein synthesis. Additional data supporting such a hypothesis is shown in Table 2. In these studies, a two stage incubation was used. During the first stage, PI was incubated with a fixed amount of lysate. After a 10 min incubation, additional lysate or water was added. Addition of more lysate in the second stage almost completely eliminated the inhibition by PI. This would suggest that once PI interacted with the endogenous factor, it is not recycled for further interaction. Because inhibition by PI can be reversed by eIF-2, G6P, or cAMP, and because eIF-2 forms a complex with GTP and Met-tRNA$_f$, we studied the effect of PI on ternary complex formation. The results in Table 3 show that ternary complex formation was not inhibited by PI. Instead, it was

Table 2

Effects of lysate concentrations on the inhibition of protein synthesis by PI

Addition at stage 1		Addition at stage 2		Polypeptide[a] synthesis (cpm)	% Inhibition
Lysate (μl)	PI (μg)	Lysate (μl)	H_2O (μl)		
10	0		6	6,500	0
10	2.0		6	3,200	51
10	3.5		6	1,830	72
10	0	6		17,690	0
10	2.0	6		14,060	20
10	3.5	6		10,590	39
16	0			17,030	0
16	2.0			15,190	15
16	3.5			12,080	29

[a]Lysates were preincubated with PI for 10 min at 30°C (stage 1) before the addition of more lysate or water. Master mix was then added and the total reaction mixture was incubated for an additional 60 min (stage 2).

Table 3

Effect of PI on Ternary Complex Formation by eIF-2

Addition		[^{35}S]Met-tRNA$_f$ bound (cpm)
eIF-2	PI	
Experiment 1[a]		
-	-	380
-	+	370
+	-	6,450
+	+	7,930
Experiment 2[b]		
+	-	2,470
+	+	6,240

[a]Partially purified eIF-2 (1.55 μg) was used. The PI concentration was 3.4 μg. Results are the average of three independent experiments (standard deviation 8%). Bovine serum albumin (10 μg) was added to each reaction mixture.

[b]Highly purified eIF-2 (1.4 μg) was added. PI concentration was 3.4 μg. Results are the average of two independent experiments. Bovine serum albumin was not included in the assay mixture.

significantly increased by PI. The magnitude of the increase varied with the purify of eIF-2; with PI showing a greater stimulation using a more purified eIF-2 (Table 3).

In summary, a corneal extract possessing trypsin inhibitory activity (PI) inhibited peptide initiation in rabbit reticulocyte lysates. We propose that PI interferes with the turnover of the complex between eIF-2, GTP, and Met-tRNA$_f$ and eIF-2 recycling.

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