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HIGH pO_2-ACTIVATED INHIBITOR OF PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES: ITS RELATIONSHIP TO GLUTATHIONE DISULFIDE-INDUCED INHIBITOR AND TO A $\sim$23,000-M_r SULFHYDRYL PROTEIN

G.Kanıgür, B.Kan, D.Tiryaki and E.Bermek

Biyofizik Bilim Dalı, Istanbul Tıp Fakültesi
Çapa, Istanbul, Turkey

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Summary: The treatment of reticulocyte post-ribosomal supernatant containing ribosome wash with high pO_2 or glutathione disulfide resulted in the activation of an inhibitor of protein synthesis of $\sim$ 23,000-M_r as implicated by its elution from Sephadex G-100. This inhibitor could also be directly activated by exposure of the $\sim$23,000-M_r fractions of the control eluate to high pO_2 or glutathione disulfide. The high pO_2-dependent activation of the inhibitor was blocked by the presence of glucose-6-phosphate or cAMP (2 mM). The inhibitor was stable (and activable) during a 5 minute incubation at 80°C. The analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the G-100 ($\sim$23,000-M_r) fractions treated with [^{14}C]N-ethylmaleimide revealed the abolishment of the label in a $\sim$ 23,000-M_r protein band in parallel to high pO_2-dependent inhibitor activation.

Different inhibitors of protein synthesis thus far found in reticulocytes seem to involve at some stage of their activation process oxidation or modification of SH-groups (1-6). This suggests that the oxidation state of SH-groups may play an important role in the control of protein synthesis in reticulocytes. As recently shown, high pO_2 also promotes in whole reticulocytes or in their lysates the activation of an inhibitor (hpO_2I) of $\sim$23,000-M_r which shares some common features with the previously reported inhibitors (7). In this paper we report results showing the oxidation of SH-groups of a $\sim$23,000-M_r protein in parallel to the activation of hpO_2I and the possible relationship of hpO_2I to the inhibitor activated in the presence of GSSG (GSSGI).

Materials and Methods

Preparations of reticulocytes, reticulocyte lysates and of post-ribosomal supernatant containing ribosome salt wash (S-100(W)) were as described (7). All steps were carried out at 4°C and all fractions were frozen and kept in liquid nitrogen, although hpO_2I proved to be

Abbreviations: hpO_2I, high oxygen partial pressure-activated inhibitor; GSSGI, glutathione disulfide-activated inhibitor; PAI,pressure-activated inhibitor; G6P, glucose-6-phosphate; NEM, N-ethylmaleimide; SDS, sodium dodecyl sulfate; S-100(W), post-ribosomal supernatant containing ribosomal salt wash; PPO, 2,5-diphenyloxazole; POPOP, 1,4-Bis(5-phenyl-2-oxazolyl)-benzene.

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heat-stable in the course of this work. [^{3}H]Leucine(specific activity 52 Ci/mmole, adjusted to 984 mCi/mmole) and [^{14}C]NEM (specific activity 8.2 mCi/mmole) were obtained from the Radiochemical Centre (Amersham). Hemin chloride was purchased from Boehringer (Mannheim). The hpO_2-treatment of S-100(W) fractions were as described (7). The inhibitor effect on protein synthesis in rabbit reticulocytes was performed as described (7). SDS-polyacrylamide gel electrophoresis was performed (8). After staining and destaining, the gel discs were sliced and the radioactivity in the slices solubilized in 0.5 ml H_2O_2 (8) were counted in 8.4 ml scintillation liquid (9) composed of PPO (16 g), POPOP (400 mg), Triton X-100 (2 l), Toluene (2 l). Counting efficiencies for ^{3}H (7) and ^{14}C (9) in the corresponding scintillation liquids in Packard Tricarb Spectrometer were 16 % and 75 % respectively.

Results and Discussion

Our previous data (7) suggested that the activation of hpO_2I might occur through oxidation of some sulfhydryl group(s) e.g. via formation of GSSG in the reticulocytes and their lysates exposed to high pO_2. To test this possibility, that is- a possible identity of hpO_2I with GSSGI, the S-100(W) fraction treated with GSSG (5) was passed through a Sephadex G-100 column and the column fractions assayed for inhibitor activity in protein synthesis. The GSSGI activity was found, like the hpO_2I, in the 23,000-M_r region of the eluate (Fig.1). The inhibitory activity eluting behind the void volume (7) was found also in this case. Moreover, also consistent with previous findings(7), a third peak of inhibitor activity eluting with column volume was routinely found after the hpO_2-or GSSG-treatment of the S-100(W) fraction. When the S-100(W) fraction was without treatment directly applied to Sephadex G-100 and the column fractions, thereafter, treated with high pO_2 or GSSG, the inhibitory activities were again found in the fractions corresponding to the three regions (Fig.1).

The activation of the $\sim$23,000-M_r inhibitor by direct exposure of the corresponding G-100 fractions to high pO_2 or GSSG did not take place in the presence of G6P or of high concentrations of cAMP (2 mM)(Table 1). Thus, G6P and cAMP (2 mM) which have been shown to prevent the activation of different inhibitors (3,5,10-13) appeared to exert here this effect by somewhat direct interaction with the proinhibitor, and not over NADPH generation. The $\sim$ 23,000-M_r hpO_2I, like the heat-stable (HS) component of PAI (14) was activated and stable during a 5 min incubation at 80^{o}C.

These results indicated that the effect of hpO_2 and of GSSG on protein synthesis might indeed occur through the same mechanisms, that is, through activation of a $\sim$ 23,000-M_r protein. The similarities in chromatographic behaviour and the heat stability of the $\sim$ 23,000-M_r

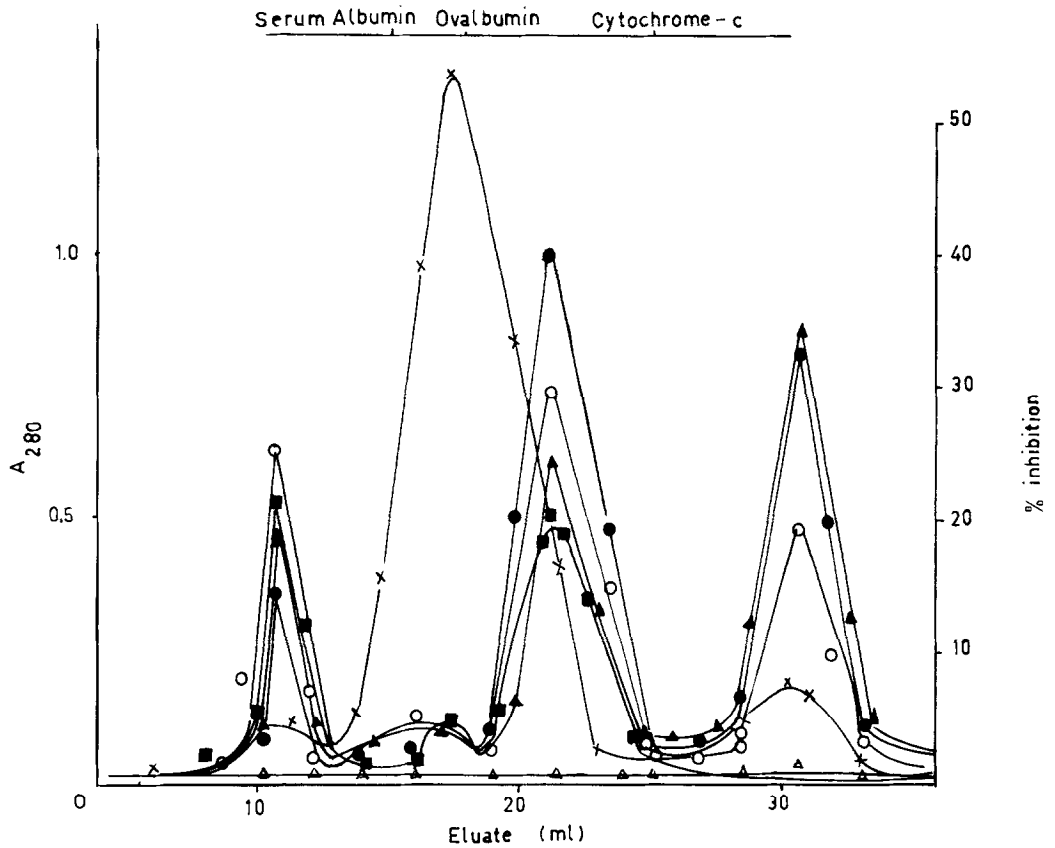

Fig 1. Chromatography of hpO_2I and GSSGI on Sephadex G-100. 9.0 mg S-100(W) fraction proteins were treated either with hpO_2 (7) or with 2.5 mM GSSG (5) and, thereafter, gel-filtered on Sephadex G-100 as described (7). Alternatively, the S-100(W) fraction without treatment was directly applied to G-100 column (control eluate). G-100 fractions of the control eluate, whenever indicated, were subsequently treated with hpO_2 or GSSG. 15 µl aliquots from G-100 fractions were assayed for inhibitory activity in protein synthesis (7). Control activity (0% inhibition) was 16.500∓800 cpm/50 µl reaction mixture containing 1 A_{260} unit lysate fraction. (Δ-Δ), control eluate; (●-●), inhibitory activity of the fractions from hpO_2-S-100(W); (▲-▲) inhibitory activity of the fractions from the control eluate , subsequently treated with hpO_2; (■-■), inhibitory activity of the fractions from the (GSSG)S-100(W); (○-○), inhibitory activity of fractions from the control eluate , subsequently treated with GSSG; (X-X), A_{280}.

inhibitor indicated also a close relationship to PAI (HS)(14). Moreover, the data suggested a remarkable parallelism between the ∿ 23,000-M_r inhibitor and a SH-protein of similar molecular weight recently reported (15): the inactivation of the G-25 gel filtered reticulocyte lysates in protein synthesis seemed to be accompanied by oxidation of the SH-group(s) of this latter protein. We decided, therefore, to investigate the relationship of the activation of hpO_2I to this SH-protein (15). For this purpose, the S-100(W) or the ∿ 23,000-M_r G-100 fractions of the

Table 1: Activation of the ∿23,000-M_r inhibitor of protein synthesis by hpO_2 or GSSG (2.5 mM). Effects of G6P, ATP, cAMP or of heat treatment upon activation.

System	[^{3}H]Leucine incorporated cpm/1 A_{260} unit lysate
	Experiment I
Control lysate	18993
+∿23,000-M_r fraction 5 min 37°C (+ATP)	21283
+ " " " " " " hpO_2	12330
	Experiment II
Control lysate	7588
+∿23,000-M_r fraction 5 min 37°C	6667
+ " " " " hpO_2	5972
+ " " " " (+ATP), hpO_2	1880
+ " " " " (+G6P), hpO_2	6663
+ " " " " (+cAMP), hpO_2	8783
+ " " " " (+GSSG)	4272
+ " " " " (+GSSG+ATP)	2957
+ " " " " (+GSSG+G6P)	7428
+ " " " 80°C	5473
	Experiment III
Control lysate	13703
+∿23,000-M_r fraction from hpO_2-S-100(W)	7400
+ " " " "^{2} 5 min 80	7908

The ∿23,000-M_r fraction obtained by gel-filtration on Sephadex G-100 was treated with hpO_2 (7) or GSSG (5). Whenever added to the ∿23,000-M_r fraction during treatment, the concentrations of G6P, ATP and cAMP were 3.0, 1.0, and 2.0 mM, respectively. (G6P added after treatment failed to reverse the inhibitor activation (not shown)). The effect of the samples on protein synthesis in the lysates were assayed as described (7). In Experiments II and III, the heat treated G-100 fractions were cleared by centrifugation for 10 min at 10,000xg and the supernatants assayed for inhibitory effect on protein synthesis. The precipitate from such samples after resuspension revealed no inhibitory activity; nevertheless, in crude and concentrated fractions like S-100(W), the inhibitory activity was found to be trapped in the heat precipitate.

control eluate either directly or after treatment with hpO_2 and the corresponding G-100 fractions from the hpO_2-S-100(W) were treated with [^{14}C]N-ethylmaleimide. The SDS-polyacrylamide gel electrophoresis of these samples and the subsequent determination of the radioactivity in the gel slices showed a high ^{14}C radioactivity peak and its hpO_2-dependent loss in the 23,000-M_r region in parallel to inhibitor activation (Fig.2). This ∿23,000-M_r protein which, in its oxidized state, is possibly identical with hpO_2I (and with GSSGI, respectively) might be involved in the activation of heme-controlled translational inhibitor in a subsequent step (16). The apparent involvement of cAMP, ATP and of G6P in the activation of the ∿23,000-M_r inhibitor might provide a certain differentiation in the response of protein synthetic activity to the oxidation state of the (erythroid) cell: it might warrant on additional modulation of the (SH-group oxidation-mediated) control of protein synthesis through the cellular levels of phosphorylated compounds.

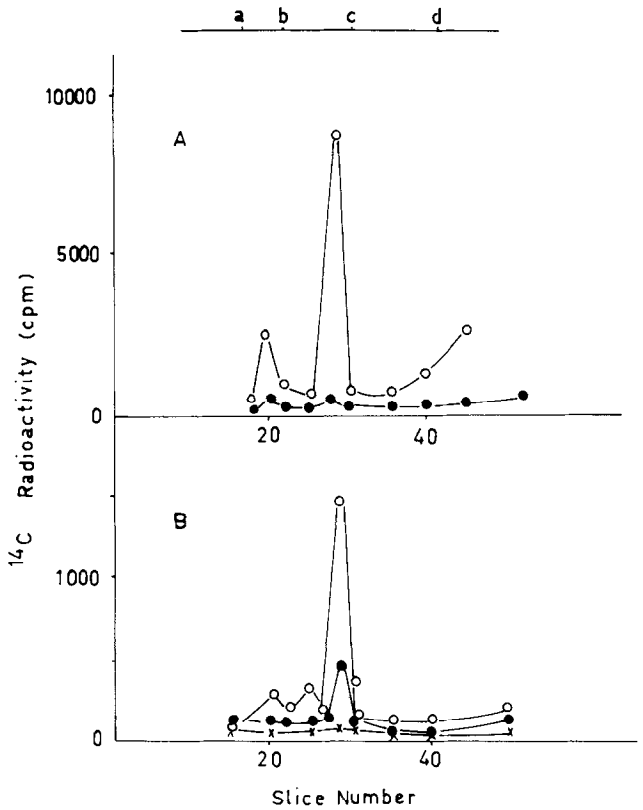

Fig 2. High pO_2-promoted oxidation of SH-group(s) in S-100(W) and ∿23,000-M_r G-100 fractions. Untreated (control) S-100(W) and hpO_2-S-100(W) fractions were dialyzed against TKMM (7) containing 1 mM instead of 7 mM 2-mercaptoethanol. Alternatively, they were gel-filtrated in the same buffer on Sephadex G-100 and the fractions corresponding to 23,000-M_r region were saved. Aliquots from the control eluate were subsequently treated with hpO_2. The inhibitory activity of the hpO_2 samples on protein synthesis was assayed. After incubation for 15 min at 37°C with 1.2 mM [^{14}C]NEM, the samples containing 450 µg S-100(W) proteins or 45 µg 23,000-M_r fraction proteins were electrophoresed on SDS-polyacrylamide gels and the ^{14}C activity in the gels determined as described under Methods. A) (O-O), control S-100(W); (●-●), hpO_2-S-100(W). B) (O-O), control ∿23,000-M_r fraction; (X-X), control 23,000-M_r fraction subsequently treated with hpO_2; (●-●), ∿23,000-M_r fraction from hpO_2-S-100(W). a) catalase; b) ovalbumin; c) chymotrypsinogen; d) cytochrome C.

Acknowledgements

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