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Irreversible inactivation of calcium-dependent proteinases from rat liver by biological disulfides

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The effect of low-molecular-mass biological disulfides and their related reduced compounds on the activity of two calcium-dependent neutral proteinases (calpains) from rat liver has been investigated. L-Cystine and L-cystamine bring about the inactivation of both enzymes, while the related reduced compounds L-cysteine and L-cysteamine are without effect. Calpain II is more sensitive to the inactivating effect of glutatione disulfide in comparison with calpain I. The inactivation rates of both calpains depend on the concentration of glutathione disulfide. Reduced glutathione, added at physiological concentration (5 mM), neither affects the proteinase activities nor protects the enzymes from the inactivating effect of glutathione disulfide. The enzymes inactivated by biological disulfides cannot be restored by a large excess of a reducing thiolic compound (dithiothreitol). It is suggested that calcium-dependent proteinases might be inactivated also in vivo by enhanced level of glutathione disulfide.

Calpain; Proteolysis; Disulfide compound; Proteinase; Ca^{2+} dependence; Glutathione; (Rat liver)

1. INTRODUCTION

Neutral calcium-dependent endoproteinases (calpains) have been identified in liver [1], heart [2], muscle [3], erythrocytes [4], kidney [5] and platelets [6].

All these proteinases seem to be characterized by some common properties such as dimeric structure with different subunits, inhibition by specific natural endogenous protein inhibitor and the localization in the cytosolic fraction of the cell [7].

Although a large number of recent studies have led to an increased knowledge about the properties and structures of calpains, the system(s) regulating the enzyme activity and the exact role of calpains in the cell are still uncertain.

Several different factors seem to potentially affect the enzyme activity in vivo such as: the concentration of calcium in a micro-environment [1]; the concentration of endogenous inhibitor and activator proteins [8,9]; the presence of susceptible substrate proteins which may activate the enzyme [10]; the presence of biological membranes and their phospholipids [11,12]. In addition, since calpains are thiol-dependent enzymes [7], a modulation of the enzyme activity could be obtained by compounds affecting the integrity of the essential sulphydryl group(s).

Although it is well known that typical chemical reagents of thiol groups [13] inhibit the calpain activity, and several enzymes are modulated by biological disulfides [14], the effect of these latter compounds on the calpain activities has not been investigated.

In the present paper we demonstrate the irreversible inactivation of calpains from rat liver by low-molecular-mass biological disulfides suggesting a possible role of glutathione disulfide in the control of the enzyme activity in vivo.

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2. MATERIALS AND METHODS

2.1. *Purification of calcium-dependent neutral proteinases*

Livers from male Wistar rats of 250–300 g body wt, fed on commercial pellet diet, were first perfused with 0.15 M NaCl to remove blood, then homogenized in a teflon-glass Potter type homogenizer with 5 mM potassium phosphate buffer, pH 7.6, containing 0.25 M sucrose and 1 mM EDTA. From the cytosolic fraction two different forms of enzymes (type I and type II, activated by micro- and milli-molar calcium concentration, respectively) were isolated as described in [1]. Isolated enzymes were equilibrated with 0.05 M borate buffer, pH 7.6, containing 1 mM EDTA and 0.4 mM dithiothreitol (buffer A) by a gel filtration on G-25 Sephadex equilibrated and eluted with the same buffer A. The enzymes were concentrated by an Amicon apparatus on PM-30 membranes and stored at −80°C with negligible loss of activity up to two months.

2.2. *Enzyme activity*

Both enzymes were assayed with succinyl-casein as substrate prepared according to Mellgren et al. [15], measuring the liberation of free α-amino group. The proteinase substrate solution containing 5 mg/ml of succinylated casein, was incubated at 25°C in 50 mM sodium borate buffer, pH 7.5, provided with 1 mM dithiothreitol and 4 mM $CaCl_2$. 10–30 μl of the protease was added to 0.3 ml of substrate solution and at different times 40 μl samples were removed and diluted in 2.7 ml of 0.5 M borate buffer, pH 8.5. The samples were rapidly mixed with 0.3 ml of 0.03% solution of fluorescamine in acetone. Fluorescence was determined in a Perkin Elmer spectrofluorometer (excitation wavelength = 390 nm; analyzer wavelength = 475 nm). The enzyme activity was calculated by a standard curve constructed using various amounts of glycine diluted in borate buffer and reacted with fluorescamine as previously described. The reaction is linear up to 8 min incubation. A unit of protease activity is defined as the amount of the activity which releases 1 μmol of glycine-equivalent free amino group from succinyl-casein per min under the standard reaction conditions.

2.3. *Enzyme inactivation*

All disulfides and thiols except L-cystine were directly dissolved in 0.05 M borate buffer, pH 7.6 (buffer B). L-Cystine was initially dissolved in a few microliters of 0.2 M NaOH, diluted in the buffer B, adjusted to pH 7.6 with 0.2 M HCl and immediately used.

Suitable enzyme aliquots were thawed and immediately used. The inactivation mixtures contained: 0.2 ml of calpain I (0.3 units) or calpain II (0.8 units); 0.2 ml of buffer B in which disulfides or thiols had been dissolved at twice final concentration. The mixtures were incubated at 25°C and at different times samples were withdrawn to assay activity as described above.

Since the fluorescence background produced by disulfides in the assay mixtures decreases depending on the time of incubation of disulfides in the buffer A, all measurements were corrected by a control blank constituted with disulfides and buffer A incubated as described for the samples containing the enzyme.

Reduced glutathione and glutathione disulfide were estimated according to [16,17], respectively.

3. RESULTS AND DISCUSSION

The effect of low-molecular-mass biological disulfides and their related thiols on the activity of calpains I and II is summarized in table 1. All disulfides at 2 mM concentration bring about the partial inactivation of both proteinases. However, while L-cystamine and L-cystine cause the inactivation of both enzymes to a similar extent, glutathione disulfide (GSSG) is a less effective inhibitor of calpain I in comparison with calpain II. Related reduced compounds, L-cysteine, L-cysteamine and reduced glutathione (GSH), do not affect the proteinase activities, suggesting that the inactivation should be ascribed to the presence of the disulfide bridge in the inhibitory molecule.

The inactivation kinetics of both calpains were further investigated at different concentrations of glutathione disulfide which is the only one, among the disulfides tested, that occurs in the cell in any appreciable amount. The rates of inactivation of both calpains are dependent on the concentration of the disulfide, however, while calpain II is affected at every concentration, calpain I requires at

Table 1

Inactivation of calpain I and II by biological disulfides and related thiols

Compounds	Calpain I	Calpain II
Cystamine	29	33
Cystine	38	39
GSSG	70	30
Cysteamine	94	96
Cysteine	95	98
GSH	100	103

All compounds were added at 2 mM. Activity measurements, expressed as percentage of enzyme residual activity with respect to untreated control sample, were performed after 60 min incubation at 25°C. All measurements were corrected by fluorescence background produced by disulfides as described in section 2. Data are mean values of three experiments varying within 5% of the mean

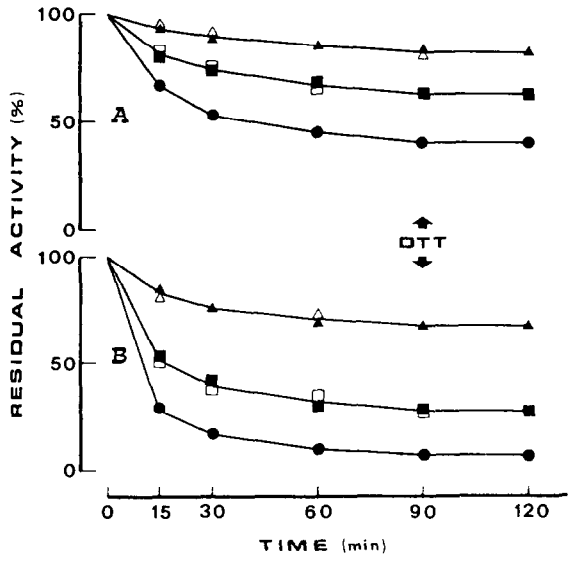

Fig.1. Effect of GSH and GSSG on calpain I (A) and calpain II (B). All reaction mixtures contained 0.2 ml of enzyme (0.3 units of calpain I or 0.8 units of calpain II) and 0.2 ml of 0.05 M sodium borate buffer, pH 7.5, containing appropriate amounts of reagents to give the final concentrations indicated. (●) 5 mM GSSG; (■) 1 mM GSSG; (▲) 0.5 mM GSSG; (□) 5 mM GSH + 1 mM GSSG; (△) 5 mM GSH + 0.5 mM GSSG. At the times indicated suitable aliquots were withdrawn and added to a substrate mixture as described in section 2. The arrow indicates the addition of 15 mM dithiothreitol in the incubation mixtures. Other experimental details are reported in table 1.

least 1 mM GSSG concentration to be inactivated to a significant extent.

In order to evaluate whether the kinetics of calpain inactivation might be influenced by the simultaneous addition of GSH, similar experiments were also performed in the presence of GSH at 5 mM concentration. Identical inactivation curves, obtained in the presence or absence of GSH (fig.1), indicate that calpain inactivation is not modulated by the GSH/GSSG ratio, but only depends on the concentration of glutathione disulfide.

To exclude that the GSH/GSSG ratio could be modified during the course of incubation, essentially owing to GSH oxidation, the concentration of both compounds was measured after 90 min incubation. No significant modification of the relative initial concentrations was observed, since more than 97% of the original GSH was recovered in the reduced form and GSSG slightly increased up to 112% of initial value.

As shown in fig.1 the loss of enzyme activity cannot be restored by addition of 15 mM dithiothreitol to the incubation mixture. Similarly, the enzyme inactivation brought about by L-cystamine or L-cystine cannot be reverted by dithiothreitol (not shown).

It is interesting to observe that the inactivation of calpains, mainly calpain II, takes place at a relatively low concentration of GSSG, even in the presence of a large excess of GSH. Such experimental conditions resemble the status of living cells. Indeed, GSSG generally occurs at a concentration lower than the one used [18], however in several circumstances the cellular level can be enhanced to values close to those reported herein [19]. Therefore, it is possible that the calcium-dependent proteinase activity could be controlled also in vivo by GSSG concentration. The exact mechanism of the enzyme inactivation remains to be investigated. Although several other enzymes have been reported to be inactivated by a thiol-disulfide exchange [14], the process is generally reversible. In our case, the irreversible inactivation provides evidence against a simple thiol-disulfide exchange suggesting a more complex phenomenon.

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