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Short Communication

AUTOPHOSPHORYLATION INHIBITS THE ACTIVITY OF γ-GLUTAMYLCYSTEINE SYNTHETASE*

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INTRODUCTION

The tripeptide, glutathione (GSH) is synthesized from its constituent amino acids in two sequential, ATP-dependent enzymatic reactions, catalyzed by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase.[1] γ-GCS catalyzes the first and rate-limiting step in GSH synthesis. The activity of this enzyme is feedback-inhibited by GSH.[2] γ-GCS purified from rat kidney is composed of two subunits; a catalytic subunit (Glclc), Mr ~ 73,000, and a

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regulatory subunit (Glclr), Mr ~ 27,700. The holoenzyme can be dissociated into its subunits under reducing conditions.[3] The heavy subunit possesses all of the catalytic activity and is the site of GSH feedback-inhibition, while the light subunit has been shown to perform a regulatory function, greatly influencing the activity of the holoenzyme.[4] In addition to its synthetase activity, γ-GCS catalyzes several partial reactions, including hydrolysis of ATP (ATPase activity), phosphorylation by ATP of inorganic phosphate to yield inorganic pyrophosphate, and cyclization of glutamate to pyrrolidone carboxylate.[5] It has also been shown that the ATPase activity is inhibited by glutamate and glutamate analogs. The optimal pH for ATPase activity is pH 9.0 in contrast to the synthetase activity which is maximal at pH 8.0. The ATPase activity is approximately 1–10% of total synthetase activity.[5]

Recently it has been shown that γ-GCS is phosphorylated by several protein kinases including cAMP kinase, Ca^{2+}/calmodulin-dependent kinase II and protein kinase C.[6] In the present communication, we have shown that γ-GCS can be autophosphorylated and that this autophosphorylation can be inhibited by L-glutamate.

METHODS AND MATERIALS

Sprague-Dawley rat kidneys were purchased from PelFreeze (Rogers, AZ) and stored at −70°C. [γ-^{32}P]ATP was obtained from Amersham (Arlington Heights, IL). L-Glutamate, L-α-aminobutyrate, EDTA, activated charcoal, and ATP were purchased from Sigma (St. Louis, MO).

Purification of γ-GCS

γ-GCS was purified from rat kidneys as described by Seelig and Meister[7] with slight modification; 2-mercaptoethanol was omitted in the first step of purification. The kidneys were homogenized in 5 volumes of buffer containing 150 mM KCl and 1 mM $MgCl_2$ for 2 min. Further purification steps include, (1) Ammonium sulfate precipitation, (2) DEAE cellulose column, and (3) ATP agarose affinity column purification, as described by Seelig and Meister[7]. The enzyme fractions from the affinity column purification, were pooled, concentrated and dialyzed against 10 mM imidazole, pH 8.4 containing 1 mM EDTA. The purified enzyme was stored at 4°C. The purity of the enzyme was confirmed by gel electrophoresis. No contaminating proteins were observed on SDS-PAGE or native PAGE.

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Autophosphorylation of γ-GCS in the Presence of Synthetase Mix and ATPase Mix

Ten μl of γ-GCS (0.13–1.5 μg) was added to 10 μl, 2 × synthetase mix containing 200 mM Tris HCl, pH 8.0 at 37°C, 300 mM KCl, 20 mM L-glutamate, 20 mM L-α-aminobutyrate, 40 mM $MgCl_2$, 4 mM EDTA followed by 20 μCi of [γ-^{32}P]ATP and the mixture was incubated at 37°C for 30 min. ATPase mix contained only Tris HCl, pH 9.0 at 37°C, $MgCl_2$, and [γ-^{32}P]ATP. Reactions were stopped by placing on ice, adding sample buffer, which contained 62 mM Tris HCl, pH 6.8 and 10% glycerol and bromophenol blue dye. Samples were electrophoresed on 9.5% native polyacrylamide gel or 9.5% or 12% SDS-PAGE (in this case sample buffer contained 2% SDS and 5% mercaptoethanol) at constant current of 15 mA. The gel was stained with Coomassie blue, destained, and dried and exposed to X-ray film to visualize the autophosphorylated γ-GCS.

For determination of activity of autophosphorylated γ-GCS, after the reaction with ATPase mix as described above, the contents were cooled on ice immediately, dialyzed against imidazole buffer containing 10 mM imidazole and 1 mM EDTA, and assayed for activity and for K_m determinations for Mg^{2+} and L-glutamate, as described below.

Assay Methods

1. Synthetase Activity

γ-GCS synthetase activity was determined with slight modification of the methods reported in the literature.[8,9] This modified method is useful with low quantities of enzyme and can be performed rapidly. The enzyme (5 μl, 0.65 μg) was added to 95 μl of assay mix containing 100 mM Tris HCl, pH 8.0 at 37°C, 150 mM KCl, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 2 mM EDTA, and 5 mM ATP spiked with [γ-^{32}P]ATP. The mixture was incubated at 37°C for 30 min. The reaction tubes were immediately placed on ice and 900 μl of 0.1 M EDTA was added followed by 50–100 mg of hydrochloric acid-washed activated charcoal. The contents were vortexed, and centrifuged at 10,000 rpm for 4 min. The inorganic phosphate was measured by counting 100 μl of supernatant in scintillant (EcoLite from ICN Biomedicals, Irvine, CA). The enzyme activity is defined as the amount that catalyzes the formation of one μmol of inorganic phosphate per hour per mg of protein at 37°C.

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2. *ATPase Activity*

The ATPase activity was measured in the absence of substrates, L-glutamate and L-α-aminobutyrate. The optimum pH for ATPase activity is pH 9.0 at 37°C. The ATPase mix contains only 100 mM Tris HCl, pH 9.0, 20 mM $MgCl_2$, and 5 mM ATP spiked with [γ-^{32}P]ATP. The inorganic phosphate was measured as described above.

RESULTS AND DISCUSSION

γ-GCS catalyzes several partial reactions as described in the Introduction. It hydrolyzes ATP and couples the two amino acids, L-glutamate and cysteine to form γ-glutamylcysteine. Magnesium is required for these two reactions. We have designed and developed a new assay method for the activity studies of γ-GCS. After the incubation, the reaction was terminated by cooling immediately on ice, and then adding a large excess of cooled (4°C) EDTA buffer, which is a good chelator of Mg^{2+}. The inorganic phosphate is measured by treating with hydrochloric acid-washed activated charcoal.

The ATPase activity of γ-GCS was determined in the presence and absence of substrates. The synthetase activity (1022 μmol h^{-1} mg^{-1}) was also determined to compare it with the ATPase activity (3 μmol h^{-1} mg^{-1}). The ATPase activity was found to be less than 1% of total synthetase activity, which is consistent with the literature.[10]

Autophosphorylation of γ-GCS

In the present communication, we showed that in the absence of substrate L-glutamate, γ-GCS (0.13 μg of enzyme present in each lane) was autophosphorylated (Figure 1) (lane 3). In Panel B, the Coomassie stained protein bands are shown, and in Panel A, the autoradiograph of the corresponding gel is shown. It is obvious from this figure that γ-GCS was not autophosphorylated in the presence of synthetase mix, which contained both substrates (lane 2), or in the presence of ATPase mix if it contained both substrates (lane 6). In the presence of L-α-aminobutyrate (lane 5), γ-GCS was autophosphorylated, and in the presence of L-glutamate, a weak band (lane 4) was noticed in the autoradiograph. The band observed in lane 4 could be due to partial autophosphorylation of γ-GCS. Autophosphorylation of γ-GCS occurred in the presence of L-glutamate, when ten fold excess of γ-GCS was used. In Figure 2, it was shown that in the presence of various concentrations of L-glutamate, autophosphorylation of the

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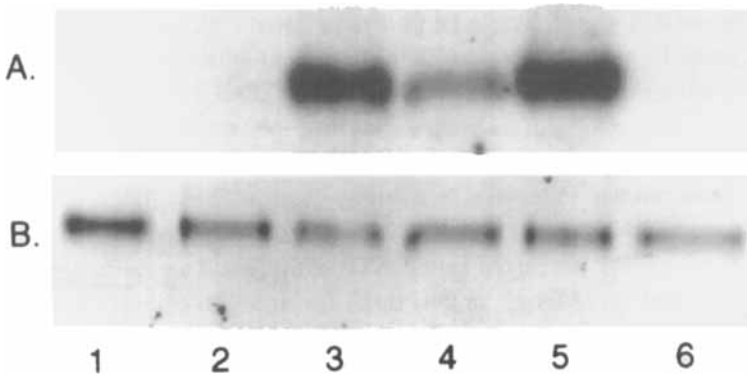

FIGURE 1 Autophosphorylation of γ-GCS in the presence and absence of substrates. Panel A represents the autoradiograph and Panel B is corresponding Coomassie stained native PAGE. Lane 1, native γ-GCS; lane 2, γ-GCS with synthetase mix; lane 3, γ-GCS with ATPase mix; lane 4, γ-GCS with ATPase mix and L-glutamate; lane 5, γ-GCS with ATPase mix and L-α-aminobutyrate; lane 6, γ-GCS with ATPase mix, L-glutamate, and L-α-aminobutyrate.

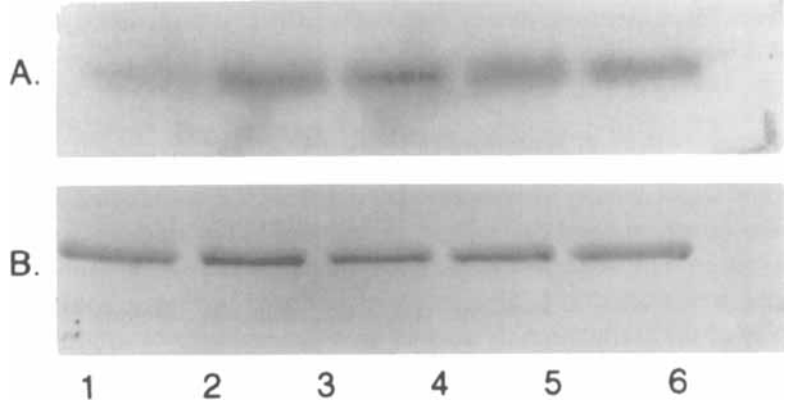

FIGURE 2 Effect of L-glutamate on the autophosphorylation of γ-GCS. Panel A represents the autoradiograph and Panel B is the corresponding Coomassie stained SDS-PAGE. Lane 1, 1 mM L-glutamate; lane 2, 0.1 mM L-glutamate; lane 3, 0.01 mM L-glutamate; lane 4, 0.001 mM L-glutamate; lane 5, no L-glutamate.

catalytic subunit (Glclc) was observed (SDS-PAGE was used). However, the extent of autophosphorylation of Glclc was less in the presence of 1 mM L-glutamate (lane 1). Based on this result, we propose that some of the autophosphorylation sites are located in the glutamate binding site. The regulatory subunit (Glclr) was not autophosphorylated in these experiments.

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Recently, γ-GCS was shown to be phosphorylated [6] by protein kinase A, protein kinase C, and Ca^{2+}/calmodulin-dependent kinase II. It was also shown that phosphorylation of γ-GCS altered its catalytic activity. It was reported that the regulatory subunit was not phosphorylated by these kinases.

It has been shown that γ-GCS exhibits high affinity for ATP.[5] To prove that the band in lane 3 (Figure 1) is not due to non-specific binding of [γ-^{32}P]ATP to γ-GCS, we mixed [γ-^{32}P]ATP with γ-GCS in the absence of Mg^{2+} and treated the enzyme as was done for autophosphorylation. The enzyme does not exhibit activity in the absence of Mg^{2+}. After the reaction, 100 µl of 250 mM cold ATP was added to the samples, which were left on ice for 1 h and then dialyzed against Tris HCl, pH 7.5 containing 10 mM $MgCl_2$ and 5 mM ATP. The protein was precipitated with 90% ammonium sulfate, washed twice with 50% ammonium sulfate, and dissolved in sample buffer, which contained 62 mM Tris HCl, pH 6.8, 10% glycerol, 5% mercaptoethanol, and 2% SDS, and electrophoresed on 9.5% SDS-PAGE. The resulting autoradiograph demonstrated that binding of [γ-^{32}P]ATP to γ-GCS did not occur in the absence of Mg^{2+}, where enzyme activity was inhibited (data not shown). This experiment clearly demonstrated that autophosphorylation of γ-GCS was an enzymatic reaction.

Synthetase Activity of Autophosphorylated γ-GCS

The synthetase activity of autophosphorylated γ-GCS was studied by isolating the autophosphorylated enzyme as described in the Methods and Materials section. The synthetase activity of both native and autophosphorylated enzyme was studied and the results are presented in Figure 3. Synthetase activity of the autophosphorylated enzyme was inhibited by approximately 50% compared to native γ-GCS. We have also determined the K_m values for Mg^{2+} and L-glutamate and found no change in their values compared to those of native γ-GCS. This suggests that the change in the activity observed for autophosphorylated γ-GCS is the result of a decrease in V_{max} for Mg^{2+} and L-glutamate without changing its K_m. These results are consistent with those obtained by Sun *et al.*[6] using kinase-induced phosphorylation of γ-GCS.

pH-dependency of Autophosphorylation of γ-GCS

To determine whether autophosphorylation of γ-GCS was strictly dependent upon its ATPase activity, the pH-dependency of the enzyme activities

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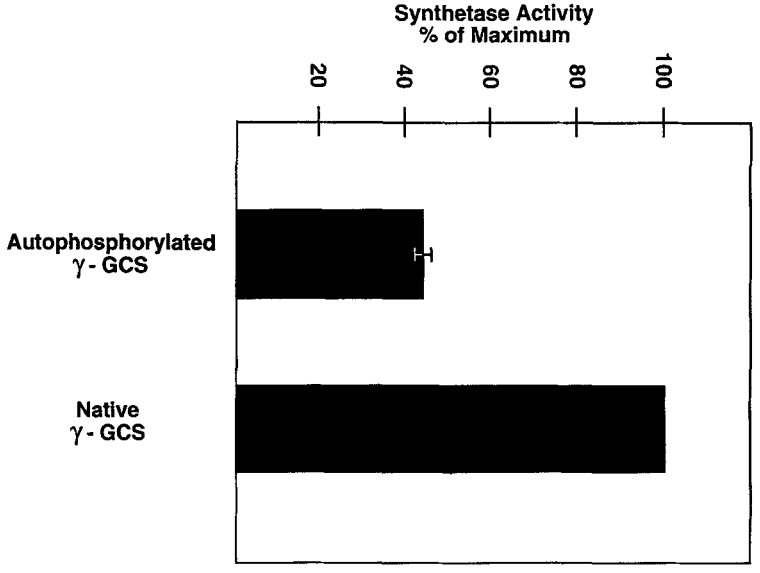

FIGURE 3 Histogram showing synthetase activity of native γ-GCS and autophosphorylated γ-GCS. The isolation of autophosphorylated γ-GCS is discussed in the Methods and Materials.

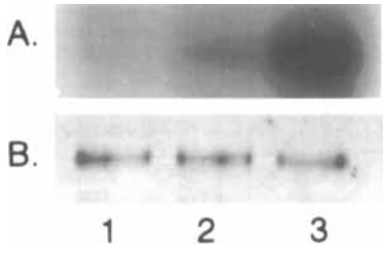

FIGURE 4 Effect of pH on the autophosphorylation of γ-GCS. Panel A shows the autoradiograph and Panel B is the corresponding Coomassie stained native PAGE. Lane 1, autophosphorylation of γ-GCS at pH 7.0; lane 2, pH 8.0; and lane 3, pH 9.0.

(Figure 4) was compared. Three different pHs, pH 7 (lane 1), pH 8 (lane 2), and pH 9 (lane 3), were used. A direct correlation between ATPase activity and autophosphorylation was observed. Maximum ATPase activity was observed at pH 9.0 and diminished with decreasing pH (data not shown). Similarly, decreasing the pH from 9.0 to 7.0, decreased autophosphorylation. This experiment indicates that ATPase activity is essential for autophosphorylation.

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CONCLUSIONS

Though the physiological significance of autophosphorylation is not well understood, it is possible that it may play an important role in glutamate-deficient diseases such as Huntington's disease.[11] In those situations, the activity of the enzyme may be decreased by autophosphorylation, and inhibit glutathione synthesis. This could be exceptionally deleterious if exposed to peroxides or toxic xenobiotics such as phenol, benzoquinone, bromosulfophthalein, or other glutathione S-transferase substrates. Inhibition of GSH synthesis would potentiate the degree of GSH depletion, and increase the accumulation of toxic and carcinogenic materials.

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