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Alteration in arginine activation of *N*-acetylglutamate synthetase in vitro by disulfide or thiol compounds

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Abstract

N-acetyl-L-glutamate synthetase (EC 2.3.1.1) is a regulatory enzyme involved in the control of carbamoyl-phosphate synthesis in the mammalian liver. The enzyme is activated specifically by arginine, and the sensitivity of acetylglutamate synthetase to arginine undergoes marked changes after food ingestion. Since the extent of arginine activation of the synthetase — high for the enzyme from fed mice and low for the enzyme from fasted mice — remained much the same during partial purification, these changes appear to be due to a modification of the enzyme molecule itself. When the enzyme preparation with a low sensitivity to arginine activation, partially purified from the liver of starved mice, was incubated with thiol compounds, the sensitivity increased. When the enzyme preparation with a high sensitivity, obtained from the liver of fed mice, was incubated with disulfide compounds, the sensitivity decreased. Diminution and enhancement of arginine sensitivity of a single enzyme preparation were also achieved by the disulfide and thiol treatments, thereby revealing the reversibility of the process. These results suggest that thiol/disulfide interchange may be involved in the regulation of arginine sensitivity of acetylglutamate synthetase in vivo.

Keywords: Urea synthesis; Acetylglutamate synthetase; Arginine; Thiol/disulfide interchange; Carbamoyl-phosphate synthetase I

1. Introduction

N-acetyl-L-glutamate (acetylglutamate) synthetase (EC 2.3.1.1) [1] catalyzes the synthesis of acetylglutamate, which is required [2,3], although not absolutely [4,5], as an activator for carbamoyl-phosphate synthetase I (EC 6.3.4.16) in liver mitochondria of ureotelic animals from acetyl-CoA and glutamate [6]. We have proposed [7–12] that the hepatic concentration of acetylglutamate — the product of acetylglutamate synthetase — is a control factor of urea biosynthesis. This proposal has been supported by other investigators (Refs. [13,14]; for a review, see Refs. [15,16]). Acetylglutamate synthetase, which was purified from the liver of rats [17–19] and humans [20], is activated

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specifically by arginine. We reported that the extent of increase in the activity of the synthetase by a saturating level of arginine undergoes marked changes after ingestion of food [21] or injection of inhibitors of nucleic acid and protein synthesis [22]. Two forms of acetylglutamate synthetase, low and high sensitivity types, were obtained from liver mitochondria of starved and well-fed mice, respectively [21], and the extent of arginine activation of each synthetase preparation remained much the same after partial purification. The results suggested that changes in arginine sensitivity were due to a modification of the enzyme molecule itself.

We now report on the enhancement of arginine sensitivity of the low-sensitivity enzyme and the diminution of the sensitivity of the high-sensitivity enzyme in vitro by treatment with thiol and disulfide compounds, respectively. The extent of arginine activation was diminished or enhanced by these treatments and there was a partial reversion by opposite treatments. Thus, thiol/disulfide interchange may possibly be involved in the regulation of arginine sensitivity of acetylglutamate synthetase, in vivo, and there is a possibility that redox signaling may be one of the possible mechanisms of regulating urea synthesis.

2. Experimental

2.1. Materials

Male dd/Y strain mice, weighing about 20 g, were used. Oxidized glutathione (free acid, grade III) and DL-dithiothreitol were obtained from Sigma (St. Louis, MO, USA) and L-cystine and reduced glutathione were from Kyowa Hakko Kogyo (Tokyo, Japan). 2-Mercaptoethanol and Sephadex G-25 (fine grade) were from Nakarai Chemicals (Kyoto, Japan) and Pharmacia Fine Chemicals (Uppsala, Sweden), respectively. L-[U-^{14}C]Glutamic acid (290 Ci/mol) was obtained from New England Nuclear (Boston, MA, USA).

2.2. Enzyme preparation and enzyme assay

Acetylglutamate synthetase was partially purified to the step of ammonium sulfate fractionation by a modification of the method of Sonoda and Tatibana [19]. The buffer solution (50 mM potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA) was outgassed under vacuum prior to use, and in the case of the purification of a high sensitivity type, the solution contained 0.1 mM dithiothreitol. The enzyme preparation (0.2–0.3 mg protein in a final volume of 50 μl) was incubated with disulfide or thiol compounds in a medium (pH 7.9) containing 40 mM potassium phosphate buffer, 12.5 mM Tris/HCl and 0.08 mM EDTA. Conditions for the purification and the incubation are specified in the legend of each table and figure. Five microliters was removed from the mixture and acetylglutamate synthetase activity was assayed essentially as described [18]. The system contained, unless stated otherwise, 1.0 mM L-[^{14}C]glutamic acid (5 Ci/mol), 0.5 mM acetyl-CoA, 1.0 mM EDTA, 50 mM Tris/HCl (pH 8.2), the enzyme and 1 mM L-arginine, where indicated, in a final volume of 100 μl. Since the concentration of arginine gives a full or maximal activation of the enzyme [19], the extent of arginine activation of the enzyme was determined under these conditions. The incubation was carried out for 10 min at 25°C and acetyl[^{14}C]glutamate formed was isolated by chromatography on Dowex-50 (H^+ form) resin. Protein was determined by the method of Lowry et al. [23] with crystalline bovine serum albumin as a standard.

3. Results and discussion

Acetylglutamate synthetase was partially purified from liver mitochondria of 72-h starved mice and the preparation was only slightly sensitive to arginine activation; the activity in the presence of a saturating concentration of arginine (1 mM) was only 1.2 times higher than the

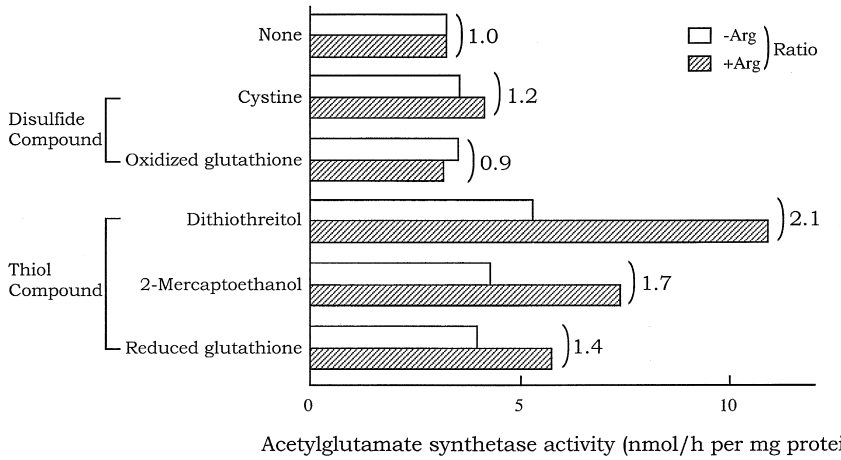

Fig. 1. Changes in the maximal extent of arginine activation of acetylglutamate synthetase with a low arginine sensitivity by treatment with disulfide or thiol compounds. Livers were taken from 72-h starved mice. Acetylglutamate synthetase in the extract of sonicated mitochondria of the liver was purified by two cycles of ammonium sulfate fractionation (0–40% and 0–37%) and desalted on a Sephadex G-25 column. The activities in the presence and absence of arginine were 3.83 and 3.20 nmol h mg protein^{-1}, respectively (arginine activation ratio, 1.2). The preparation was incubated, without (''None'') or with a disulfide compound (cystine, oxidized glutathione) or a thiol compound (dithiothreitol, 2-mercaptoethanol, reduced glutathione), as indicated, at a concentration of 10 mM at 25°C for 15 min. A portion of the mixture was used for the assay of acetylglutamate synthetase activity, as described in Section 2. The activity was measured in the presence (hatched column) and absence (blank column) of 1 mM arginine, giving the value for arginine activation ratio on the right side of each pair of columns.

activity without arginine. The preparation was then incubated with several disulfide or thiol compounds and examined for changes in the activation ratio (Fig. 1). After treatment with a disulfide compound, the activity in the absence of arginine was little changed, while the activity in the presence of arginine slightly increased (cystine) or remained the same (oxidized glutathione), thus not significantly affecting the activation ratio. On the other hand, when the preparation was incubated with a thiol compound, such as dithiothreitol, 2-mercaptoethanol or reduced glutathione, both the activities with and without arginine increased to various degrees. Since the increase in activity with arginine is greater than that without, a value of 1.4–2.1 was obtained for the activation ratio. Thus, the treatment increased the sensitivity of the synthetase having a low activation ratio.

Using an acetylglutamate synthetase preparation with a low sensitivity to arginine activation, effects of varying concentrations of dithiothreitol (0, 5, 10 and 50 mM) on the enzyme activities in the presence and absence of arginine were examined (Fig. 2). Both the activities, with or without arginine, increased with increasing concentrations of dithiothreitol up to 10 mM; the activity without arginine increased slightly and the activity with arginine increased

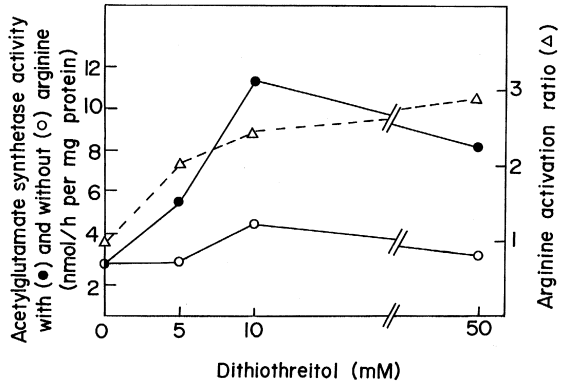

Fig. 2. Effects of treatments with varying concentrations of dithiothreitol on acetylglutamate synthetase with low arginine sensitivity. Acetylglutamate synthetase was purified as in Fig. 1. The preparation was incubated with dithiothreitol at the concentrations indicated at 25°C for 20 min. Assay was performed as in Section 2. The activity was measured in the presence (●) and absence (○) of 1 mM arginine, giving the value for arginine activation ratio (Δ).

Table 1
Kinetic properties of acetylglutamate synthetase preparations with low and high sensitivities to arginine activation[a]

Preparation of enzyme	Extent of arginine activation (fold)	Apparent K_m (mM) for		V_{max} (nmol h mg protein^{-1})	K_a (μM)
		Glutamate	Acetyl-CoA		
(A) Non-treated	1.6	6.3	2.0	45.2	143
(B) Treated with dithiothreitol	2.5	6.3	2.0	47.5	87

[a]Livers were taken from 72–h starved mice. Acetylglutamate synthetase in the extract of sonicated mitochondria of the liver was purified by ammonium sulfate fractionation (0–40%) and dialyzed. The preparation (designated as A) was treated with 20 mM dithiothreitol at 25°C for 20 min to make preparation B. Both preparations A and B were dialyzed and used for enzyme assay. The apparent K_m value for glutamate was determined with a fixed acetyl-CoA concentration of 2.5 mM and the K_m value for acetyl-CoA and V_{max} value were determined with a glutamate concentration fixed at 10 mM. These values were determined in the absence of arginine. Extent of arginine (1 mM) activation and arginine concentration that gave a half-maximal activation (K_a) were determined with 1.0 mM glutamate and 0.5 mM acetyl-CoA.

to a higher extent. In consequence, the arginine activation ratio increased to 2–3. Of the thiol compounds examined, dithiothreitol was most effective in elevating the activation ratio.

The kinetic properties of the synthetase preparation with a low sensitivity (activation ratio, 1.6) and after treatment with dithiothreitol (activation ratio, 2.5) were examined (Table 1). There was no difference between the K_m values both for glutamate and for acetyl-CoA of the two preparations. V_{max} value of the synthetase with a low sensitivity was slightly lower than that of the enzyme with a high sensitivity. The arginine concentration that gave a half-maximal activation (K_a) of the synthetase with a low sensitivity was higher than that of the enzyme with a high sensitivity. The finding suggests that the change in enzyme activity in response to treatment of the synthetase with thiol or disulfide compounds is accompanied by change in the binding of an allosteric effector, arginine.

Acetylglutamate synthetase, partially purified from liver mitochondria of fed mice and having a relatively high extent of arginine activation of 2.7, was incubated with several disulfide or thiol compounds and changes in sensitivity to activation by arginine were examined (Fig. 3). With disulfide compounds, the activity in the

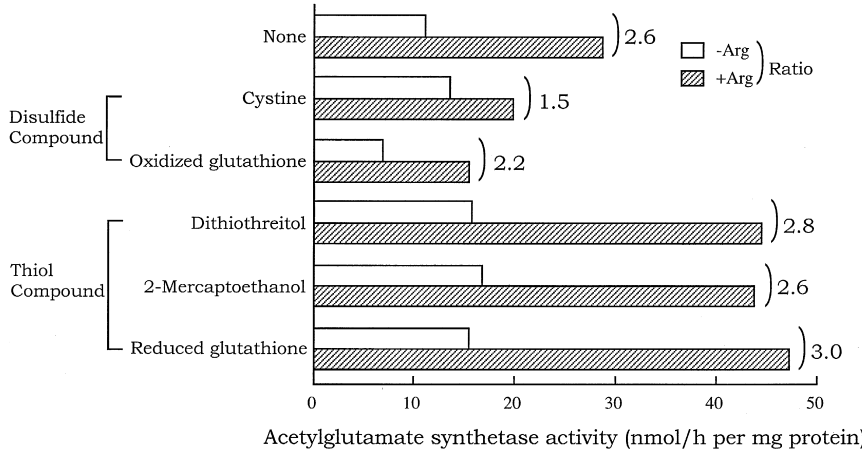

Fig. 3. Changes in the maximal extent of arginine activation of acetylglutamate synthetase with high arginine sensitivity by treatment with disulfide or thiol compounds. Mice were allowed only 3 h access to food (normal laboratory chow) for consecutive 3 days. On day 4, they were fed for 3 h, and killed at 8 h after the start of feeding. Acetylglutamate synthetase was purified as in Fig. 1. The activities in the presence and absence of arginine were 38.4 and 14.2 nmol h mg protein^{-1}, respectively (arginine activation ratio, 2.7). The preparation was incubated and assayed as described in Fig. 1.

absence of arginine slightly increased (cystine) or decreased (oxidized glutathione), while the activity in the presence of arginine decreased markedly. Consequently, the extent of arginine activation decreased to 1.5–2.2. On the other hand, treatment with thiol compounds, including dithiothreitol, 2-mercaptoethanol and reduced glutathione, moderately increased both activities with and without arginine. After the treatment, the activation ratio was slightly increased.

To examine the reversibility of altered arginine sensitivity, the synthetase was incubated with 20 mM oxidized glutathione for 15 min, dialyzed and then incubated with 20 mM reduced glutathione for an additional 15 min. Initial activities of the preparation, with and without arginine, were 19.5 and 11.1 nmol h mg protein^{-1}, respectively (arginine activation ratio, 1.8). After treatment with oxidized glutathione, the respective activities were 13.7 and 11.6 nmol h mg protein^{-1} (arginine activation ratio, 1.2), and after the treatment with reduced glutathione, the respective activities changed to 20.8 and 10.6 nmol h mg protein^{-1} (arginine activation ratio, 2.0). Thus, when the synthetase desensitized by oxidized glutathione was incubated with reduced glutathione, the decreased sensitivity was restored. These results show that diminution of arginine sensitivity in this case is a reversible process. Enhancement of arginine sensitivity of the low-sensitivity enzyme by reduced glutathione was also reversible (data not shown). The reversibility observed may favor the view that the thiol/disulfide interchange is involved in the regulation of arginine sensitivity of acetylglutamate synthetase in vivo. Oxidation–reduction-dependent reactions have proven to be important in regulating numerous physiological and pathophysiological functions of cells and tissues, and it is well known that activities of many enzymes are regulated by thiol/disulfide interchange in the living cell [24]. The presence of a mitochondrial pool of glutathione has been noted [25] and protein thiol oxidation and reduction is thought to occur in mitochondria [26]. Thus, it is likely that the thiol/disulfide interchange plays a role, at least to some extent, in the regulation of arginine sensitivity of acetylglutamate synthetase in mitochondria.

The sulfhydryl groups of proteins can be modified by the disulfide compounds and alkylating reagents. The disulfide compound (RS–SR), such as cystine or oxidized glutathione, is thought to react with the sulfhydryl group(s) of the enzyme molecule (Ⓔ-SH, Ⓔ$\overset{SH}{SH}$) to generate the mixed disulfide (Ⓔ-SH + RS-SR ➡Ⓔ-S-SR) or intramolecular disulfide (Ⓔ$\overset{SH}{SH}$ + RS-SR➡Ⓔ$\overset{S}{S}$) To test whether the disulfide formation or loss of the free sulfhydryl group is required for the diminution of arginine sensitivity of the enzyme, the alkylating reagents (R), which could modify sulfhydryl groups irreversibly (Ⓔ-SH + R ➡Ⓔ-SR) were used (Fig. 4). When the enzyme preparation with a high arginine sensitivity was treated with iodoacetamide (Fig. 4A) or *N*-ethylmaleimide (Fig. 4B) at various concentrations, arginine sensitivity decreased remarkably. Alkylation of the sulfhydryl group of the enzyme irreversibly resulted in the diminution of arginine sensitivity, suggesting that loss of the free sulfhydryl group, not disulfide formation, should be important. When the

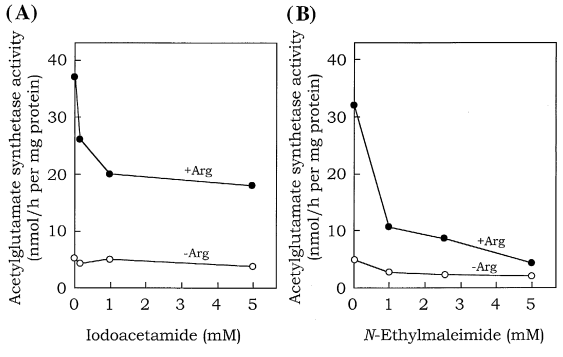

Fig. 4. Effects of treatments with alkylating reagents on acetylglutamate synthetase with high arginine sensitivity. Acetylglutamate synthetase was purified as in Fig. 1. The preparation was incubated with monoiodoacetamide (A) or *N*-ethylmaleimide (B) at the concentrations indicated at 25°C for 20 min. Assay was preformed as in Section 2. The activity was measured in the presence (●) and absence (○) of 1 mM arginine.

enzyme preparation with the diminished arginine sensitivity after alkylation was incubated with the sulfhydryl reagent in excess, enhancement of arginine sensitivity was not observed. These results suggested that the sulfhydryl reagents enhanced the arginine sensitivity of the enzyme by reducing the sulfhydryl group of the enzyme.

Based on the data of estimates of total cellular concentrations of thiol and disulfide compounds in rat liver under conditions of feeding and fasting, it is suggested that the fed state is more reduced than the fasted state [24,27]. Several enzymes, such as enzymes of glycogen synthesis, glycolysis and fatty acid synthesis, which are active in the fed state, were reported to lose activity on oxidation [24]. On the other hand, some enzymes of glycogen degradation and gluconeogenesis, which function in the fasted state, were activated by oxidation [24, 28]. The response of these enzyme activities to oxidation by the thiol/disulfide interchange is consistent with the metabolic functions of the enzymes. Furthermore, fructose 1,6-bisphosphatase was isolated from the rat liver in two forms, separately, an oxidized and, the other, a reduced form [29,30]. If the fed state is more reduced than the fasted state in mitochondria of mouse liver, as is the case in the whole liver of rats, then changes in arginine sensitivity of acetylglutamate synthetase in vivo, in response to starvation and feeding of mice, may be caused or facilitated by oxidation and reduction states. Further work needs to be done to quantitate and compare the actual intramitochondrial oxidation/reduction levels of fasted and fed mice.

The synthetase with a low and a high arginine sensitivity was obtained from liver mitochondria of starved and fed mouse, respectively [21]. In the present study, we found that the two forms of the enzyme are distinguishable, in oxidation and reduction states. It remains to be determined if other post-translational modifications or some other factors are involved in the change of arginine sensitivity and whether oxidation and reduction of acetylglutamate synthetase take place nonenzymatically (depending upon environmental thiol and disulfide concentrations) or enzymatically (by the catalysis of an enzyme system such as thiol:protein disulfide oxidoreductase [31]). Since interconversion of the two forms of the synthetase requires a relatively high concentration of thiol and disulfide compounds, it may proceed enzymatically. The possibility has not yet been excluded that the arginine sensitivity of the acetylglutamate synthetase was affected, either directly or indirectly, by contaminated protein(s) in our enzyme preparation. Acetylglutamate synthetase from rat liver has been purified to an apparent homogeneity [19]; nevertheless, amounts of the purified preparation sufficient for studies of the abovementioned factors have yet to be prepared. The future molecular cloning of the enzyme and the expression of its recombinant protein will be helpful in elucidating that point.

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