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Short communication

Chemical Modification of Brain Glutamate Dehydrogenase Isoproteins with Phenylglyoxal

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Incubation of two types of glutamate dehydrogenase isoproteins from bovine brain with the arginine-specific dicarbonyl reagent phenylglyoxal resulted in a biphasic loss of enzyme activity. Reaction of the glutamate dehydrogenase isoproteins with phenylglyoxal caused a rapid loss of 53~62% of the enzyme activities and modification of two residues of arginine per enzyme subunit. Prolonged incubation of the glutamate dehydrogenase isoproteins with phenylglyoxal resulted in the modification of an additional four residues of arginine per enzyme subunit without further loss of the residual activities. Partial protection against inactivation was provided by the coenzyme NADH or substrate 2-oxoglutarate. The most marked decrease in the rate of inactivation was observed by the combined addition of NADH and 2-oxoglutarate, suggesting that the first two modified arginine residues are in the vicinity of the catalytic site. However, inactivation of the glutamate dehydrogenase isoproteins by phenylglyoxal appears to be partial with approximately 40% activity remained after an extended reaction time with excess reagent, suggesting that the modified arginine residues may not be directly involved in catalysis. The lack of complete protection by substrates also suggest the possibility that the modified arginine residues are not directly involved at the active site, and the partial loss of activity by the modification of arginine residues may be due to a conformational change. There were no significant differences between the two glutamate dehydrogenase isoproteins in sensitivities to inactivation by phenylglyoxal, indicating that the microenvironmental structures of the glutamate dehydrogenase isoproteins are very similar to each other.

Keywords: Glutamate dehydrogenase, Phenylglyoxal, Reactive arginine.

Introduction

Glutamate dehydrogenase is a family of enzymes which catalyze the reversible deamination of L-glutamate to 2-oxoglutarate using NAD^+, $NADP^+$, or both, as coenzymes (Fisher, 1985). Since the pathology of the disorders associated with glutamate dehydrogenase defects is restricted to the brain, the enzyme may be of particular importance in the biology of the nervous system. The importance of the pathophysiological nature of the glutamate dehydrogenase-deficient neurological disorders has attracted considerable interest (McGeer and McGeer, 1976; Plaitakis *et al.*, 1982). Hussain *et al.* (1989) detected four different forms of glutamate dehydrogenase isoproteins from the human cerebellum, and the enzyme isolated from one of the patients with a variant form of multisystem atrophy displayed a marked reduction of one of the glutamate dehydrogenase isoproteins. Although the origin of the glutamate dehydrogenase polymorphism is not known, it has been reported that four differently sized mRNAs and multiple gene copies for glutamate dehydrogenase occur in humans (Plaitakis *et al.*, 1993; Shashidharan *et al.*, 1994). It is not known whether the distinct properties of the glutamate dehydrogenase isoproteins are essential for the regulation of glutamate metabolism.

It was only in recent years that the three-dimensional structure of glutamate dehydrogenase from microorganisms became available (Baker *et al.*, 1992; Yip *et al.*, 1995). Recently, crystallization of mammalian

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glutamate dehydrogenase was also reported (Peterson *et al.*, 1997). However, little is known about the detailed structure of mammalian glutamate dehydrogenase. We have isolated and characterized two types of glutamate dehydrogenase isoproteins (designated glutamate dehydrogenase I and glutamate dehydrogenase II) from bovine brain (Cho *et al.*, 1995; 1999; Cho and Lee, 1996; Lee *et al.*, 1997) and have reported the regulatory properties of the glutamate dehydrogenase isoproteins (Cho *et al.*, 1996; 1998a; 1998b; 1999; Kim *et al.*, 1997; Ahn *et al.*, 1999; Cho and Yoon, 1999). Our work led to the finding that glutamate dehydrogenases are present in bovine brain in "heat-labile" (glutamate dehydrogenase I) and "heat-stable" (glutamate dehydrogenase II) forms (Cho *et al.*, 1995). It has been reported that nerve tissue-specific human glutamate dehydrogenase is thermolabile and highly regulated by ADP (Shashidharan *et al.*, 1997). Similar results were reported, showing that reduction in glutamate dehydrogenase activity in patients with neurodegenerative disorders was largely limited to the heat-labile form (Plaitakis *et al.*, 1984; Abe *et al.*, 1992). Recently, Stanley *et al.* (1998) have reported that the hyperinsulinism-hyperammonemia syndrome is caused by mutations in the glutamate dehydrogenase gene that affects enzyme sensitivity to GTP-induced inhibition. The mutations identified in the patients with hyperinsulinism and hyperammonemia (Stanley *et al.*, 1998) laid exactly within the peptide of amino acids that we had previously suggested to be a part of the GTP binding site of the brain glutamate dehydrogenase isoproteins (Cho *et al.*, 1996). To our knowledge, however, comparisons of the detailed structure and function of glutamate dehydrogenase isoproteins have been rarely reported.

In this paper, we describe the modification of glutamate dehydrogenase isoproteins by the arginine-specific dicarbonyl reagent phenylglyoxal, with the view that arginine residues might play a general role in the binding of coenzyme throughout the family of pyridine nucleotide-dependent dehydrogenases (Yang and Schwert, 1972; Foster and Harrison, 1974; Lange *et al.*, 1974).

Materials and Methods

Materials NADH, 2-oxoglutarate, glutamate, ADP, and phenylglyoxal were purchased from Sigma Chemical Co. (St. Louis, USA) Bovine brains were obtained from Majang Slaughterhouse, Seoul, Korea. Two types of glutamate dehydrogenase isoproteins were purified from bovine brain by the method developed at our laboratory (Cho *et al.*, 1995) and were homogeneous as judged by Coomassie-stained gradient SDS-polyacrylamide gel electrophoresis. All other chemicals and solvents were of reagent grade or better.

Enzyme assay Glutamate dehydrogenase activity was measured spectrophotometrically in the direction of glutamate oxidation by following the increase in absorbance at 340 nm, as described before (Cho *et al.*, 1995). Glutamate dehydrogenase concentrations were adjusted to give a measured rate of about 0.04 absorbance units per min. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 μmol of NADH per min at 25°C. The protein content was determined as described elsewhere (Bradford, 1976).

Modification of arginine residues with phenylglyoxal Phenylglyoxal was prepared by dissolving the solid in a minimum volume of methanol and then adding water to give a 20 mM solution. The concentration of phenylglyoxal was determined from the absorbance in methanol (ε_{247nm} = 11,300 $M^{-1}cm^{-1}$) as described elsewhere (Kohlbrenner and Cross, 1978). The extent of arginine modification by phenylglyoxal in phosphate buffer was determined from the difference at 247 nm using the estimated molar extinction coefficient for the diphenylglyoxal adduct of 11,300 $M^{-1}cm^{-1}$. Enzymes in 50 mM sodium phosphate, pH 7.4, containing 1 mM EDTA and 1 mM dithiothreitol, were incubated for 1 h at 25°C with 0.1~2.0 mM phenylglyoxal. Reaction mixtures were dialyzed for 24 h at 4°C against 50 mM sodium phosphate buffer, pH 7.4. Solutions were assayed for enzyme activity and protein. Controls without phenylglyoxal were included and the remaining activities were expressed as percentage of each control activity. For protection experiments, the enzymes were preincubated with varying concentrations of 2-oxoglutarate or NADH prior to the addition of phenylglyoxal. Aliquots were withdrawn from the mixture to determine the remaining activity.

Stoichiometric studies For stoichiometric studies, the enzymes were treated with phenylglyoxal as described in the figure legend (Fig. 3). At different time intervals, the increase in absorbance at 247 nm, which is characteristic of a diphenylglyoxal adduct (Kohlbrenner and Cross, 1978), was recorded along with simultaneous measurement of the loss of enzyme activities.

Results and Discussion

The common feature of the enzymes for which arginine is essential seems to be the catalysis of a reaction involving a negatively-charged substrate or coenzyme. There are several instances where the modification of guanidinium groups of arginine residues has been shown to play an essential role in the action of certain pyridine nucleotide-dependent dehydrogenases (Yang and Schwert, 1972; Foster and Harrison, 1974; Lange *et al.*, 1974). Glutamate dehydrogenase not only has negatively-charged substrates and coenzymes, but is also regulated by negatively-charged purine nucleotides which bind at distinct allosteric sites. Therefore, it has several regions that might be postulated to include arginine.

Purified glutamate dehydrogenase isoproteins from bovine brain were inactivated by the arginine-specific dicarbonyl reagent phenylglyoxal at 25°C. The time course of inactivation is shown in Figs. 1A and 1B for glutamate dehydrogenase I and glutamate dehydrogenase II, respectively. After 20 min of incubation with 0.5 mM phenylglyoxal, about 62% and 53% of the original activities of the glutamate dehydrogenase I and glutamate dehydrogenase II, respectively, were lost. Protection

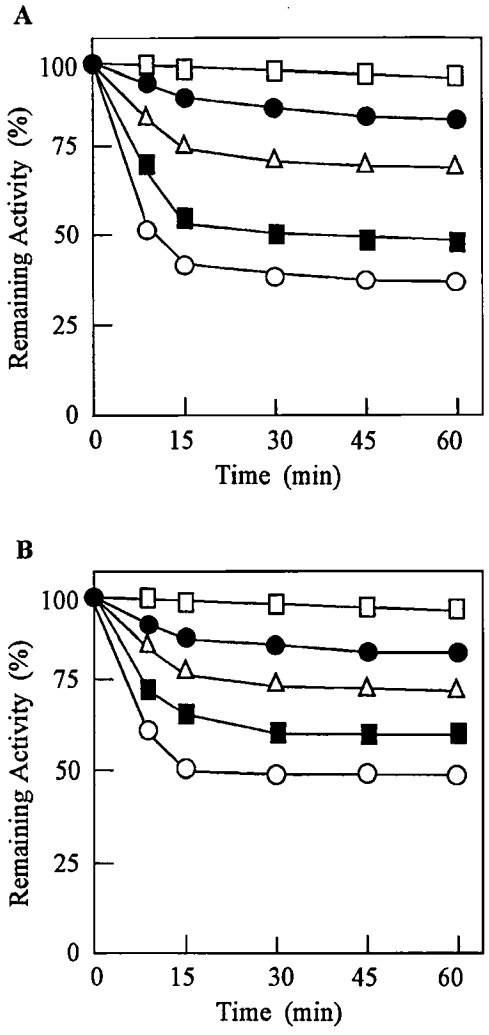

Fig. 1. Protection of 2-oxoglutarate and NADH against phenylglyoxal-induced inactivation. Glutamate dehydrogenase I (**A**) and glutamate dehydrogenase II (**B**) were treated with phenylglyoxal in the presence and absence of 2-oxoglutarate or NADH in 50 mM sodium phosphate, pH 7.4, at 25°C. At indicated times, the remaining activities were assayed by the addition of the standard assay mixture. This diluted the concentrations of phenylglyoxal, 2-oxoglutarate, and NADH to 0.5 mM, 10 mM, and 1 mM, respectively, during the assay. □ (glutamate dehydrogenase only); ○ (glutamate dehydrogenase + phenylglyoxal); ■ (glutamate dehydrogenase + 2-oxoglutarate + phenylglyoxal); △ (glutamate dehydrogenase + NADH + phenylglyoxal); ● (glutamate dehydrogenase + NADH + 2-oxoglutarate + phenylglyoxal).

experiments were performed to locate the site of phenylglyoxal reaction in glutamate dehydrogenase. We studied the effect of 2-oxoglutarate and NADH on inactivation by phenylglyoxal. The inactivation was partially prevented by preincubation of the glutamate dehydrogenase isoproteins with NADH (Fig. 1). This result indicates that the glutamate dehydrogenase isoproteins saturated with NADH are still accessible to attack by phenylglyoxal. 2-oxoglutarate also gave a partial protection against the inactivation caused by phenylglyoxal, but to a lesser extent than NADH (Fig. 1). Thus, it appears that 2-oxoglutarate, as well as NADH, may bind to the enzyme in the presence of phenylglyoxal. The most marked decrease in the rate of inactivation was observed by the combined addition of NADH (1 mM) and 2-oxoglutarate (10 mM) as shown in Fig. 1, suggesting that the modified arginine residues are near to the catalytic site. There were no significant differences between the two glutamate dehydrogenase isoproteins in the sensitivities against inactivation by phenylglyoxal, indicating that the microenvironmental structures of the glutamate dehydrogenase isoproteins are very similar to each other.

Considering that the reaction between phenylglyoxal and arginine is particularly reversible at neutral pH (Bond *et al.*, 1980), conditions which promote the reversal of phenylglyoxal from treated enzyme can be anticipated to restore enzyme activity. As shown in Fig. 2, the addition of hydroxylamine or arginine to enzyme which had been inactivated to the extent of 60% by phenylglyoxal, resulted in a substantial restoration of activity 30 min after the the addition of these reagents. This reversible interaction of phenylglyoxal with proteins is responsible for the difficulty in locating phenylglyoxal modified arginine residues in isolated peptides from glutamate dehydrogenase isoproteins.

Incubation of two types of glutamate dehydrogenase isoproteins with phenylglyoxal resulted in a biphasic modification of arginine residues. The extent of modification of glutamate dehydrogenase isoproteins by phenylglyoxal was quantitated from its absorbance at 247 nm, which is characteristic of a diphenylglyoxal adduct. When the extent of modification determined by the spectrophotometric titration method was plotted versus the percentage activity remaining, a rapid loss of 54~59% of the enzyme activities and modification of two residues of arginine per enzyme subunit was observed (Fig. 3). Prolonged incubation of the glutamate dehydrogenase isoproteins with phenylglyoxal resulted in the modification of an additional four residues of arginine per enzyme subunit without further loss of the residual activities, suggesting that the first two modified arginine residues are in the region of the catalytic site (Fig. 3). Therefore, it is suggested that phenylglyoxal produces two distinguishable modifications on glutamate dehydrogenase isoproteins: a relatively specific modification at or near the active center and a distinct modification of the other arginine residues which are not directly involved in catalysis. However, phenylglyoxal appears to partially inactivate glutamate dehydrogenase isoproteins as only about 40% of activity remained even after extended incubation times in the presence of excess amount of reagent (data not shown), suggesting that the modified arginine residues are not directly involved in catalysis. The incomplete protection by substrates (Fig. 1) also suggested the possibility that the modified arginine residues were not directly involved at

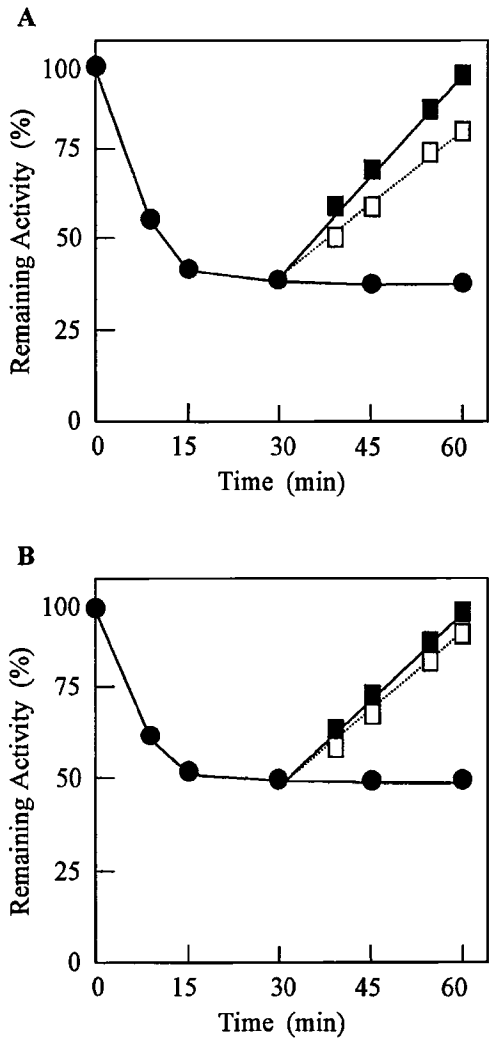

Fig. 2. Reactivation of phenylglyoxal-treated glutamate dehydrogenase isoproteins. Glutamate dehydrogenase I (**A**) and glutamate dehydrogenase II (**B**) were treated with phenylglyoxal in 50 mM sodium phosphate, pH 7.4, at 25°C. After 30 min, hydroxylamine (■) or arginine (□) were added to aliquots of unmodified and phenylglyoxal treated enzymes to a final concentration of 0.1 M. In each case, activities are expressed relative to those of unmodified enzymes.

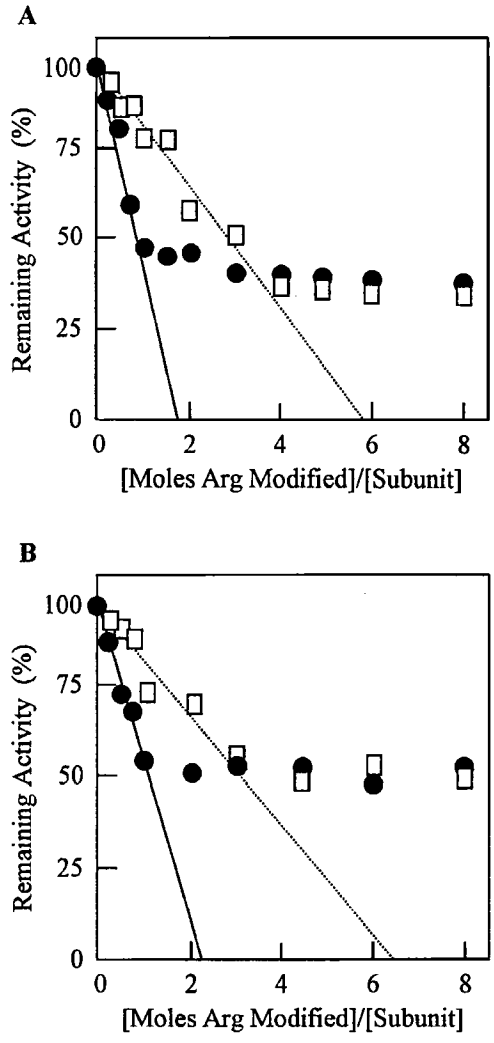

Fig. 3. Effects of the extent of modification of glutamate dehydrogenase isoproteins by phenylglyoxal on activity. Glutamate dehydrogenase I (**A**) and glutamate dehydrogenase II (**B**) were treated with 0.5 mM phenylglyoxal as described in Materials and Methods for 20 min (●) and for 60 min (□). The modification of arginine residues by phenylglyoxal was determined from the change in absorbance at 247 nm using ε_{247nm} = 11,300 $M^{-1}cm^{-1}$.

the active site, but rather that the protection was due to an environmental conformational change.

As many proteins have functions distinct from those for which they were originally identified, other roles of glutamate dehydrogenase isoproteins have been reported. For instance, a membrane-bound form of glutamate dehydrogenase possesses a microtubule-binding activity (Rajas *et al.*, 1996) and glutamate dehydrogenase reacts as an RNA-binding protein and shows a possible role in regulation of transcription (Preiss *et al.*, 1993; McDaniel, 1995; Bringaud *et al.*, 1997). Recently, Cavallaro *et al.* (1997) identified glutamate dehydrogenase as one of the late memory-related genes in the hippocampus. It would appear that we have just begun to unravel the mystery of glutamate dehydrogenase isoproteins and their role in neurobiology. It is, therefore, essential to have a detailed structural and functional description of the various types of brain glutamate dehydrogenases to elucidate the physiological nature of the glutamate dehydrogenase-deficient disorders.

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