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Brain Succinic Semialdehyde Dehydrogenase; Reaction of Arginine Residues Connected with Catalytic Activities

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The succinic semialdehyde dehydrogenase from bovine brain was inactivated by treatment with phenylglyoxal, a reagent that specifically modifies arginine residues. The inhibition at various phenylglyoxal concentrations shows pseudo-first-order kinetics with an apparent second-order rate constant of 30 $M^{-1}min^{-1}$ for inactivation. Partial protection against inactivation was provided by the coenzyme NAD^+, but not by the substrate succinic semialdehyde. Spectrophotometric studies indicated that complete inactivation of the enzyme resulted from the binding of 2 mol phenylglyoxal per mol of enzyme. These results suggest that essential arginine residues, located at or near the coenzyme-binding site, are connected with the catalytic activity of brain succinic semialdehyde dehydrogenase.

Keywords: Arginine residue, Phenylglyoxal, Succinic semialdehyde dehydrogenase.

Introduction

GABA (γ-aminobutyric acid) is produced from glutamate in a reaction catalyzed by glutamate decarboxylase (EC 4.1.1.15) and further metabolized to succinate by the successive action of GABA transaminase (EC 2.6.1.19) and succinic semialdehyde dehydrogenase (EC 1.2.1.24). The carbon skeletal of GABA thus enters the tricarboxylic acid cycle in the form of succinate. GABA metabolism has been well characterized in the mammalian central nervous system where GABA functions as a major inhibitory neurotransmitter. Succinic semialdehyde dehydrogenase (SSADH), the final enzyme in GABA metabolism, has been purified from the mammalian species (Blaner and Churchich, 1980; Ryzlak and Pietruszko, 1988; Chambliss and Gibson, 1992; Lee *et al.*, 1995) and microorganisms (Donnelly and Cooper, 1981; Sanchez *et al.*, 1989; Koh *et al.*, 1994). Succinic semialdehyde dehydrogenase is also the site of an inborn error of human metabolism (Jakobs *et al.*, 1993). In autosomal recessively inherited succinic semialdehyde dehydrogenase deficiency, now identified in more than 45 patients who manifest varying degrees of psychomotor retardation with speech delay, the normal oxidative pathway is blocked, thereby resulting in the accumulation of succinic semialdehyde (Gibson *et al.*, 1997). Metabolite patterns in physiologic fluids derived from patients show a large increase in γ-hydroxybutyrate, the reduction product of succinic semialdehyde by succinic semialdehyde reductase (Cho *et al.*, 1993). γ-Hydroxybutyrate, the biochemical hallmark of succinic semialdehyde dehydrogenase deficiency, produces central nervous system effects that includes altered motor activity and behavior disturbances when administered to animals and humans at pharmacological levels (Snead, 1978). Recently, the cDNA clones of rat brain and human liver SSADH were isolated (Chambliss *et al.*, 1995) and chromosomal localization was identified (Trettel *et al.*, 1997).

In view of the importance of succinic semialdehyde dehydrogenase in the metabolism of GABA, we investigated the functional roles of arginine residues in succinic semialdehyde dehydrogenase by using chemical modification with the arginine specific dicarbonyl reagent phenylglyoxal.

Materials and Methods

Materials NAD^+, succinic semialdehyde, phenylglyoxal, EDTA, bovine serum albumin, hydroxylamine, and β-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, USA). CM-Sepharose, Blue-Sepharose, 5'-AMP-Sepharose was obtained from Phamacia (Milwaukee, USA). Bovine brains were obtained

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from Majang-dong slaughterhouse (Seoul, Korea). All other materials were the purest commercial grade available.

Enzyme purification and assay Succinic semialdehyde dehydrogenase from bovine brain was purified by the method developed in our laboratory (Lee *et al.*, 1995). The method involves four column chromatographic steps: CM-Sepharose, Blue-Sepharose, hydroxyapatite and 5'-AMP-Sepharose. The formation of NADH was measured by the increase in absorbance at 340 nm. All assays were performed in duplicate and the initial velocity data was correlated with a standard assay mixture containing 30 μM succinic semialdehyde and 5 mM NAD^+ in 0.01 M potassium phosphate (pH 7.0) at 25°C.

Spectroscopy Spectrophotometric measurements were carried out using a Kontron UVIKON 930 double beam spectrophotometer.

Modification of succinic semialdehyde dehydrogenase with phenylglyoxal Phenylglyoxal was prepared by dissolving the solid in a minimum volume of methanol and adding water to give a 40 mM solution. The concentration of phenylglyoxal was determined from the absorbance in methanol ($\varepsilon_{247\,nm}$= 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 by using the molar extinction coefficient for the diphenylglyoxal adduct of 11,300 $M^{-1}cm^{-1}$ at 247 nm (Takahashi, 1977). Enzymes in 10 mM potassium phosphate, pH 7.0, containing 0.1 mM EDTA and 1 mM β-mercaptoethanol were incubated for 40 min at 25°C with 0.25-1.5 mM phenylglyoxal. Reaction mixtures were dialyzed for 24 h at 4°C against 10 mM potassium phosphate, pH 7.0. Solutions were assayed for enzyme activity, protein concentration and absorbance at 247 nm. Controls without phenylglyoxal were included and the remaining activities were expressed as a percentage of each control activity. Protection experiments were performed in a similar manner except that the enzyme was preincubated with a substrate or coenzyme for 20 min before addition of phenylglyoxal. In reactivation experiments the inactivated succinic semialdehyde dehydrogenase was treated with neutralized 0.1 M hydroxylamine, which on dilution during assay had no effect on the activity of the control enzyme.

Stoichiometric studies For stoichiometric studies, the enzymes were treated with 1.5 mM phenylglyoxal. 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 the simultaneous measurement of the loss of enzyme activities.

Results and Discussion

Phenylglyoxal has been shown to act as a specific reagent for arginine residues. The common feature of the enzyme 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 a guanidinium group of arginine residues have been shown to play an essential role in the action of certain pyridine nucleotide-dependent dehydrogenase (Foster and Harrison, 1974; Lange *et al.*, 1974; Jörnvall *et al.*, 1977).

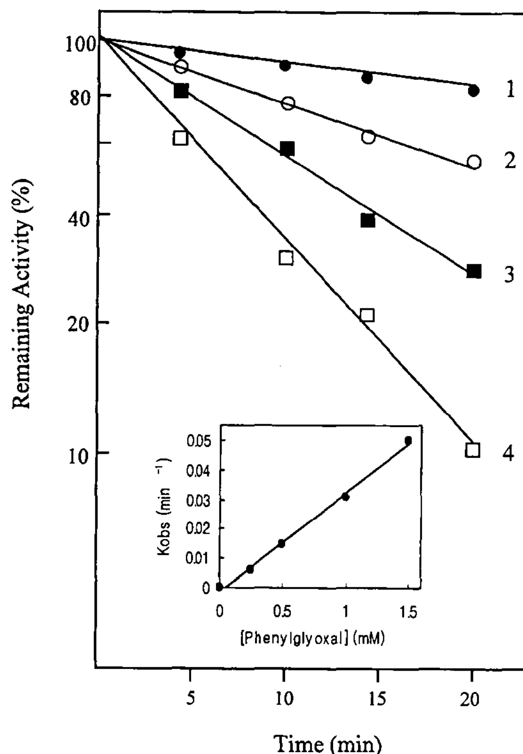

Fig. 1. Effect of phenylglyoxal concentration on the rate of inactivation of succinic semialdehyde dehydrogenase. The enzyme (3 μM) was incubated with 0.25 mM (1), 0.5 mM (2), 1 mM (3) and 1.5 mM (4) phenylglyoxal in 0.1 M potassium phosphate, pH 7.0, at 37°C. Inset, replot of pseudo-first order rate constants versus the concentrations of phenylglyoxal. Aliquots withdrawn from the incubation mixtures were tested for enzymatic activity.

Purified succinic semialdehyde dehydrogenase from bovine brain was inactivated by the arginine-specific dicarbonyl reagent phenylglyoxal with a time- and dose-dependent manner (Fig. 1). The linear plots of the logarithm of residual enzyme activity *versus* the reaction time indicate that the time-dependent decrease in activity displayed pseudo-first-order kinetics. The inactivation followed pseudo-first-order kinetics with a concentration of phenylglyoxal in the range 0.25-1.5 mM. The pseudo-first-order rate constants obtained at each phenylglyoxal concentration are replotted as a function of phenylglyoxal concentration. (Fig. 1, inset). The second-order rate constant for the inactivation of the enzyme by phenylglyoxal was 30 $M^{-1}min^{-1}$ as determined from the slope of this plot. The plot of the reciprocal first-order rate constants vs. the reciprocal concentration of phenylglyoxal gives a dissociation constant for phenylglyoxal (K_d= 8.3 mM) as shown in Fig. 2. These results indicate that the inactivation of succinic semialdehyde dehydrogenase by phenylglyoxal appears to follow initial reversible binding of phenylglyoxal to succinic semialdehyde dehydrogenase since inactivation by

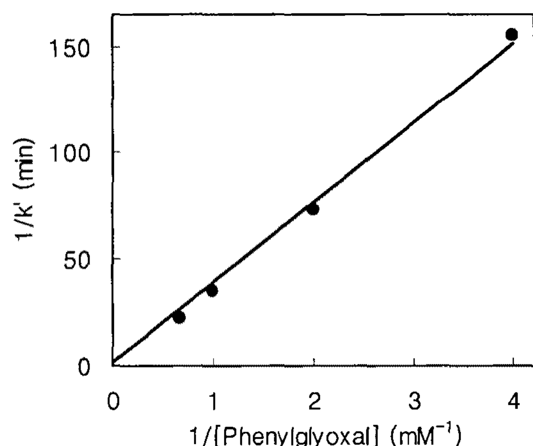

Fig. 2. Reciprocal first-order rate constants (k') calculated from Fig. 1 are plotted versus the reciprocal concentrations of phenylglyoxal. The dissociation constant for phenylglyoxal calculated from the intercept on the abscissa ($1/K_d$) is 8.3 mM.

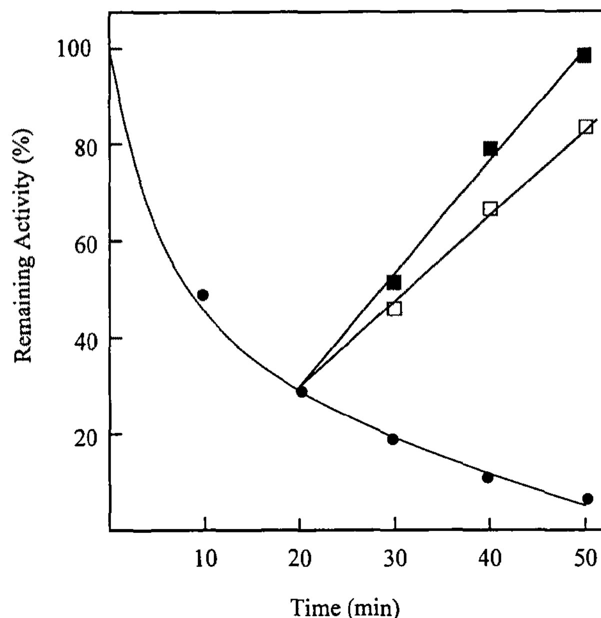

Fig. 3. Reactivation of phenylglyoxal treated succinic semialdehyde dehydrogenase. The enzyme was inactivated with 1.5 mM phenylglyoxal at 37°C (closed circle). After 20 min, hydroxylamine (closed square) or arginine (open Square) were added to aliquots of unmodified and phenylglyoxal treated enzyme to a final concentration of 0.1 M. In each case, activities are expressed relative to those of unmodified enzymes.

phenylglyoxal shows saturation kinetics.

The nature of the inhibitory effect exerted by phenylglyoxal was studied in detail. The possibility that phenylglyoxal inhibition is the result of the reaction of essential arginine residues critically connected with catalysis was investigated by performing inhibition studies in the presence and absence of succinic semialdehyde or NAD^+. The reaction of succinic semialdehyde dehydrogenase with phenylglyoxal was partially prevented by preincubation of the enzyme with the coenzyme NAD^+, but not by the succinic semialdehyde (Table 1). These results indicate that the inactivation of succinic semialdehyde dehydrogenase by phenylglyoxal resulted from the modification of amino acid residues that might be located at or close to the NAD^+ binding site. It could not, however, exclude the possibility that the modified residues are in the region whose conformation is altered upon the inactivation of the enzyme with its coenzyme and inhibitor.

A specific test for modification of essential arginine residue(s) is the regeneration of activity in the presence of hydroxylamine (Patthy *et al.*, 1975). In view of the finding that the reaction of phenylglyoxal with model arginine compounds is particularly reversible at neutral pH (Bond and Chiu, 1980), conditions that promote the removal of phenylglyoxal from treated enzyme can be anticipated in order to restore enzyme activity. As shown in Fig. 3, the addition of hydroxylamine to enzyme, which had been inactivated to 70% extent by phenylglyoxal, resulted in a substantial restoration of activity 30 min after 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 succinic semialdehyde dehydrogenase.

To establish the stoichiometry of phenylglyoxal to arginine as the number of arginine residues that are modified during the process of inactivation, a Spectrophotometric analysis was carried out. The extent of modification of succinic semialdehyde dehydrogenase by phenylglyoxal was quantitated from its absorbance at 247 nm, which is characteristic of a diphenylglyoxal adduct (Fig. 4). Extrapolation of the line from the data shows that two arginine residues should be modified for total inactivation, suggesting that two arginine residues located at or near the coenzyme binding site are important for succinic semialdehyde dehydrogenase activity.

The localization of phenylglyoxal-modified arginine residues still needs to be identified. Since there is no X-ray crystallographic structural data, it would be difficult to discuss modified residues that are located in the active site or in the

Table 1. Inactivation of succinic semialdehyde dehydrogenase by phenylglyoxal

Reaction mixture	Remaining activity (%)
Enzyme (3 μM)	100
Enzyme (3 μM) + phenylglyoxal (1.5 mM)	10
Enzyme (3 μM) + NAD^+ (3 mM) + phenylglyoxal (1.5 mM)	70
Enzyme (3 μM) + succinic semialdehyde (3 mM) + phenylglyoxal (1.5 mM)	13

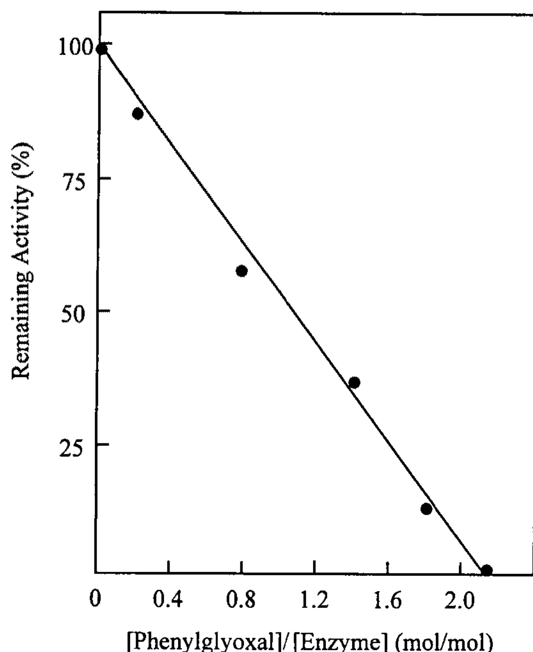

Fig. 4. Effects of the extent of modification of succinic semialdehyde dehydrogenase by phenylglyoxal. Succinic semialdehyde dehydrogenase was treated with 1.5 mM phenylglyoxal as described in Materials and Methods for 40 min. The modification of arginine residues by phenylglyoxal was determined from the change in absorbance at 247 nm using $\varepsilon_{247\,nm} = 11{,}300\ M^{-1}cm^{-1}$.

region whose conformation is altered upon the interaction of the enzyme with its coenzyme and inhibitor. Studies on the multiple sites mutants using site-directed mutagenesis will be necessary in order to prove the rôles of conserved arginine residues for binding catalysis.

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