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Chemical Modification of Bovine Brain Succinic Semialdehyde Reductase by Diethylpyrocarbonate

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The NADPH-dependent succinic semialdehyde reductase is one of the key enzymes in the brain GABA shunt, and it catalyzes the formation of the neuromodulator γ-hydroxybutyrate from succinic semialdehyde. This enzyme was inactivated by diethylpyrocarbonate (DEP) with the second-order rate constant of 1.1×10^3 $M^{-1}min^{-1}$ at pH 7.0, 25°C, showing a concomitant increase in absorbance at 242 nm due to the formation of N-carbethoxyhistidyl derivatives. Complete inactivation of succinic semialdehyde reductase required the modification of five histidyl residues per molecule of enzyme. However, only one residue was calculated to be essential for enzyme activity by a statistical analysis of the residual enzyme activity. The inactivation of the enzyme by DEP was prevented by preincubation of the enzyme with the coenzyme NADPH but not with the substrate succinic semialdehyde. These results suggest that an essential histidyl residue involved in the catalytic activity is located at or near the coenzyme binding site of the brain succinic semialdehyde reductase.

Keywords: Brain GABA shunt, Diethylpyrocarbonate, Histidyl residue, γ-Hydroxybutyrate, Succinic semialdehyde reductase.

Introduction

Succinic semialdehyde (SSA) is an intermediate of the γ-aminobutyrate (GABA) shunt. SSA is formed by transamination of GABA, a major inhibitory neurotransmitter, by GABA transaminase and is primarily oxidized to succinate by a specific dehydrogenase in the brain (Pitts and Quick, 1965; Cash *et al.*, 1977; 1978). However, SSA can also be reduced to γ-hydroxybutyrate (GHB) in brain tissue (Fishbein and Bessman, 1964). Several oxidoreductases which catalyze the reduction of SSA to GHB have been identified as NADPH-dependent aldehyde reductases (Tabakoff and von Wartburg, 1975; Cash *et al.*, 1979; Kaufman *et al.*, 1979; Hearl and Churchich, 1985).

Compared to the transamination of GABA to SSA, the reduction of SSA to GHB has not received considerable attention because the mechanism by which this reductive pathway operates *in vivo* is not yet well understood. However, a specific binding site for GHB with high affinity has been detected in synaptic membrane preparations (Benavides *et al.*, 1982), and a fairly specific succinic semialdehyde reductase has been isolated from brain tissues (Cash *et al.*, 1979). In addition, the studies of reduction of SSA to GHB, using rat or pig brain as the enzyme source, strongly support the proposal that GHB biosynthesis may be an important step in the GABA shunt of pharmacological interest (Rivett *et al.*, 1981; Hearl and Churchich, 1985). For instance, effects induced by administration of the compound include anesthesia, evoking the sleep state, and an increase in brain dopamine levels (Gessa *et al.*, 1966; Godschalk *et al.*, 1977; Snead, 1977).

Studies of the structure and function of succinic semialdehyde reductase are very limited. No crystal structure is available for any succinic semialdehyde reductase and thus little is known about the chemistry of the active site of the enzyme. Further characterization of the structure and function of succinic semialdehyde reductase, especially the brain enzyme, is needed to elucidate its physiological nature.

We have previously purified and characterized an NADPH-dependent succinic semialdehyde reductase from

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bovine brain (Cho *et al.*, 1993). By means of monoclonal antibodies raised against brain succinic semialdehyde reductase, we reported that the brain succinic semialdehyde reductase is distinct from other aldehyde reductases and that mammalian brains contain only one succinic semialdehyde reductase (Choi *et al.*, 1995). More recently, we reported that *o*-phthalaldehyde inactivates the brain succinic semialdehyde reductase by cross-linking proximal cysteinyl and lysyl residues to form fluorescent isoindole derivatives (Cho *et al.*, 1996), and the microenvironmental structure around the lysyl residues was identified by a combination of labeling with pyridoxal-5′-phosphate and peptide analysis (Hong *et al.*, 1997a). The enzyme was also modified at the tryptophan residues by N-bromosuccinimide (Hong *et al.*, 1997b).

Even though the critical lysyl and tryptophan residues are known to be involved in enzyme activity, little else is known about the chemical nature of the amino acid residues critically involved in catalytic activity.

The present investigation was undertaken to examine the involvement of other residues in the function of succinic semialdehyde reductase using diethylpyrocarbonate (DEP) as a selective reagent for the modification of the histidyl residues. We report that DEP inactivates succinic semialdehyde reductase by forming an N-carbethoxy histidine and we describe the results of kinetic analysis of the reaction of the enzyme with DEP.

Materials and Methods

Materials Ammonium sulfate, succinic semialdehyde, bovine serum albumin, EDTA, 2-mercaptoethanol, NADPH, $NADP^+$, phenylmethylsulfonyl fluoride, and diethylpyrocarbonate were purchased from Sigma Chemical Co. (St. Louis, USA). Mono-Q, Superose-12, CM-Sepharose, and Blue-Sepharose were obtained from Pharmacia (Milwaukee, USA). Bovine brains were obtained from Majang-dong slaughterhouse (Seoul, Korea).

Enzyme purification and assay Succinic semialdehyde reductase from bovine brain was purified by the method developed in our laboratory (Cho *et al,*. 1993). The method involves four column chromatographic steps: CM-Sepharose, Blue-Sepharose, hydroxyapatite, and Mono-Q. For measuring the activity of succinic semialdehyde reductase, the oxidation of NADPH to $NADP^+$ was measured by following the reaction at 340 nm as reported previously (Cho *et al*, 1993). All assays were performed in duplicate and initial velocity data were correlated with a standard assay mixture containing succinic semialdehyde (120 μM) and NADPH (50 μM) in 0.01 M potassium phosphate buffer, pH 7.0 at 25°C. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol NADPH/min at 25°C. Protein concentration was estimated by the procedure of Bradford with bovine serum albumin as a standard (Bradford, 1976).

Spectroscopy Spectrophotometric measurements were carried out using a Kontron UVIKON 930 double beam spectrophotometer. Fluorescence spectra were recorded on a Kontron SFM 25 spectrofluorimeter.

Modification of succinic semialdehyde reductase with diethylpyrocarbonate The purified enzyme was modified by the addition of diluted DEP in ethanol to the enzyme in 0.01 M potassium phosphate buffer, pH 7.0. The final concentration of ethanol in the reaction mixture did not exceed 5% (v/v) and had no effect on the activity and stability of the enzyme. After an appropriate time, an aliquot of the incubation mixture was taken and added to a 100 mM imidazole, quenching buffer, pH 7.5. The remaining enzyme activity of the aliquot was measured at 25°C.

In some experiments, the capacity of succinic semialdehyde or NADPH to protect the enzymes from inactivation was tested by including them in the incubation mixture.

Results

Inactivation of the succinic semialdehyde reductase by DEP Inactivation of the enzyme by increasing amounts of DEP resulted in a time-dependent loss of enzyme activity (Fig. 1). The inactivation followed pseudo-first-order kinetics with concentrations of DEP in the range 0.2 – 1.0 mM. The plot of the pseudo-first-order rate constant for inactivation (k_{obs}) vs the different concentration of DEP was linear (Fig. 2). From the slope of the plot, the second-order rate constant (k_2) of 1.1×10^3 $M^{-1}min^{-1}$ for the inactivation was calculated. The apparent first-order rate constant (k_{obs}) for inactivation depends upon the concentration of DEP. It may be expressed by the following equation

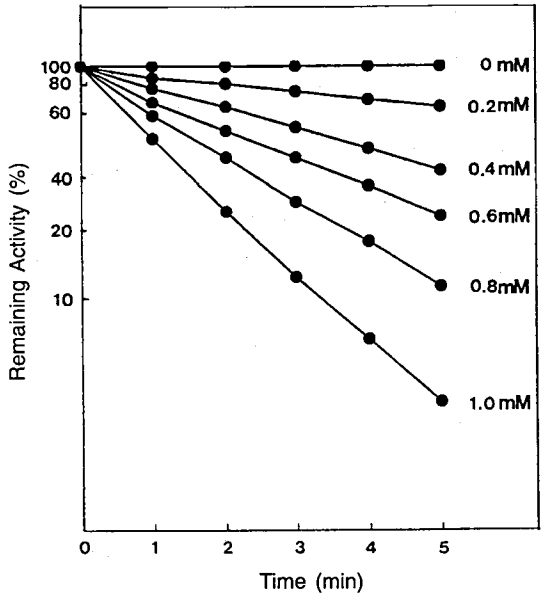

Fig. 1. Inactivation of succinic semialdehyde reductase by DEP. The enzyme (5 μM) was incubated with different concentrations of the reagent in 0.01 M potassium phosphate buffer, pH 7.0, at 25°C. At one-minute intervals, aliquots were removed for measurement of the residual enzyme activity.

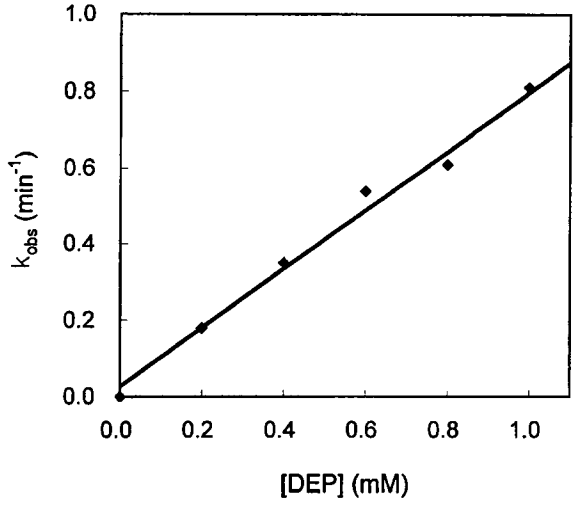

Fig. 2. Plot of apparent first-order rate constant for inactivation (k_{obs}) obtained at various concentration of DEP reagent. From this plot, the second-order rate constant (k_2) was calculated.

$$k_{obs} = k_2(DEP)^n$$

This equation can be converted to

$$\log k_{obs} = \log k_2 + n \log(DEP)$$

The plot of log k_{obs} vs log[DEP] for succinic semialdehyde reductase was linear (data not shown). From the slope of the plot, the reaction order of 1.03 was obtained, indicating that an average of at least one molecule of DEP binds to one molecule of enzyme when inactivation occurs. The inactivation studies were carried out in the presence of substrate or coenzyme NADPH to define the site of DEP modification. The reaction of succinic semialdehyde reductase with DEP was completely blocked by the coenzyme NADPH but not by the substrate (Table 1).

Characterization of DEP-modified residue Although the reaction of DEP is reasonably specific for histidyl residues in the range from pH 5.5 to 7.5, reaction can also occur with other nucleophiles such as cysteine, tyrosine, and other primary amino groups.

Spectral analysis for the modification of succinic semialdehyde reductase by DEP revealed a concomitant increase in absorbance at 242 nm (Fig. 3). There was no change in absorbance at 280 nm, indicating the carbethoxylation of the imidazole nitrogen of the histidyl residue but no reaction with the tyrosyl residue.

In order to determine the number of sulfhydryl groups modified by DEP, the number of sulfhydryl groups in the native and the DEP-modified enzymes were determined by using 5,5′-dithiobis-(2-nitrobenzoate) as described by Choi and Kim (1991). The number of sulfhydryl groups per molecule of enzyme was calculated to be two in both cases. This result suggest that the cysteinyl residues do not react with DEP.

Table 1. Inactivation of succinic semialdehyde reductase by DEP.

Reaction mixture	Remaining Activity (%)
Enzyme (5 μM)	100
Enzyme (5 μM) + DEP (400 μM)	11
Enzyme (5 μM) + SSA (3 mM) + DEP (400 μM)	21
Enzyme (5 μM) + NADPH (3 mM) + DEP (400 μM)	93

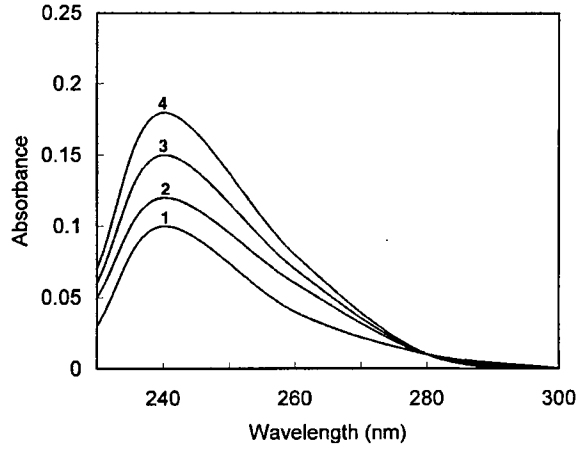

Fig. 3. Absorption spectra for modification of succinic semialdehyde reductase by DEP within the UV region. Enzyme (5 μM) was divided equally between the sample and the reference cuvettes in the spectrophotometer, and the base line was recorded. Spectra were recorded every minute (1: 0 min; 2: 1 min; 3: 2 min; 4: 3 min) after the addition of DEP. The absorbance at 242 nm increased as a function of time.

Stoichiometry of inactivation by DEP The number of the modified histidyl residues can be estimated by measuring the absorbance change at 242 nm (ε = 3200 $M^{-1}m^{-1}$) upon reaction with DEP. Figure 4 shows the relationship between the remaining enzyme activity and the number of histidyl residues modified by DEP. Extrapolation of the linear plot shows that the modification of five residues is required for complete inactivation. However, by the statistical method of Tsou (1962), only one of the five residues is essential for the enzyme activity. That is, with all the modifiable residues, n, equally reactive but only i residues essential for activity, the number of residues modified at a given stage of modification, m, is related to the residual activity by:

$$m = n\{1-(A/A°)^{1/i}\}$$

A°: Enzymatic activity at time 0 after modification of enzyme with DEP

A: Enzymatic activity at time t after modification of enzyme with DEP

The line in Fig. 4, calculated from the equation above with n=5 and i=1, shows a satisfactory fit to the experimental data.

Discussion

Little is known about the chemistry of the active site of succinic semialdehyde reductase, partly because no crystal structure of the enzyme is available from any source. It is therefore essential to obtain a detailed structural description of succinic semialdehyde reductase by other means. Further characterization of the structure and function of succinic semialdehyde reductase, especially the brain enzyme, is needed to elucidate the physiological nature of the succinic semialdehyde reductase. By means of monoclonal antibodies against bovine brain succinic semialdehyde reductase, we previously reported that the brain succinic semialdehyde reductase is distinct from other aldehyde reductases and that mammalian brains, including the human brain, contain one specific succinic semialdehyde reductase (Cho *et al.*, 1993; Choi *et al.*, 1995). An investigation of the catalytic role of specific amino acid residues of bovine brain succinic semialdehyde reductase indicated the involvement of lysyl and tryptophanyl residues in the enzymatic activity (Cho *et al.*, 1996; Hong *et al.*, 1997a; 1997b).

Chemical modification of histidine residues by DEP in brain succinic semialdehyde reductase is shown here to bring original information concerning the microenvironmental structure of the active site and the role of catalytically essential histidyl residues in enzyme catalysis. DEP reacts with several amino acid side chains including imidazole, phenolates, and sulfhydryls (Melchior and Fahrney, 1970), but under neutral or slightly acid conditions the specificity for histidine is greatly increased. The inactivation of proteins by modification of histidyl residues with DEP has generally occurred with a second-order rate constant for inactivation greater than 10 $M^{-1}min^{-1}$ at pH 6.0, ranging from about 10 $M^{-1}min^{-1}$ for the inactivation of thermolysin (Burstein. *et al.*, 1974) to 1800 $M^{-1}min^{-1}$ for the modification of pig heart lactate dehydrogenase (Holbrook and Ingram, 1973).

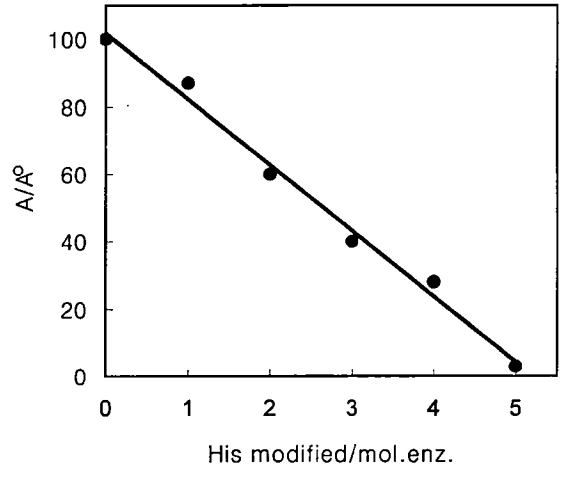

Fig. 4. Relationship between the residual activity and the number of histidyl residue modified. The enzyme (5 μM) was incubated with 0.4 mM DEP. Aliquots were removed at intervals to determine the enzyme activity and the amount of carbethoxyhistidine formed was measured as described in the text.

In this study, bovine brain succinic semialdehyde reductase was inactivated by DEP in a pseudo-first order process. Replot of the kinetic data yielded a second-order rate constant of 1.1×10^3 $M^{-1}min^{-1}$ for the inactivation process at pH 7.0 and 25°C. Even though the inactivation with DEP was carried out at pH 7.0, the rate constant for the inactivation of brain succinic semialdehyde reductase was sufficiently high to lead to the conclusion that modification of a histidyl residue is essential. The specificity of the DEP reaction for histidyl residues was supported by a concomitant increase in absorbance at 242 nm but no change at 280 nm. The increase in absorbance at 242 nm by formation of N-carbethoxy imidazole has been well studied (Miles, 1977). The reaction of tyrosyl residues with DEP is expected to show a decrease in absorbance at around 280 nm (Burstein *et al.*, 1974). Therefore, no change in absorbance at 280 nm means that no tyrosyl residues in succinic semialdehyde reductase were modified by DEP. The involvement of a cysteinyl residue in the inactivation by DEP could be ruled out because the number of sulfhydryl groups of the native and modified enzyme were identical. The stoichiometry of adduct formation was approximately five moles of histidyl residues per mole of enzyme. However, by the statistical method of Tsou (1962), only one of five residues was found essential for the enzyme catalysis.

The nature of the inhibitory effect exerted by DEP was studied in detail. The possibility that DEP inhibition is the result of the reaction of essential histidyl residues critically connected with catalysis was investigated by performing inhibition studies in the presence and absence of substrate succinic semialdehyde or coenzyme NADPH. The inhibitory effect of DEP was influenced by coenzyme NADPH at a concentration of 3 mM (Table 1). The nearly complete protection afforded by coenzyme NADPH strongly suggests that inactivation occurred due to interaction with histidyl residues located at or near the coenzyme binding site.

The results of the present investigation provide evidence for the presence of a catalytically essential histidyl residue in the active site of the enzyme and only one histidyl residue is highly reactive to DEP. There is a need for further studies of the exact reaction site including the isolation of histidyl residue-DEP adducts from the

inactivated enzyme and the sequence of DEP-containing peptides. Further analysis of isolated peptides labeled with DEP should provide additional microenvironmental structural data about the active site of brain succinic semialdehyde reductase.

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