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Chemical Modification of Tryptophan Residue in Bovine Brain Succinic Semialdehyde Reductase

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Incubation of an NADPH-dependent succinic semialdehyde reductase from bovine brain with N-bromosuccinimide (NBS) resulted in a time-dependent loss of enzyme activity. The inactivation followed pseudo-first-order kinetics with the second-order rate constant of 6.8×10^3 $M^{-1}min^{-1}$. The inactivation was prevented by preincubation of the enzyme with substrate succinic semialdehyde, but not with coenzyme NADPH. There was a linear relationship between oxindole formation and the loss of enzyme activity. Spectrophotometric studies indicated that about one oxindole group per molecule of the enzyme was formed following complete loss of enzymatic activity. It is suggested that the catalytic function of succinic semialdehyde reductase is modulated by binding of NBS to a specific tryptophan residue at or near the substrate binding site of the enzyme.

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 primarily oxidized to succinate by a specific dehydrogenase in 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 that 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 with transamination of GABA to SSA, 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 SSA 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 state of sleep, and an increase in brain dopamine level (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 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 the mammalian brains contained 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). We also identified the regulatory site of the enzyme by a combination of labeling with pyridoxal-5'-phosphate and peptide analysis (Hong et al., 1997).

Even though the critical lysyl residues are involved in enzyme activity, little is known about the chemical nature of the amino acid residues critically connected with catalysis. In the present study, we investigated the role of the tryptophan residue using chemical

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modification with the tryptophan-specific reagent NBS.

Materials and Methods

Materials

Ammonium sulfate, SSA, bovine serum albumin, EDTA, DL-glyceraldehyde, GHB, 2-mercaptoethanol, NADPH, $NADP^+$, phenylmethylsulfonyl fluoride, and NBS were purchased from Sigma. Mono-Q, Superose-12, CM-Sepharose, and Blue-Sepharose were obtained from Pharmacia (U.S.A.), and hydroxyapatite was from Bio-Rad. Bovine brains were obtained from Majang Slaughterhouse (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. The oxidation of NADPH to $NADP^+$ was measured by following the decrease in absorbance at 340 nm as reported previously (Cho et al., 1993). 1 unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol NADPH/min at 25℃. 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.

Chemical modification of succinic semialdehyde reductase with NBS

The purified enzyme was dialyzed against 0.05 M potassium phosphate, pH 6.5 and then used immediately. NBS (200 mM) was freshly prepared in 0.05 M potassium phosphate, pH 6.5 and kept on ice. The incubation mixture (1 ml) contained succinic semialdehyde reductase (5 μM). NBS (10-100 μM) and 0.05 M potassium phosphate, pH 6.5. The reaction was initiated by addition of NBS in the dark at 25℃. All solutions containing NBS were protected from photolytic destruction with metal foil. At intervals after the initiation of the inactivation, aliquots were withdrawn for the assay of activity. Whenever possible, a small sample volume (2 μl) was taken to minimize artifactual blanks due to the transfer of NBS.

In some experiments, the capacity of succinic semialdehyde or NADPH to protect the enzymes against inactivation was tested by including them in the incubation mixture. If their concentration was sufficient for the small amounts transferred to the assay mixture to affect activity, separate control experiments were included to determine the appropriate value of the 100% 'zero time' activity.

The number of tryptophan residues oxidized per mole of enzyme (n) was calculated from Equation 1, designed by Spande and Witkop (1967).

$$n = \frac{1.13 \times \triangle A_{280}}{5{,}500 \times \text{molarity of enzyme}} \qquad \text{(Equation 1)}$$

Where $\triangle A_{280}$=corrected optical density decreased at 280 nm, 1.13 is an empirical factor which corrects for absorption at 280 nm of the oxidation product of tryptophan, oxindole, and 5,500 is the molar extinction coefficient for tryptophan.

Results

Inactivation of the succinic semialdehyde reductase by NBS

Incubation of succinic semialdehyde reductase with increasing concentrations of NBS resulted in a progressive decrease in enzyme activity (Fig. 1). The inactivation followed pseudo-first-order kinetics with NBS in the concentration range of 10 μM-100 μM. The pseudo-first-order rate constants obtained at each NBS concentration were replotted as a function of NBS

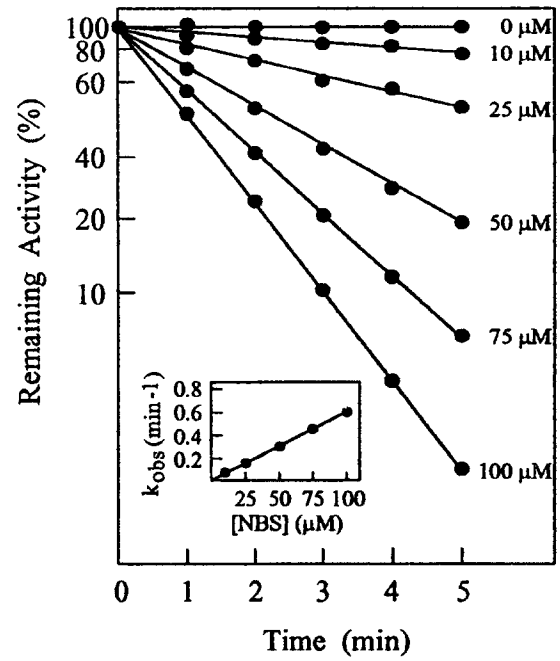

Fig. 1. Determination of the rate constant (Kobs) for the inactivation of succinic semialdehyde reductase at different concentrations of NBS. The enzyme (5 μM) was incubated with 10 μM, 25 μM, 50 μM, 75 μM, and 100 μM NBS in 0.05 M potassium phosphate, pH 6.5 at 25℃, respectively. Aliquots withdrawn from the incubation mixtures were tested for enzymatic activity. The inset shows the dependence of the observed rate constant (Kobs) on NBS concentrations.

Table 1. Inactivation of succinic semialdehyde reductase by NBS at pH 6.5

Reaction mixture	Remaining activity (%)
Enzyme (5 μM)	100
Enzyme (5 μM)+NBS (200 μM)	10
Enzyme (5 μM)+NADPH (3 mM)+NBS (200 μM)	18
Enzyme (5 μM)+SSA (3 mM)+NBS (200 μM)	92

concentration (Fig. 1, inset). The second-order rate constant for the inactivation of the enzyme by NBS was 6.8×10^3 $M^{-1}min^{-1}$ as determined from the slope of this plot.

The inactivation studies were carried out in the presence of substrate or coenzyme NADPH to define the sites of NBS modification. The reaction of succinic semialdehyde reductase with NBS was completely prevented by incubation of the enzyme with the substrate but not with the coenzyme NADPH (Table 1).

Spectroscopic properties

In an effort to demonstrate that the indole chromophore of tryptophan, absorbing strongly at 280 nm, is converted, on oxidation with NBS, to oxindole, a much weaker chromophore at this wavelength, the enzyme (10 μM) was incubated with different concentrations of NBS (100 μM, 200 μM) at pH 6.5 for 30 min. The ultraviolet absorbance peak at 280 nm was lowered, whereas an increased absorbance was observed at 250 nm (Fig. 2). A pronounced effect was observed on the emission spectrum of intrinsic fluorescence, characterized by a large decrease in intensity at 330 nm-340 nm, the maximal emission wavelength that remained apparently unchanged (Fig. 3).

The values for incorporation of reagents were measured using an extinction coefficient of 5,500 $M^{-1}cm^{-1}$ at 280 nm. NBS gave values of overall incorporation, about 0.94 mol/enzyme monomer, that indicated the masking of 1 mol tryptophan. Correlation between oxindole formation and enzyme activity is shown in Fig. 4. During the inactivation process there was a linear relationship between oxindole formation and the loss of enzyme activity, which extrapolates to a stoichiometry of 1.0 mol of oxidized tryptophan/mol enzyme, based on the decreased absorbance at 280 nm. This result indicates that a single tryptophan residue participates in the catalytic function of the enzyme.

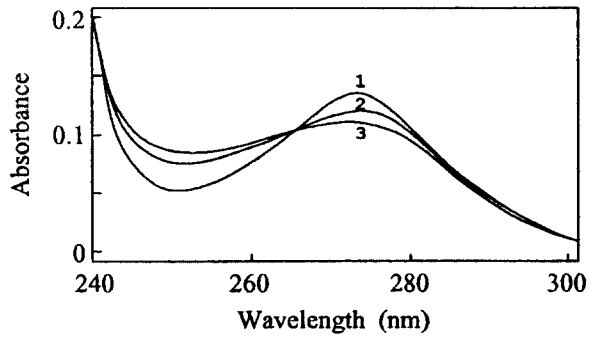

Fig. 2. Absorption spectra of native and modified succinic semialdehyde reductase. The enzyme (10 μM) was incubated without NBS (1) or with 100 μM NBS (2), 200 μM NBS (3) in 0.05 M potassium phosphate, pH 6.5, at 25℃. The reaction mixture was dialyzed against the same buffer, and the absorption spectra were determined as described in Materials and Methods.

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 sources. It is, therefore, essential to have a detailed structural description of succinic semialdehyde reductase. By means of monolconal antibodies against the bovine brain succinic semialdehyde reductase, we reported that 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 residue in the enzymatic activity (Cho et al., 1996; Hong et al., 1997). These conclusions were reached based on evidence obtained from chemical modification of the enzyme with *o*-phthalaldehyde, a possible cross-linking reagent and identification of a

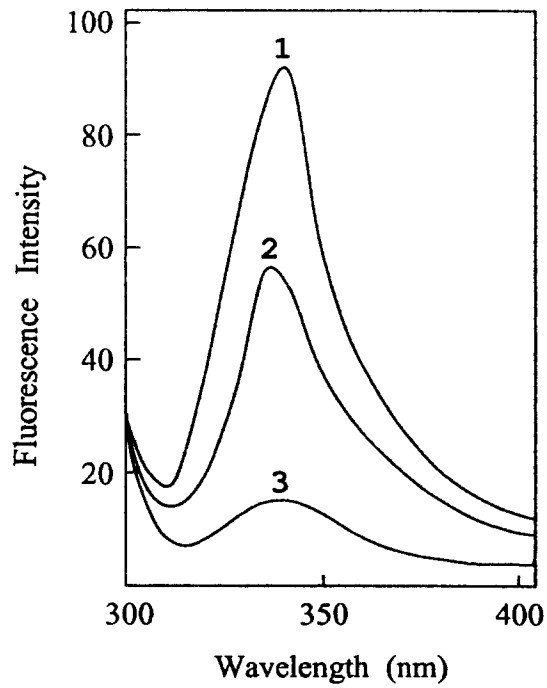

Fig. 3. Fluorescence emission spectra of native and modified succinic semialdehyde reductase. The enzyme (2 μM) was incubated without NBS (1) or with 100 μM NBS (2), or 200 μM NBS (3) in 0.05 M potassium phosphate, pH 6.5 at 25℃. (Excitation wavelength : 280 nm).

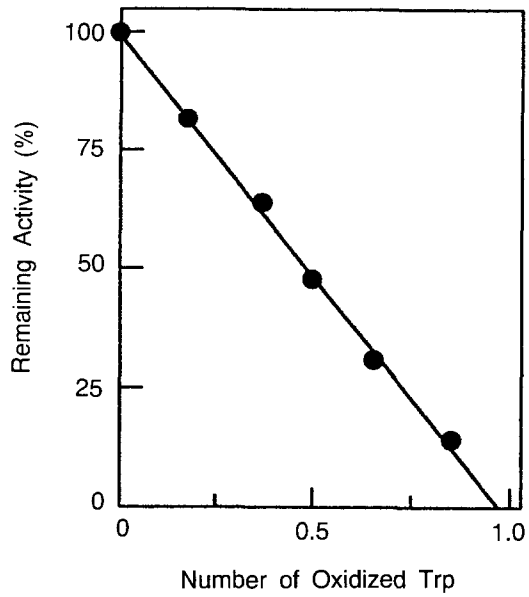

Fig. 4. Quantitative relationship between oxidized tryptophan residues and succinic semialdehyde reductase activity. Succinic semialdehyde reductase (5 μM) was incubated with 200 μM NBS in 0.05 M potassium phosphate, pH 6.5, at 25℃. At various times, aliquots were withdrawn and diluted with an assay mixture to assay the enzyme activity or with the same buffer to measure the modified enzyme induced spectral changes as described in Materials and Methods.

regulatory site by labeling with pyridoxal-5'-phosphate. Chemical modification of tryptophan residues in brain succinic semialdehyde reductase is shown here to bring original information concerning their microenvironmental structure of the active site, contribution to intrinsic fluorescence, and role in enzyme catalysis.

Oxidation of the tryptophan indole moiety into oxindole by NBS is particularly interesting since the additional steric hindrance of the substituted group is very limited, contrary to other chemical modifiers, and the modified residue becomes totally nonfluorescent (Imoto et al., 1971). Tryptophan residues may be rapidly (Ohnishi et al., 1989) and specifically modified at acidic pH, which increases both reactivity and selectivity (Spande and Witkop, 1967). NBS at 200 μM inhibits the reaction catalyzed by succinic semialdehyde reductase. The second-order rate constant for inactivation was 6.8×10^3 $M^{-1}min^{-1}$ and stoichiometry of the inactivation of the enzyme with NBS showed that one tryptophan residue participates in the catalytic function.

Tryptophan residues are generally considered to play an important role in stabilizing protein conformation by virtue of the hydrophobic chracteristics of the indole ring, but the residues are usually not involved in protonation-deprotonation or the charge relay system (Nishihira et al., 1992). The observed loss of enzyme activity with NBS modification may be due either to an oxidation of essential tryptophan residue or to a consequence of extensive conformational change in the enzyme. If a conformational change occurred due to the modification, then both large changes in fluorescence and a shift in the emission maximum should be observed. According to the results of absorption and emission spectra, the tryptophan residue of the enzyme reacted with NBS was oxidized with the linear decrease in absorbance at 280 nm and increase simultaneously in absorbance at 250 nm which might be attributed to the retention of oxindole derivative (Spande and Witkop, 1967). The fluorescent intensity was almost completely quenched without any change in the emission maximum, indicating that the modification did cause an extensive alteration in the structure of succinic semialdehyde reductase.

The nature of the inhibitory effect exerted by NBS was studied in detail. The possibility that NBS inhibition is the result of the reaction of essential tryptophan residues critically connected with catalysis was investigated by performing inhibition studies in the presence and absence of substrate SSA or coenzyme NADPH. At pH 6.5, the inhibitory effect of NBS was influenced by substrate SSA at 3.0 mM (Table 1). The nearly complete protection afforded by succinic semialdehyde strongly suggests that the inactivation occurred due to interaction with tryptophan residues located at or near the substrate binding site. In marked contrast to SSA, the coenzyme, NADPH, did not afford any protection against inactivation by NBS.

The results of our studies raise some questions about the role of the reactive tryptophan residue in enzyme function. Two distinct possibilities emerge. The tryptophan residue is essential for catalysis, i.e., it participates in some catalytic events. Alternatively, blocking of the reactive tryptophan residue triggers a conformational change which affects the catalytic site domain. The first interpretation seems to be consistent with the protective effect exerted by SSA, but binding of SSA can also result in stabilization of protein conformations which are no longer accessible to attacking tryptophanyl attacking reagent NBS.

In summary, NBS inactivates bovine brain succinic semialdehyde reductase by conversion of the indole chromophore of tryptophan residue in the active site to oxindole and conformational changes of the enzyme structure. Although it is generally accepted that tryptophan residues are usually located on the hydrophobic pocket in protein molecules, our chemical modification results show that only one tryptophan residue has a high reactivity with NBS.

Analysis of isolated peptides labeled with NBS is not established in this work. The absence of the primary sequence and X-ray crystallographic structural data make it difficult to discuss whether the modified residue is located within the active site or in the region whose conformation is altered upon the interaction of the enzyme with substrate and inhibitor.

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