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Inactivation of Brain Succinic Semialdehyde Reductase by o-Phthalaldehyde

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Abstract: Succinic semialdehyde reductase was inactivated by o-phthalaldehyde. The inactivation followed pseudo-first order kinetics, and the second-order rate constant for the inactivation process was 28 $M^{-1}s^{-1}$ at pH 7.4 and 25℃. The absorption spectrum (λ_{max} 337 nm) and fluorescence excitation (λ_{max} 340 nm) and fluorescence emission spectra (λ_{max} 409 nm) were consistent with the formation of an isoindole derivative in the catalytic site between a cysteine and a lysine residue approximately about 3 Å apart. The substrate, succinic semialdehyde, did not protect enzymatic activity against inactivation, whereas the coenzyme NADPH protected against o-phthaladehyde induced inactivation of the enzyme. About 1 isoindole group per mol of the enzyme was formed following complete loss of enzymatic activity. These results suggest that the amino acid residues of the enzyme participating in a reaction with o-phthalaldehyde are cysteinyl and lysyl residues at or near the NADPH binding site.
Key words: GABA shunt, γ-hydroxybutyrate, o-phthalaldehyde, succinic semialdehyde reductase.

Succinic semialdehyde (SSA) is an intermediate of the γ-aminobutyrate (GABA) shunt pathway. SSA is formed by transamination of the major inhibitory neurotransmitter GABA by GABA transaminase (GABA-T). The cerebral concentration of SSA is normally low in contrast to GABA (Matsuda and Hoshino, 1977). Abnormal levels of GABA in the brain have been associated with a variety of neurological disorders, including seizures, convulsion, epilepsy, Huntington's disease, and Parkinsonism (Tower, 1970; Perry *et al.*, 1973; Lloyd *et al.*, 1977). However, SSA can also be reduced to γ-hydroxybutyrate (GHB) in brain tissue (Fishbein and Bessman, 1964) and a number of 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; Hearl and Churchich, 1985). GHB is a normal constituent of the mammalian brain (Roth and Giarman, 1970; Roth, 1970) and has neurophysiological functions (Turner and Whittle, 1983). Systemically administered GHB has a number of pharmacological effects. These include an anesthetic action and induction of sleep, and anesthetic doses of GHB increase the dopamine level in the brain (Gessa *et al.*, 1966; Godchalk *et al.*, 1977).

Reduction of SSA to GHB has not received much attention, compared to reduction of GABA to SSA, since the mechanism by which this reductive pathway operates *in vitro* is not clearly known. However, a specific binding site for GHB with a high affinity has been detected in synaptic membrane preparations (Benavides *et al.*, 1982) and a fairly specific SSA reductase from brain tissue has been isolated. In addition, studies of SSA reduction to GHB using rat and pig brain as the enzyme source support the idea that GHB biosynthesis is an important step in the GABA shunt (Rivett *et al.*, 1981; Hearl and Churchich, 1985).

o-Phthalaldehyde has been used as an active-site inhibitor of succinic semialdehyde dehydrogenase (Blaner and Churchich, 1979), aldolase (Palczewski *et al.*, 1983), cylic AMP dependent protein kinase (Puri *et al.*, 1985), and hexokinase (Puri *et al.*, 1989). The compound reacts with amino and thiol groups, forming an isoindole adduct which exhibits strong fluorescence. The wavelength of the emission maximum gives information on the microenvironment of the modified residues (Churchich, 1987). Therein, o-phthalaldehyde is

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shown to be a potent inhibitor of brain succinic semialdehyde reductase.

Materials and Methods

Materials

Bovine brains were obtained from Majangdong Packing Company, Seoul. Korea. NADPH, $NADP^+$, succinic semialdehyde, bovine serum albumin, o-phthalaldehyde, β-mercaptoethanol, γ-hydroxybutyrate, DL-glyceraldehyde, EDTA, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, USA). Mono-Q, Superose-12, CM-Sepharose, and Blue-Sepharose were obtained from Phamacia/LKB (Uppsala, Sweden), Ltd. and hydroxyapatite was obtained from Bio-Rad (Richmond, USA).

Purification and enzymatic assays of succinic semialdehyde reductase

Succinic semialdehyde reductase from bovine brain was purified according to the procedure of Cho *et al.* (1993). The oxidation of NADPH to $NADP^+$ was measured by following a decrease in absorbance at 340 nm by the method of 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 0.1 M potassium phosphate, pH 7.4, at 25℃. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of NADPH per min at 25℃. Initial velocity data were fitted by a least squares method to the double reciprocal transformation of Eq. (1).

$$v=V_{max}[S](K_m+[S]) \quad (1)$$

Protein concentrations were estimated by the Bradford procedure with a bovine albumin standard (Bradford, 1976).

Chemical modification of succinic semialdehyde redutase by o-phthalaldehyde

Solutions of o-phthalaldehyde were prepared fresh daily by dissolving o-phthalaldehyde in methanol and diluting to the desired concentration with distilled water. The final concentration of methanol in the incubation mixure was no more than 1% (v/v) and had no effect on enzyme activity. The enzyme (10 μM) was preincubated with o-phthalaldehyde in 0.1 M potassium phosphate buffer (pH 7.4), containing 1 mM β-mercaptoethanol, at 25℃. Aliquots withdrawn from the incubation mixture were assayed for enzymatic activity at various times. The degree of labelling of the enzyme reacted with o-phthalaldehyde was determined from an absorption spectrum using a molar extincion coefficient of 7.66×10^3 $M^{-1}cm^{-1}$ at 340 nm (Simmons and Johnson, 1978). Protection experiments were performed in a similar manner except that the enzyme was preincubated with a substrate or coenzyme for 20 min before the modification was started by addition of o-phthalaldehyde.

Spectroscopy

Spectrophotometric measurements were carreid out using a Kontron UVIKON 930 double beam spectrophotometer. Fluorescence spectra were recorded on a Kontron SFM 25 spectrofluorimeter. Fluorescence decay measurements were made using the monophotonic technique with an Ortec model 8200 nanosecond spectrometer. Time base calibration of the multichannel analyzer was performed indirectly using a solution of quinine sulfate in 0.05 M sulfuric acid. A free-running flash lamp operating in air at 1 atmosphere was used as the excitation source. The lamp was pulsed at 10 KHz. Excitation was set at 340 nm and the emission was filtered through a Corning glass filter (C-S-3-72). Deconvolution of the data was performed with a computer program based on the least-squares method of Ware *et al.* (1973). Fluorescence life times were measured with a precision of 2%. Steady emission anisotropy (r) is related to fluorescence polarization (p) by Eq. (2).

$$r=\frac{2P}{3-P} \quad (2)$$

Results

Inactivation of succinic semialdehyde reductase by o-phthalaldehyde

Purified succinic semialdehyde reductase was inactivated upon incubation with o-phthalaldehyde at 25℃. The time course of inactivation at various concentrations of the reagent is shown in Fig. 1. A plot of the natural logarithm of percent residual activity versus time of incubation with o-phthalaldehyde gave a straight line. The slope of this line yields an apparent or observed pseudo-first-order rate constant ($K_{obs.}$) according to Eq. (3), where E is the activity at any given time and E_0 is the activity at time zero.

$$-\ln\ (E/E_0)=K_{obs}\ t \quad (3)$$

Pseudo-first order reaction rates of enzyme inactivation were linear with respect to an o-phthalaldehyde concentration in the range of 0.05~0.2 mM (Fig. 1, insert). The slope of this line yieled a second order rate constant K, equal to 28 $M^{-1}S^{-1}$.

According to the definition by Levy *et al.* (1963), stoichiometric incorporation of the ligand gave a slope

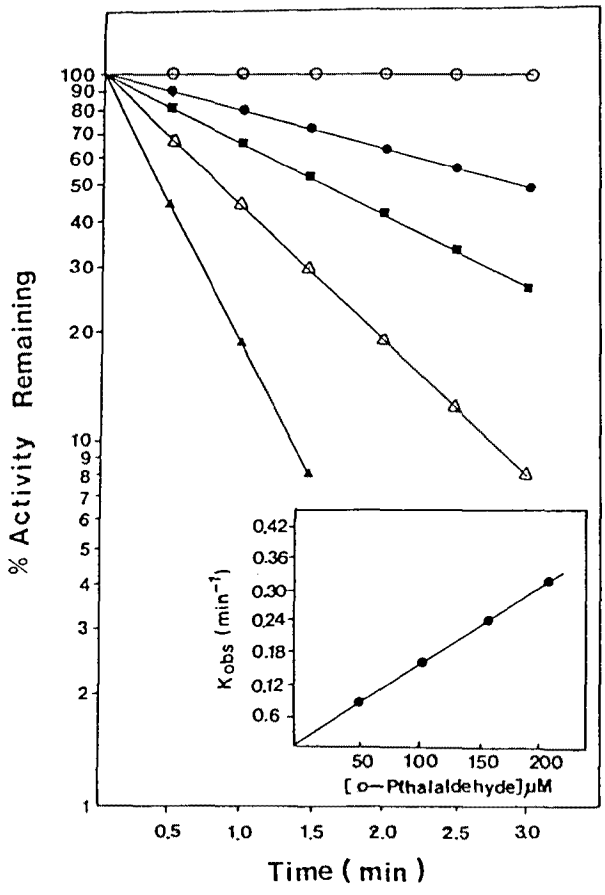

Fig. 1. Time course of inactivation of bovine brain succinic semialdehyde reductase by o-phthalaldehyde. The enzyme (10 μM) was incubated with o-phthalaldehyde in 0.1 M potassium phosphate buffer (pH 7.4) at 25℃. The data are plotted as the natural logarithm of percent activity remaining vs. time. o-phthalaldehyde concentrations were as follows: (0), 0 μM; (●), 50 μM; (■), 100 μM; (△), 150 μM; (▲), 200 μM. The insert shows a plot of pseudo-first order reaction vs. concentration of o-phthalaldehyde.

Table 1. Inactivation of Succinic semialdehyde reductase by o-phthalaldehyde at pH 7.4

Reaction mixture	Remaining activity (%)
Enzyme (7 μM)	100
Enzyme (7 μM)+OPA (100 μM)	3
Enzyme (7 μM)+NADPH (3 mM)+OPA (100 μM)	95
Enzyme (7 μM)+SSA (3 mM)+OPA (100 μM)	10

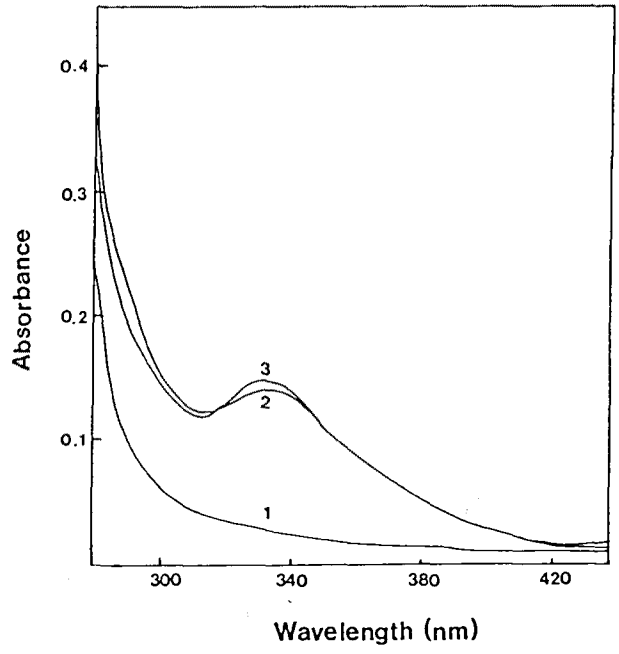

Fig. 2. Absorption spectra of succinic semialdehyde reductase (1), enzyme reacted with 10 fold molar excess of o-phthalaldehyde in the absence (2) and presence (3) of β-mercaptoethanol, respectively.

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Under the experimental conditions a loss of catalytic activity was prevented by the addition of the 3 mM coenzyme NADPH prior to reaction with o-phthalaldehyde. The addition of the substrate succinic semialdehyde prior to reaction with o-phthalaldehyde had no effect on the rate and extent of inactivation (Table 1).

Spectroscopic studies

The extent of the reaction of o-phthalaldehyde with the lysine residue ε-amino groups of the enzyme is easily determined by measuring either an increase in absorbance at 337 nm or an increase in fluorescence at 450 nm (Blaner and Churchich, 1979). Free o-phthalaldehyde does not absorb at 337 nm and it does not exhibit any fluorescence over a spectral range of 350 to 500 nm (Benson and Hare, 1975).

Treatment of succinic semialdehyde reductase with o-phthalaldehyde for 20 min completely eliminated enzymatic activity and resulted in the formation of a derivative that dispalyed an absorbance peak at 337 nm, which is a characteristic feature of an isoindole derivative (Fig. 2). o-phthalaldehyde can react with primary amines in the presence of β-mercaptoethanol (Roth, 1971).

Absorption spectra of the native and modified enzymes were recorded over a spectral range of 300~500 nm. The modified enzyme reacted with o-phthalaldehyde in the presence and absence of β-mercaptoethanol.

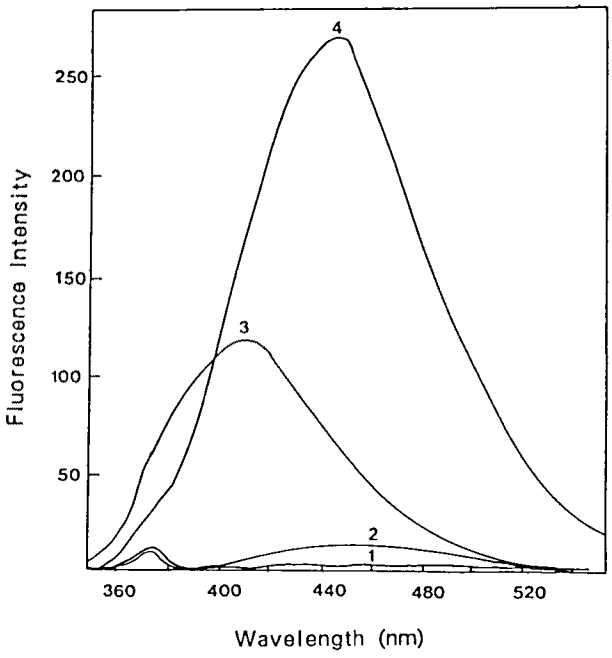

Fig. 3. Emission spectra of succinic semialdehyde reductase (1), enzyme reacted with o-phthalaldehyde in the absence (3) and presence (4) of β-mercaptoethanol. (2): o-phthalaldehyde alone. (Excitation at 340 nm).

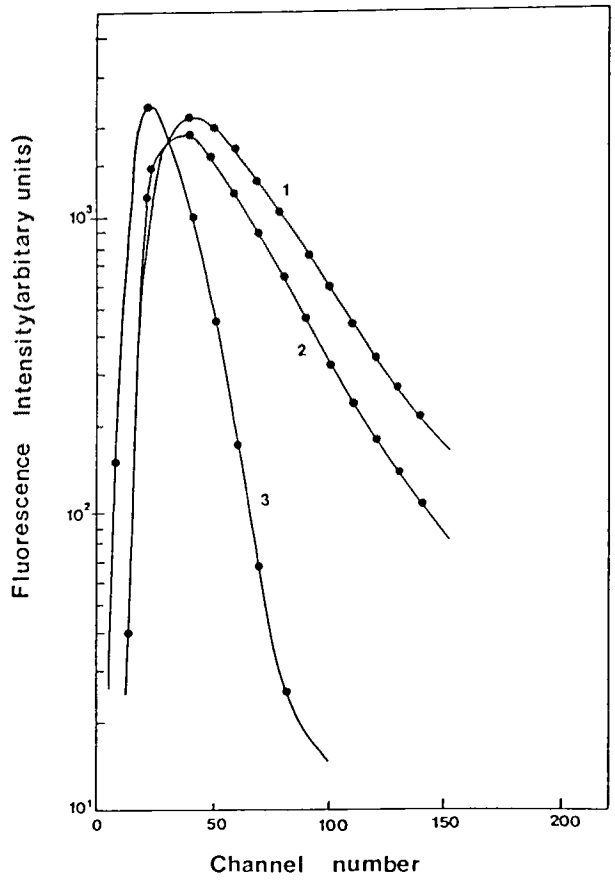

Fig. 4. Fluorescence decay kinetics of succinic semialdehyde reductase-o-phthalaldehyde adduct in the absence (1) and presence (2) of β-mercaptoethanol obtained with the monophotonic fluorescence technique. The lamp profile is included in the figure (curve 3).

These results indicate that the isoindole ring is formed by the cross-linking of Lys-NH_2 and Cys-SH groups with o-phthalaldehyde even though β-mercaptoethanol is absent from the reaction mixture.

From the absorption coefficient of the isoindole derivative (Simons and Johnson, 1978), it was calculated that 1.27 mol of isoindole/mol of enzyme was formed. A linear relationship between an increase in absorbance (hence, an increase in isoindole groups formed) and an increase in enzyme concentration was observed.

Modified succinic semialdehyde reductase was fluorescent, with the same excitation peak at 337 nm in the presence and absence of β-mercaptoethanol. However, the emission peak was shifted from 450 nm to 409 nm when β-mercaptoethanol was absent from the reaction mixture (Fig. 4). These fluorescent spectral shifts are consistent with the result of the o-phthalaldehyde reactions with adenosine cyclic 3′,5′-monophosphate dependent protein kinase (Puri *et al.*, 1985), and malonyl CoA synthetase (Lee and Kim, 1994). The enzyme reacted with o-phthalaldehyde exhibits a strong emission band when excited at 337 nm. Upon covalent binding to lysine residues of the enzyme, the fluorescent adduct emits fluorescent light which decays in a monoexponential manner with a fluorescence lifetimes of 8.70 ns and 9.87 ns in the presence and absence of β-mercaptoethanol, respectively (Fig. 5). Steady emission anisotropy values of 0.14 and 0.09 were obtained at different wavelengths in the presence and absence of β-mercaptoethanol (Table 2). Judging from the differences in the anisotropy values, it appears that o-phthalaldehyde is more rigidly trapped by the protein matrix in the absence of β-mercaptoethanol.

Discussion

NADPH-dependent succinic semialdehyde reductase was purified homogenously from bovine brain (Cho *et al.*, 1993) and monoclonal antibodies against the enzyme were produced and characterized (Choi *et al.*, 1995). In this study the inhibitory mechanism of o-phthalaldehyde with succinic semialdehyde reductase was investigated.

Succinic semialdehyde reductase is irreversibly inactivated by o-phthalaldehyde in a pseudo-first order process. Replots of kinetic data yield a second order rate constant of 28 $M^{-1}S^{-1}$ for the inactivation process at 25℃ and pH 7.4. The stoichiometry of adduct formation is approximately 1 mol of isoindole formed/mol of enzyme. o-Phthalaldehyde reacts with primary amines in the presence of β-mercaptoethanol (Roth, 1971). The

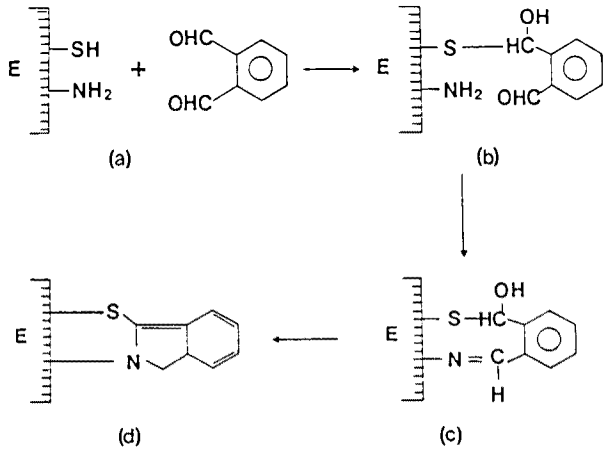

Fig. 5. Schematic representation of the reaction between the enzyme and o-phthalaldehyde.

Table 2. Anisotropy and fluorescence lifetime of succinic semialdehyde reductase labelled with o-phtalaldehyde in the absence and presence of β-mercaptoethanol, respectively

	γ (anisotropy)	τ (life time)
Reductase (10 μM)+ +OPA (100 μM)	0.14	8.74 ns
Reductase (10 μM) +OPA (100 μM) +β-mercaptoethanol (1 mM)	0.09	9.87 ns

effect of addition of o-phthalaldehyde on the enzymatic activity of pig brain 4-aminobutyrate aminotransferase was investigated in a reaction mixture containing β-mercaptoethanol (Kim and Churchich, 1981; Blaner and Churchich, 1979). o-Phthalaldehyde has been used in chemical modification studies in the absence of β-mercaptoethanol (Palczewski *et al.*, 1983; Puri *et al.*, 1985; Puri *et al.*, 1989; Rider and Hue, 1989; Huynh, 1990; Simons *et al.*, 1978). In these studies, the elimination of enzymatic activity by o-phthalaldehyde was a consequence of an intramolecular reaction between lysine -NH_2 group and cysteine -SH group of the enzyme and was not due to β-mercaptoethanol.

The present absorption and fluorescence spectrophotometric data indicate that cysteine residues are involved in the formation of the isoindole derivatives (Fig. 2, Fig. 3). These results are consistent with the formation of isoindole derivatives via the crosslinking of proximal cysteine and lysine residues (Simons and Johnson, 1978; Simons *et al.*, 1979; Palczewski *et al.*, 1983). When the enzyme was pretreated with iodoacetamide, a known -SH attacking agent, it did not show any fluorescence intensity over a spectral range 350 to 500 nm (data not shown).

The absorption spectrum of the modified enzyme resembles the spectrum of polylysine with o-phthalaldehyde. Samples of polyarginine preincubated with o-phthalaldehyde at pH 7.4 failed to show any absorption band at 337 nm as indicated in Fig. 2. These data indicate that the arginyl residues do not react with o-phthalaldehyde. This implies that the -SH and -NH_2 fractions of the cysteine and lysine residues, participating in isoindole formation, are in an optimum (3 Å) spatial disposition in the native enzyme (Fig. 5).

There is considerable evidence to suggest that at least one of the cysteine-lysine pairs modified by o-phthalaldehyde is near the coenzyme binding site. Complete protection from inactivation and isoindole formation was afforded by the coenzyme NADPH. The substrate succinic semialdehyde did not protect the enzyme from this inactivation process.

The results of fluorometric measurements (fluorescence lifetime) reflect a difference between the modified enzyme in the presence and absence of β-mercaptoethanol. A comparison of sample fluorescence polarization values reveals that the absence of β-mercaptoethanol, the enzyme reacting with o-phthalaldehyde remains relatively immobilized during the brief duration of fluorescence decay, whereas the modified enzyme in the presence of β-mercaptoethanol displays a higher degree of rotational mobility.

In summary, o-phthalaldehyde inactivates bovine brain succinic semialdehyde reductase by cross-linking proximal cysteine and lysine residues to form fluorescent isoindole derivatives. One mol of isoindole/mol of enzyme is found in the fully inactivated enzyme. Protection experiments localized at least one of the reactive cysteine-lysine pairs within the putative coenzyme binding site of the enzyme. There is need for further studies of the exact reaction site including the isolation of lysine or cysteine-o-phthalaldehyde adducts from the inactivated enzyme and the sequence of o-phthalaldehyde containing peptides. Further analysis of isolated peptides labeled with o-phthalaldehyde would provide microenvironmental structural data about the active site of brain succinic semialdehyde reductase.

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