Inactivation of an NADPH-dependent succinic semialdehyde reductase by *o*-phthalaldehyde

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Abstract **Incubation of an NADPH-dependent succinic semialdehyde reductase from bovine brain with *o*-phthalaldehyde resulted in a time-dependent loss of enzyme activity. The inactivation followed pseudo first-order kinetics with the second-order rate constant of 28 M^{-1} s^{-1}. The inactivation was prevented by preincubation of the enzyme with NADPH, but not by succinic semialdehyde. There was a linear relationship between isoindole formation and the loss of enzyme activity. Spectrophotometric studies indicated that complete inactivation of the enzyme resulted from the formation of one isoindole derivative per molecule of enzyme, which was formed from the reaction of cysteine and lysine residues with *o*-phthalaldehyde at or near the enzyme active site.**

Key words: Succinic semialdehyde reductase; *o*-Phthalaldehyde; Brain

1. Introduction

Succinic semialdehyde is an intermediate of the 4-aminobutyrate shunt pathway (GABA-shunt). Succinic semialdehyde is primarily oxidized to succinate by a specific dehydrogenase in brain [1]. However, succinic semialdehyde could also be reduced to 4-hydroxybutyrate in brain tissue [2]. 4-Hydroxybutyrate is a normal constituent of mammalian brain [3,4] and it might have a neurophysiological role [5]. Despite many interesting observations, reduction of succinic semialdehyde to 4-hydroxybutyrate has not received considerable attention because the degree to which this reductive pathway operates in vivo is not yet known.

A number of oxidoreductases which catalyze the reduction of succinic semialdehyde to 4-hydroxybutyrate have been identified as NADPH-dependent aldehyde reductases [6–9]. Recently, we reported the isolation from bovine brain of a fairly specific succinic semialdehyde reductase which can inter-convert succinic semialdehyde and 4-hydroxybutyrate [10]. These results strongly support the hypothesis that 4-hydroxybutyrate biosynthesis may be a significant pathway of pharmacological interest. Due to the lack of detailed information about the three-dimensional structure of any succinic semialdehyde reductase, remarkably little is known about the chemistry of the active site. Further characterization of the structure and function of succinic semialdehyde reductase is needed to elucidate the pathophysiological nature of the 4-hydroxybutyrate-related neurological disorders.

o-Phthalaldehyde has been used as an active-site inhibitor of many enzymes, whose sulfhydryl and ε-amino groups, respectively, are about 3 Å apart [11–15]. Most previous works suggest that *o*-phthalaldehyde forms an isoindole adduct by cross-linking ε-amino group and sulfhydryl functions of the active site lysine and cysteine residues, respectively. The isoindole adduct exhibits strong fluorescence and the wavelength of the emission maximum gives information on the microenvironment of the modified residues [16].

In this paper, we investigated the effect of *o*-phthalaldehyde, a homobifunctional cross-linking reagent, on the activity of the succinic semialdehyde reductase isolated from bovine brain. The result indicated that *o*-phthalaldehyde inactivated succinic semialdehyde reductase by forming one isoindole derivative which was formed from the reaction of lysine and cysteine residues with *o*-phthalaldehyde.

2. Materials and methods

Succinic semialdehyde, NADPH, phenylmethylsulfonylfluoride, 4-hydroxybutyrate, and *o*-phthalaldehyde were purchased from Sigma Chemical Co. Mono-Q, Superose-12, CM-Sepharose, and Blue-Sepharose were purchased from Pharmacia/LKB, Ltd. Hydroxyapatite was obtained from Bio-Rad. Bovine brains were obtained from Majang Slaughterhouse, Seoul, South Korea.

Succinic semialdehyde reductase from bovine brain was purified by the method of Cho et al. [10]. Enzyme activity in the direction of the oxidation of NADPH to $NADP^+$ was measured by following the decrease in absorbance at 340 nm as previously reported [10]. 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 7.2 at 25°C using a concentration of enzyme of 10 μg/ml. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 μmol of NADPH per min at 25°C. Protein concentration was estimated by the Bradford procedure with a bovine serum albumin standard [17].

Solutions of *o*-phthalaldehyde were prepared fresh daily as described elsewhere [11–15]. The modification was carried out at 25°C by incubating the enzyme (10 μM) with *o*-phthalaldehyde in 0.1 M potassium phosphate, pH 7.4. The final concentration of methanol in the incubation mixture was no more than 1% (v/v) and was found to have no effect on enzyme activity. At the indicated time intervals, an aliquot of the incubation mixture was assayed for succinic semialdehyde reductase as described above. The isoindole content of the inactivated enzyme was determined from the absorption at 337 nm using an extinction coefficient of 7660 $M^{-1} \cdot cm^{-1}$. 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 initiated by the addition of *o*-phthalaldehyde.

The absorption spectra were recorded on a Kontron UVIKON 930 double beam spectrophotometer. Fluorescence measurements were carried out with a Kontron SFM 25 spectrofluorimeter with an excitation at 337 nm.

3. Results and discussion

Very recently, using monoclonal antibodies against the bovine brain succinic semialdehyde reductase, we have reported that brain succinic semialdehyde reductase is distinct from

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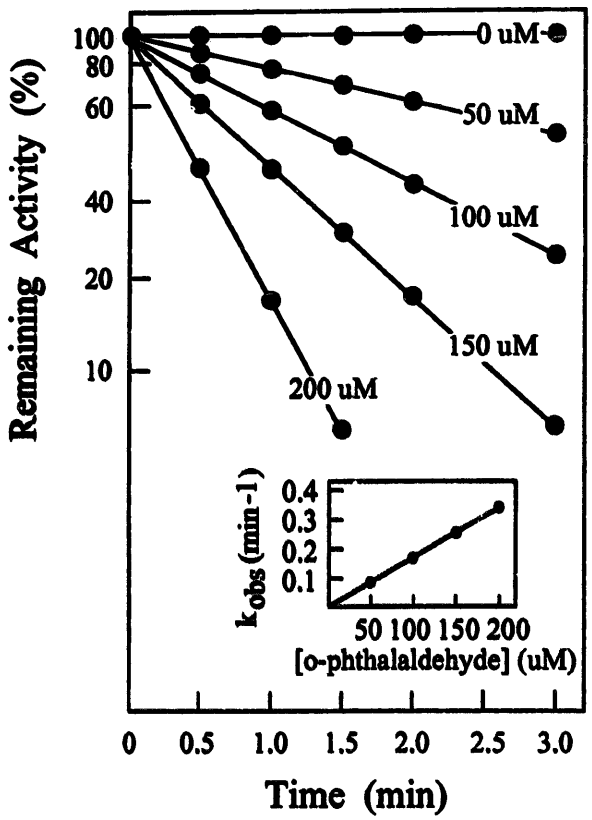

Fig. 1. Time course of inactivation of succinic semialdehyde reductase by *o*-phthalaldehyde. The enzyme (10 μM) was incubated with the indicated concentrations of *o*-phthalaldehyde in 0.1 M potassium phosphate buffer (pH 7.4) at 25°C. For details, see section 2. Data are plotted as the natural logarithm of percent of activity remaining versus time. Inset, replot of the pseudo first-order rate constants versus the concentrations of *o*-phthalaldehyde.

other aldehyde reductase and that mammalian brains including human brain contain one specific succinic semialdehyde reductase [18]. In this study, we investigated the effect of *o*-phthalaldehyde, a possible cross-linking reagent, on the activity of succinic semialdehyde reductase isolated from bovine brain [10] to obtain further information about reactive amino acid residues.

Incubation of succinic semialdehyde reductase with increasing concentrations of *o*-phthalaldehyde resulted in a progressive decrease in enzyme activity (Fig. 1). The inactivation followed pseudo-first order with various concentrations of *o*-phthalaldehyde in the range of 0.05–0.2 mM. The pseudo first-order rate constants obtained at each *o*-phthalaldehyde concentration are replotted as a function of *o*-phthalaldehyde concentration (Fig. 1, inset). The second-order rate constant for the inactivation of the enzyme by *o*-phthalaldehyde was 28 M^{-1} s^{-1} as determined from the slope of this plot. A double-reciprocal plot of these data indicates that *o*-phthalaldehyde binds weakly, if at all, to the enzyme before reaction. This value compares favorably with a value of 30 $M^{-1} \cdot s^{-1}$ for a similar reaction between fructose-1,6-bisphosphatase and *o*-phthalaldehyde [19].

The inactivation studies were carried out in the presence of substrate or coenzyme to define the sites of *o*-phthalaldehyde modification. The reaction of succinic semialdehyde reductase with *o*-phthalaldehyde was completely prevented by preincubation of the enzyme with the coenzyme NADPH but not by the substrate succinic semialdehyde (Table 1). The above results indicate that the inactivation of succinic semialdehyde reductase by *o*-phthalaldehyde resulted from the modification of amino acid residues which might be located at or close to the NADPH binding site. It could not, however, exclude the possibility that the modified residues are in the region whose conformation is altered upon the interaction of the enzyme with its coenzyme and inhibitor.

Treatment of succinic semialdehyde reductase (10 μM) with *o*-phthalaldehyde (100 μM) for 20 min completely eliminated the enzyme activity and resulted in the formation of a derivative that displayed an absorbance peak at 337 nm, which is a characteristic feature of an isoindole derivative (Fig. 2A). The absorption peak at 337 nm was not present in the spectrum of the unmodified enzyme. Excitation of the modified enzyme at 337 nm resulted in the appearance of a fluorescence emission with a maximum at 442 nm (Fig. 2B), also characteristic of isoindole derivatives. The rate of isoindole derivative formation measured by the increase in fluorescence depended on the concentration of *o*-phthalaldehyde (data not shown). The above results, indicate that modification of the enzyme by *o*-phthalaldehyde resulted in the formation of isoindole derivatives.

The polarity of the microenvironment surrounding the isoindole derivative of succinic semialdehyde reductase can be indirectly assessed by comparing the molar transition energy (E_T) of the enzyme derivative with that of a model isoindole derivative in various solutions. The molar transition energies can be calculated from the fluorescence emission maximum of an isoindole by the empirical relationship [20,21]:

$$E_T = 2.985\lambda_{em} - 1087.28$$

where λ_{em} is the wavelength of the fluorescence emission maximum of the isoindole derivative. The calculated E_T for the succinic semialdehyde reductase derivative (232 kJ/mol) is greater than those reported for most isoindole-modified proteins (116–187 kJ/mol [12–14,19]), indicating that the microenvironment of the site modified by *o*-phthalaldehyde in succinic semialdehyde reductase is less hydrophobic than in most other enzymes (see refs. [20,21] for details).

Table 1
Effects of added ligands on inactivation

Reaction mixture	Remaining activity (%)
Enzyme	100
Enzyme + *o*-phthalaldehyde	3
Enzyme + NADPH + *o*-phthalaldehyde	95
Enzyme + SSA + *o*-phthalaldehyde	10

Conditions were same as described in the legend to Fig. 1 except that the concentration of the enzyme was 7 μM throughout. Where indicated, 3 mM NADH or 3 mM succinic semialdehyde (SSA) was added 20 min prior to the addition of 100 μM *o*-phthalaldehyde. The enzyme incubated in the absence of *o*-phthalaldehyde lost no activity under the above conditions.

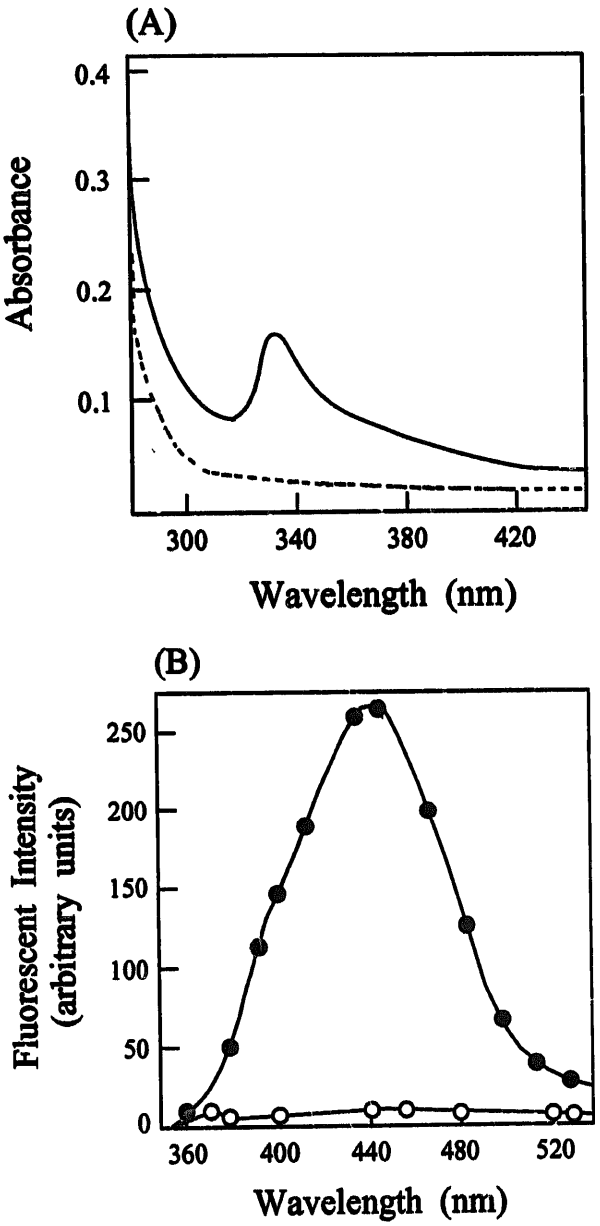

Fig. 2. Absorption and fluorescence spectra of native and modified succinic semialdehyde reductase. (A) The enzyme (10 μM) was incubated with 100 μM *o*-phthalaldehyde (solid line) or without *o*-phthalaldehyde (dashed line) in 0.1 M potassium phosphate buffer (pH 7.4) at 25°C. The reaction mixtures were then dialyzed against the above buffer and their absorption spectra were determined as described in section 2. (B) Conditions were the same as described in (A) except that emission spectra with excitation at 337 nm were determined. Closed circle, with *o*-phthalaldehyde; open circle, without *o*-phthalaldehyde.

Native and completely modified succinic semialdehyde reductase showed identical mobilities when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). No higher molecular weight species appeared from cross-linking of enzyme monomers. When succinic semialdehyde reductase was pretreated with iodoacetamide, a known -SH attacking agent, *o*-phthalaldehyde treated enzyme did not show any characteristic fluorescence intensity over a spectral range of 350 to 500 nm (data not shown). These results indicate that the modification of succinic semialdehyde reductase by *o*-phthalaldehyde results in the formation of isoindole derivatives via the cross-linking of proximal (3 Å) cysteine -SH group and lysine ε-NH_2 group within the same polypeptide chain.

Correlation between isoindole formation and enzyme activity is shown in Fig. 3. During the inactivation process there is a linear relationship between isoindole formation and the loss of enzyme activity which extrapolates to a stoichiometry of 1.0 mol of isoindole derivatives/mol of enzyme based on the increased absorbance at 337 nm. This value is corresponded well to that of stoichiometric incorporation of ligand (n = 1.16) obtained from the kinetic measurements (Fig. 1, inset) by the method of Levy et al. [22]. These results, once again, suggest that *o*-phthalaldehyde causes the inactivation of the enzyme by formation of isoindole derivatives.

In summary, *o*-phthalaldehyde inactivates bovine brain succinic semialdehyde reductase by cross-linking proximal cysteine and lysine residues to form fluorescent isoindole derivatives. Analysis of isolated peptides labeled with *o*-phthalaldehyde is not established in this work. In the absence of primary sequence and X-ray crystallographic structural data, it will be difficult to discuss as whether the modified residues are located within the active site or in the region whose conformation is altered upon the interaction of the enzyme with its coenzyme and inhibitor.

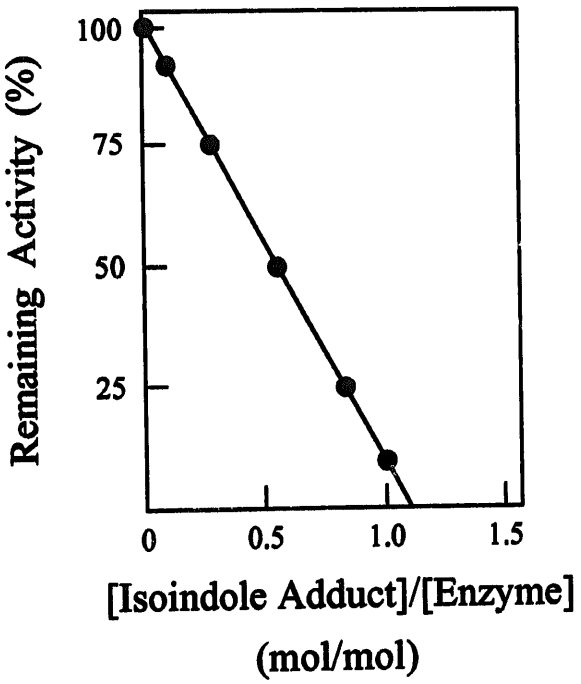

Fig. 3. Stoichiometry of *o*-phthalaldehyde inactivation. The enzyme (10 μM) was incubated with 100 μM *o*-phthalaldehyde in 0.1 M potassium phosphate buffer (pH 7.4) at 25°C. At time intervals, a portion of the reaction mixture was removed, dialyzed against the above buffer, and residual enzyme activity was determined. Isoindole formation was determined from the absorbance at 337 nm as described in section 2.

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