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Purification and Reaction Mechanism of Rat Brain Succinic Semialdehyde Dehydrogenase

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Abstract: Rat brain succinic semialdehyde dehydrogenase (EC 1.2.1.24 SSADH) activity was detected in mitochondrial, cytosolic and microsomal fractions. Brain mitochondrial soluble SSADH was purified by ammonium sulfate precipitation, DEAE Sephacel, and 5'-AMP Sepharose 4B affinity chromatography. The purified enzyme was shown to consist of four identical subunits, and the molecular weight of a subunit was 55 kD. The K_m for short chain aliphatic aldehydes and aromatic aldehydes were at the 10^{-3} M level but that for succinic semialdehyde was 2.2 μM. Either NAD^+ or $NADP^+$ can be used as a cofactor but the affinity for NAD^+ was 10 times higher than that for $NADP^+$. The brain cytosolic SSADH was also purified by ammonium sulfate precipitation, DEAE Sephacel, Blue Sepharose CL-6B and 5'-AMP Sepharose 4B affinity chromatography and its K_m for short chain aliphatic aldehydes was at the 10^{-3} level but that for succinic semialdehyde was 3.3 μM. NAD^+ can be used as a cofactor for this enzyme. We suppose that both enzyme might participate in the oxidation of succinic semialdehyde, which is produced during GABA metabolism. The activity of both cytosolic and mitochondrial SSADH was markedly inhibited when the concentration of succinic semialdehyde was high. The reciprocal plot pattern of product inhibition and initial velocity indicated a sequential ordered mechanism for mitochondrial matrix SSADH. Chemical modification data suggested that amino acid residues such as cysteine, serine and lysine might participate in the SSADH reaction.

Key words: γ-aminobutyric acid, rat brain, sequential ordered mechanism, succinic semialdehyde dehydrogenase.

Aldehyde dehydrogenase (ALDH, EC 1.2.1.3.) has a broad substrate specificity and it participates in the oxidation of aldehydes which are produced in the metabolism of ethanol, fatty acid and biogenic amines. Mammalian ALDH is distributed in liver, kidney (Danh *et al.*, 1983), brain (Weiner and Ardelt, 1984), heart (Hjelle *et al.*, 1983) and eye (Ting, 1983) but the functions of ALDH isozymes are different from each other.

The first investigation of the brain aldehyde dehydrogenase was conducted by Erwin and Deitrich (1966) on rat, monkey, and bovine brain. The investigators found that enzyme activity was present in all subcellular compartments. A few years later, Duncan and Tipton (1971a) investigated the kinetic properties of pig brain enzyme and concluded that brain ALDH was not a single enzyme, since Linweaver-Burk plots were hyperbolic. As a result of their study, and a later study of rat liver enzymes (Koivula *et al.*, 1981; Weiner and Ardelt, 1984; Tottamar *et al.*, 1973), ALDHs were divided into two broad groups: "low K_m" enzymes with micromolar K_m values for acetaldehyde and "high K_m" enzymes with millimlollar K_m values for acetaldehyde. Recently a high K_m enzyme has been purified to homogenity from human brain and identified as NAD^+-dependent succinic semialdehyde dehydrogenase (Ryzlak and Pietruszko, 1988).

It is well known that γ-aminobutyric acid (GABA), an inhibitory neurotransmitter in the the central nervous system, is synthesized from glutamate by glutamate decarboxylase and it is degraded to succinic semialdehyde by GABA transaminase (Takeuchi and Takeuchi, 1965; Kanjenvk and Schwartz, 1966). SSADH in the nervous system is of great interest since it catalyses the oxidation of succinic semialdehyde to succinate which is able to link to the TCA cycle. It has been suggested that this enzyme may have a relatively low K_m value for succinic semialdehyde. It is located in both mitochondria and synatosomes (Sims and Davis, 1973) and also uses a short chain aliphatic aldehyde as substrate (Kammeraat and Veldstra, 1968).

The reaction mechanism of ALDH has been known to be a sequential mechanism but the binding sequence of substrates, NAD^+ and aldehyde, is different from one ALDH isozyme to another. Duncan and Tipton (1971 b) indicated that pig brain ALDH followed the pattern

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of a compulsory ordered mechanism with aldehyde being the second substrate to bind and NADH being the second product to dissociate. However, yeast ALDH followed a pattern of a sequential mechanism with aldehyde being the first substrate to bind (Sidhu and Blair, 1975). In the case of human ALDH, when the concentration of substrate is high, the reaction has a random bi-bi mechanism which is produced by forming a double complex of enzyme and substrate (Bradbury and Jakoby, 1971). Studies with SSADH from pig brain, however, led Curchich and Blaner (1979) to conclude that a random-order rapid-equilibrium mechanism was obeyed.

This study describes the purification of rat brain SSADHs and discussed their kinetic properties and probable biological functions involved in aldehyde metabolism.

Materials and Methods

Materials

Male adult Sprague-Dawley rats (150~200 g) brain were used for purification.

DEAE-Sephacel, Sephacryl S-300, Blue Sepharose CL-6B was purchased from Pharmacia Fine Chemical Co. and 5'-AMP Sepharose 4B, deoxycholate, pyrazole, NAD^+, $NADP^+$, NADH, Coomassie Brilliant Blue R-250, p-nitrophenylacetate, p-hydroxybenzaldehyde, benzaldehyde, disulfiram, indole-3-acetaldehyde, succinic semialdehyde, molecular weight marker were Sigma Co. (St. Louis, USA) products. EDTA, Triton X-100, 2-mercaptoethanol, glutaraldehyde were Junsei Chemical Co. (Tokyo, Japan) products and acetaldehyde, propionaldehyde, glycoaldehyde were Fluka Co. (Buchs, Switzerland) products.

Preparation of enzyme

Enzyme preparations were carried out at 4℃. Rats were killed by decapitation and the brains were removed immediately and washed in ice-cold 0.9% NaCl. The brain tissues were cut into pieces and homogenized using Teflon-pestle Wheaton Elvehjem tissue homogenizer to make a protein concentration of 20% (w/v) in 0.25 M sucrose solution containing 10 mM Tris-HCl buffer (pH 7.4), 0.1% 2-mercaptoethanol and 1 mM EDTA. After centrifigation at $800\times g$ for 10 min to remove the nucleus and cell debris, the supernatant was centrifuged at $10,000\times g$ for 15 min to obtain the mitochondrial pellets and cytosolic supernatant. The pellets were then resuspended in same buffer. This mitochondrial fraction was used for the purification of SSADH. The supernatant was ultracentrifuged at $105,000\times g$ for 1 h to obtain microsomal pellets and the supernatant was used for the purification of SSADH.

Enzyme assay

Dehydrogenase activity of SSADH was determined by measuring spectrophotometrically the optical density at 340 nm for NADH formed during the reaction. The reation mixture(1ml) contained a final concentration of 50 mM sodium pyrophosphate (pH 8.4), 1 mM NAD^+, 40 μM succinic semialdehyde and enzyme. One unit of enzyme activity is defined as 1 nmol of NADH formed per 1 min.

Esterase activity of ALDH was determined spectrophotometrically at 400 nm as described by Duncan (1979) with p-nitrophenoxide as substrate. The K_m and maximum specific activity (V_{max}) were estimated from a straight line fitted to Lineweaver-Burk plots by least squares analysis and K_i was determined by Dixon plot.

Fluroescence measurements were carried out in a Kontron SFM-25 spectrophotometer.

Enzyme purification

The mitochondrial preparation from rat brain homogenate was resuspended in 10 mM sodium phosphate buffer (pH 6.8) containing 1mM EDTA and 0.1% 2-mercaptoethanol and sonicated using a sonicator (made by Vibra Cell,Sonics and Materials Inc., U.S.A.) and this was followed by centrifigation at $105,000\times g$ for 1 h to separate membrane and soluble fractions. The soluble fraction was 35% saturated with ammonium sulfate and centrifuged at $15,000\times g$ for 30 min to remove the precipitates. The supernatant was then 70% saturated with ammonium sulfate and centrifuged at 15,000 $\times g$ for 30 min to obtain precipitates. The precipitates were dissolved in 10 mM sodium phosphate buffer (pH 6.8) containing 1mM EDTA and 0.1% 2-mercaptoethanol and the solution was dialysed against 100 volumes of the same buffer. The dialysates were then chromatographed on a DEAE-Sephacel column. Non-bound proteins were washed out with the same buffer and the fractions containing SSADH activity were collected and concentrated by centricon (protein concentrator). The protein was applied to a column of 5'-AMP Sepharose 4B equilibrated with 100 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA, 0.1% 2-mercaptoethanol. After non-bound proteins were washed out with the same buffer, the bound proteins were eluted at the flow rate of 12.6 ml/h by the same buffer containing 0.25 mM NAD^+.

The cytosolic fraction was 30% saturated with ammonium sulfate and centrifuged at $15,000\times g$ for 30 min to remove the precipitates. The supernatant was then 65% saturated with ammonium sulfate and centri-

fuged at 15,000×g for 30 min to obtain precipitates. The precipitates were dissolved in 10 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.1% 2-mercaptoethanol and the solution was dialysed against 100 volumes of the same buffer. The dialysate were then chromatographed on DEAE-Sephacel column. Non-bound proteins were washed out with the same buffer and the fractions containing SSADH activity were collected and concentrated by centricon. The protein was applied to a column of Blue-Sepharose CL-6B equilibrated with the same buffer. After the non-bound proteins were washed out, the bound proteins were eluted by the concentration gradient of 0~1 M NaCl-phosphate solution. The fractions containing SSADH activity were collected and dialysed against 100 volumes of the same buffer. The dialysate was chromatographed on 5'-AMP Sepharose 4B equilibrated with the same buffer. After non-bound proteins were washed out with the same buffer, the bound proteins were eluted at the flow rate of 12.6 ml/h by the same buffer containing 0.25 mM NAD^+.

Protein determination

Protein concentration was determined as described by Lowry *et al.* (1951) with BSA as a standard, or spectrophotometrically at 280 nm.

Determination of the molecular weight of enzyme and subunits

The molecular weight of SSADH was determined by Sephacryl S-300 column chromatography employing molecular weight standard proteins such as thyroglobulin (660 kD), apo-ferritin (480 kD), γ-globulin (160 kD), ovalbumin (48 kD) and RNase A (18.4 kD). A void volume was measured using blue dextran. The flow rate was kept at 10 ml/h. Using the calibration curve prepared by measuring optical densities at 280 nm of eluents, the molecular weight of native SSADH was estimated. The molecular weight of a subunit was determined as follows. The purified SSADH preparation (10 μg protein) was dissolved in 10 μl of 0.0625 M Tris-HCl buffer (pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue (w/v) and heated in boiling water bath for a while to denature the protein. The heat treated SSADH and standard proteins were then subjected to polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970). The stacking gel was 4% acrylamide and the running gel was 12% acrylamide. The standard proteins were phosphorylase (97 kD), BSA (68 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD) and β-lactoglobulin (18.4 kD).

Table 1. Purification of rat brain mitochondrial matrix succinic semialdehyde dehydrogenase.

Step	Total activity (U[a])	Total protein (mg)	Specific activity (U[a]/mg)	Purification fold
Crude	283	90.6	3.1	1
$(NH_4)_2SO_4$ (30~70%)	161	19.7	8.2	2.6
DEAE-Sephacel	153	4.2	36.4	11.7
5'-AMP Sephrose 4B	145	0.025	5780	1853.7

[a]One enzyme unit was defined as the amount of enzyme to produce 1 nmol of NADH per 1 min.

Table 2. Purification of rat brain cytosolic succinic semialdehyde dehydrogenase.

Step	Total activity (U[a])	Total protein (mg)	Specific activity (U/mg)	Purification fold
Crude	2350	920	2.5	1
$(NH_4)_2SO_4$ (30~65%)	2220	203	10.9	4.4
DEAE-Sephacel	2080	31.7	65.6	26.2
Blue-Sephrose CL-6B	950	5	190	76
5'-AMP Sephrose 4B	77.1	0.027	2860	1140

[a]One enzyme unit was defined as the amount of enzyme to produce 1 nmol of NADH per 1 min.

Results and Discussion

The SSADH activity was traced using 40 μM succinic semialdehyde and we found that this activity was distributed in the various areas of rat brain with 13% in the cytosol, 75% in the mitochondrial and 12% in microsomal fraction. We have also investigated the subcellular distribution of ALDH activity of rat brain using 600 μM p-nitrobenzaldehyde and found 21.8% in the cytosol, 58.4% in the mitochondrial and 19.8% in the microsomal fraction (Joo and Kim, 1991). This suggested that ALDH isozymes present in rat brain must have different K_m values for aldehydes.

The fractions of both the mitochondrial matrix and the cytosolic SSADH obtained after 5'-AMP Sepharose chromatography were found to be homogeneous, as only one protein band with enzymatic activity was detected by polyacrylamide gel electrophoresis. The former has a specific activity of 5780 U/mg protein and the latter has a specific activity of 2860 U/mg protein. Molecular weights of the mitochondrial matrix SSADH and cytosolic SSADH appeared to be 220 kD and 200 kD, respectively by Sephacryl S-300 gel filtration. With SDS-PAGE of both mitochondrial matrix SSADH

Table 3. Kinetic data of rat brain mitochondrial matrix succinic semialdehyde dehydrogenase.

Substrate	K_m (M)	V_{max} (U[a]/mg)	V_{max}/K_m
Acetaldehyde	3.9×10^{-3}	680	1.7×10^{5}
Indole-3-acetaldehyde	1.8×10^{-4}	280	1.6×10^{6}
Succinic semialdehyde	2.2×10^{-6}	434000	2.0×10^{11}
Glycoaldehyde	8.3×10^{-4}	590	7.1×10^{5}
NAD^+	3.1×10^{-5}	19900	6.4×10^{8}
$NADP^+$	2.7×10^{-4}	3850	1.4×10^{7}

[a]One enzyme unit was defined as the amount of enzyme to produce 1 nmol of NADH per 1 min.

Table 4. Kinetic data of rat brain cytosolic succinic semialdehyde dehydrogenase.

Substrate	K_m (K)	V_{max} (U[a]/mg)	V_{max}/K_m
Acetaldehyde	3.4×10^{-3}	89.3	2.6×10^{4}
Propionaldehyde	6.0×10^{-4}	194	3.2×10^{5}
Succinic semialdehyde	3.3×10^{-6}	94000	2.8×10^{10}
NAD^+	1.7×10^{-5}	4890	2.9×10^{8}

[a]One enzyme unit was defined as the amount of enzyme to produce 1 nmole of NADH per 1 min.

and cytosolic SSADH, each showed a single band of molecular weight 55kD and 52 kD, respectively. This suggested that both enzymes are homologous tetramers.

It can be seen that the K_m of mitochondrial matrix SSADH for succinic semialdehyde is extremely low. The K_m for NAD^+ resembles that of the majority of other pyridine nucleotide-dependent dehydrogenases. Either NAD^+ or $NADP^+$ can be used as a cofactor but the affinity for NAD^+ was 10 times higher than that for $NADP^+$. This enzyme is also active with short chain aliphatic aldehydes but its K_{ms} are larger than that for succinic semialdehyde. The enzyme is subject to substrate inhibition with succinic semialdehyde and is activated by high NAD^+ concentration but no substrate inhibition occured with acetaldehyde and propionaldehyde in the range of concentrations shown in Table 3. The K_m of the cytosolic SSADH for short chain aliphatic aldehydes was at the 10^{-3} M level but that for succinic semialdehyde was 3.3 μM, and only NAD^+ can be used as a cofactor for this enzyme. These experiments showed that the reaction velocity with succinic semialdehyde was high as compared with other aldehydes (Table 4). The enzyme has a very low K_m for succinic semialdehyde: it exhibits strong substrate inhibition by succinic semialdehyde and substrate activation

Table 5. Inhibitory effect of modification reagents on rat brain mitocondrial matrix succinic semialdehyde dehydrogenase.

Modifier	Concentration (μM)	Activity remaining (%)	SSA protection (%)	NAD^+ protection (%)
N-ethylmaleimide (Cys)	50	0	54	23
Phenylmethylsulfonyl fluoride (Ser)	50	16	81.0	85.4
Pyridoxal phosphate (Lys)	50	50	80	70.1

[a]Each modification reagent was added to the enzyme reaction mixture and incubated for 1 min at 25℃. and the aliquot was taken and assayed. At protection experiment, succinic semialdehyde or NAD^+ was added to the reaction mixture and incubated for 5 min followed by the addition of modification reagent. Additional incubation was continued another 5 min. Values were expressed as percent assuming that the control value being 100. The concentrations of modification reagents were the final concentration in the reaction mixture.

by NAD^+. From the above experimental results both enzymes might participate in the oxidation of succinic semialdehyde which is produced during the GABA metabolism.

The mitochondrial matrix SSADH is active at pH 6.0, but its maximum activity is reached at pH 9.5. The enzyme is stable at 25~30℃ while the activity remained at 50% for 30 min at 45℃, and remained at 10% for 5 min at 55℃.

It is known that ALDH also hydrolyzes ester such as nitrophenylacetate. It has been suggested that ALDH, like glycealdehyde-3-phosphate dehydrogenase, forms covalent thioester intermediate during its catalytic process (Weiner, 1979). Feldman and Weiner (1972) proposed that the same active site was responsible for both esterase and dehydrogenase reactions. On the other hand, Blackwell *et al.* (1983) belived that the two activities of ALDH are located at nonidentical catalytic domains. However, the SSADH did not possess esterase activity as expected suggesting that this enzyme differs from other ALDH isozymes.

However, SSADH, like ALDH, was sensitive to disulfiram by which it was irreversibly inactivated. Both mitochondrial matrix SSADH and cytosolic SSADH were completely inhibited at 25 μM of disulfiram.

The mitochondrial matrix SSADH activity was inhibited completely by 50 μM N-ethylmaleimide (NEM) suggesting that cysteinyl residue are located nearby the active site of the enzyme where the activity remained at 54% and 23% during the pre-incubation with succinic semialdehyde and NAD^+, respectively. Also, this

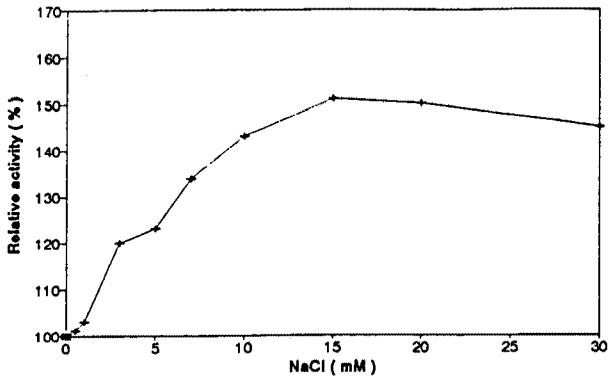


Fig. 1. Effect of monovalent ions on the activity of rat brain mitochondrial matrix SSADH. Variation of the enzyme activity in the presence of monovalent ions such as Na^+, K^+, NH_4^+ was assayed at 25℃, 0.1 M Tris-HCl (pH 8.6). Values were expressed as percent assuming that the control value being 100.

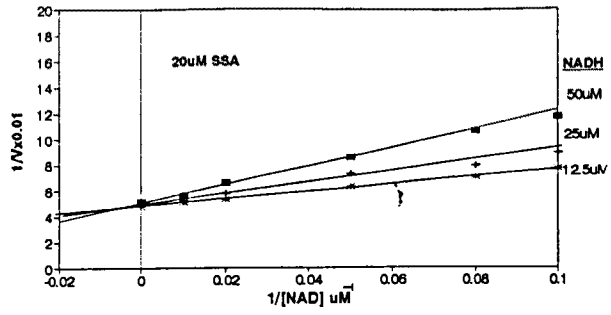


Fig. 2. Double reciprocal plot of initial velocity of the rat brain mitochondrial matrix SSADH versus NAD^+ concentration at different fixed NADH (12.5, 25, 50 μM) and SSA (20 μM) concentrations.

enzyme was inhibited 94% by 50 μM phenylmethylsulfonyl fluoride (PMSF) suggesting that one of the active sites is serine, while the activity remainedat 81% and 85.4% during the pre-incubation with succinic semialdehyde and NAD^+, respectively. This enzyme is also inactivated by incubation with pyridoxal phosphate (PLP). The effect of the addition of PLP on the activity of SSADH was investicated by preincubating the enzyme with 50 μM PLP at a temperature of 25℃. Aliquots of the pre-incubation mixtures of chemical modification reagents show effects on the enzymatic activity as demonstrated in Table 5. In ALDH catalysis of aldehyde oxidation, like all other $NAD^+(P^+)$-dependent dehydrogenases, a hydride ion is removed from the substrate and an acylonium ion would have to be formed. However, it was postulated and proved later that with glycealdehyde phosphate dehydrogenase, a cysteine residue first reacts to form a thio adduct with aldehyde and this thiohemiacetal is oxidized to produce the stable thioester. This same intermediate was postulated to exist in the overall reaction of ALDH (Feldmand and Weiner, 1972). However, no actual evidence for its existence has been presented. Chemical modification data suggested that amino acid residues such as cysteine, serine and lysine might participate in the SSADH reaction.

Pitts and Quick (1965) demonstrated an activation of SSADH from mammalian brain by NaCl. We found that rat brain mitochondrial matrix SSADH was activated 10% by 10 mM NaCl in 0.1 M sodiumpyrophosphate. Beacause the concentration of monovalent ions (Na^+ or Cl^-) was not known exactly in this assay buffer system, the enzyme was assayed in Tris-HCl buffer (pH 8.6). The enzyme activity was activated by 10 mM NaCl, KCl and NH_4Cl but was inhibited when the concentration of NaCl, KCl and NH_4Cl was over 0.1 M (Fig. 1, Data for NaCl shown). However, it is not known whether the activation was due to sodium or chloride ions, or increased salt concentration of the incubation medium. Rivett and Tipton (1981) suggested that sodium chloride had no effects on the activity of the enzyme when assayed in pyrophosphate buffer, although apparently it does in Tris buffer. In K^+-activated ALDH, Dickinson and Haywood (1987) demonstrated that the principal effect of K^+ on the dehydrogenase reaction is to accelerate a step (possibly acyl-enzyme hydrolysis) that occurs after hydride transfer and before NADH dissociation from the terminal E-NADH complex. On the other hand, Takahashi and Weiner (1980) suggested that the addition of Mg^{2+} ions enhances the specific activity of horse liver ALDH by a factor of 2 and the enzyme exhibits half-of-sites reactivity in the absence of Mg^{2+} and all-of-sites reactivity in its presence. Therefore they demonstrated that Mg^{2+} ions caused a dissociation of the tetrameric enzyme into dimers to enhance enzyme activity. Actually, it can be observed that purified rat brain mitochondrial matrix SSADH was more active in 100 mM NaCl than in its absence. Also, native 220 kD enzyme in Sephacryl S-300 gel chromatography appeared to have a native molecular weight of 110 kD eluted with the same buffer containing 0.1 M NaCl, and the enzyme had an enhanced specific activity by a factor of 2. Therefore it seemed that NaCl might have caused a dissociation of tetrameric enzyme into dimers to enhance enzyme activity. But this requires further research.

A double-reciprocal 1/v *vs.* 1/[s] plot of the initial velocity of the mitochondrial matrix SSADH versus the NAD^+ concentration at different fixed succinic semialdehyde concentrations, and a double reciprocal plot of the initial velocity of the enzyme versus succinic semialdehyde at different fixed NAD^+ concentrations produced a family of intersecting lines. Though this pattern would be obtained in case of either a sequential ordered or a rapid equilibrium random mechanism, it

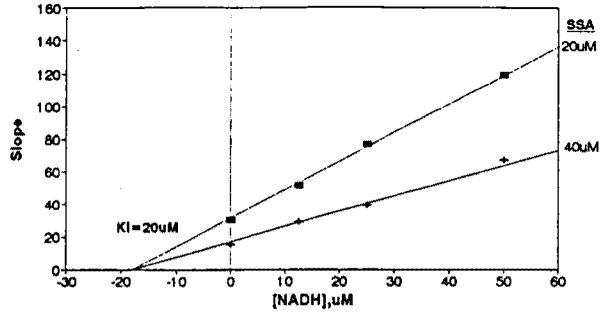


Fig. 3. Slopes obtained from the reciprocal plots of the rat brain mitochondrial matrix SSADH were replotted against NADH concentration. After the slope of the lines of the double reciprocal plot was calculated at each succinic semialdehyde concentration, the slopes were replotted against NADH at fixed succinic semialdehyde concentrations. The competitive inhibition constant (K_i value) of NADH for NAD^+ was identical at all concentrations of SSA.

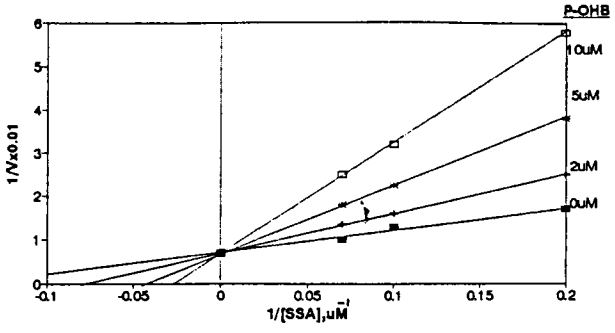


Fig. 4. Double reciprocal plot of initial velocity of the rat brain mitochondrial matrix SSADH versus SSA concentration at different fixed p-hydroxybenzaldehyde concentrations (0, 2, 5, 10 μM).

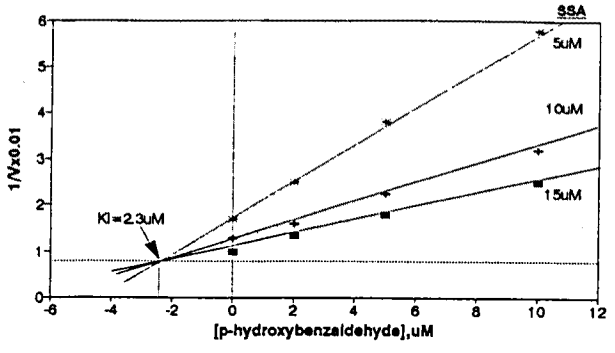


Fig. 5. Dixon plot of initial velocity of the rat brain mitochondrial matrix SSADH versus p-hydroxybenzaldehyde concentration at different fixed SSA concentrations (5, 10, 15 μM).

must be investigated further by studies of product inhibition, because the mechanism would not be discriminated examining only by initial velocities (Cleland, 1963). Because 20 mM succinate did not cause detectable inhibition at either pH 8.4 or pH 7.4, and this enzyme reaction has no reversibility, we made a double reciprocal plot of the initial velocity of the enzyme versus

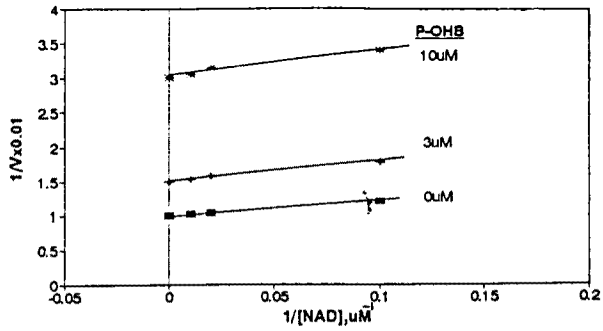


Fig. 6. Double reciprocal plot of initial velocity of the rat brain mitochondrial matrix SSADH versus NAD^+ concentration at different fixed p-hydroxybenzaldehyde concentrations (0, 3, 10 μM).

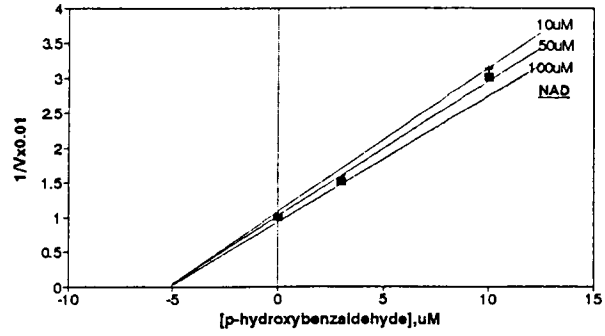


Fig. 7. Dixon plot of initial velocity of the rat brain mitochondrial matrix SSADH versus p-hydroxybenzaldehyde concentration at different fixed NAD^+ concentrations(10, 50, 100 μM).

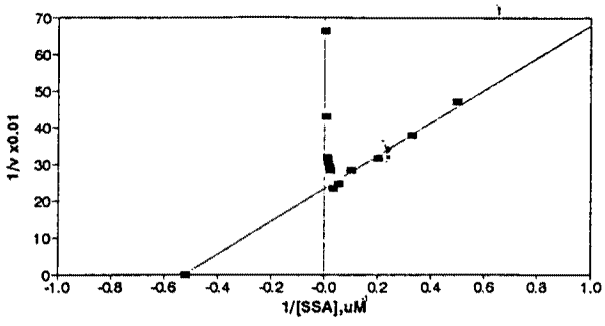


Fig. 8. Double reciprocal plot of initial velocity of the rat brain mitochondrial matrix SSADH versus different SSA concentrations. This plot shows an inhibition of the enzyme by succinic semialdehyde of high concentrations.

NAD^+ concentration at different fixed NADH (12.5, 25, 50 μM) and succinic semialdehyde (20, 40 μM) concentrations (Fig. 2.). In mitochondrial matrix SSADH, inhibition by NADH was found to be competitive with respect to NAD^+ at either 20 μM succinic semialdehyde or 40 μM (fully saturated) succinic semialdehyde. The K_i value for inhibition with respect to different fixed NAD^+ concentrations was calculated to be 20 μM. After the slope of the lines of the double reciprocal plot was calculated at each succinic semialdehyde con-

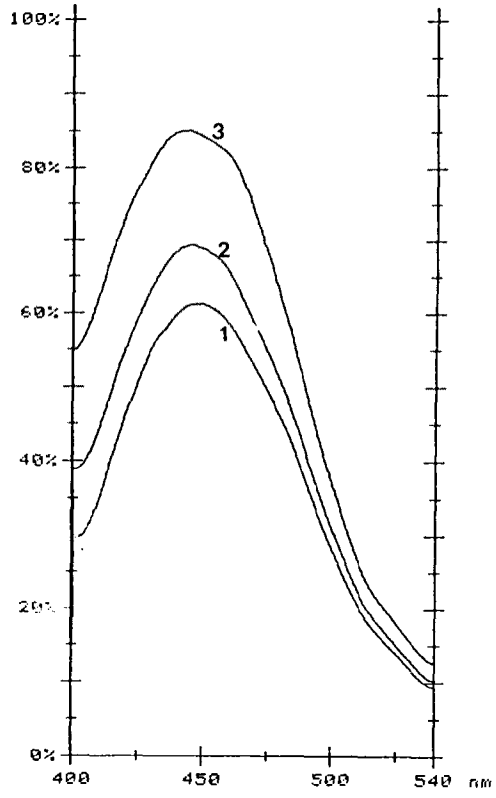
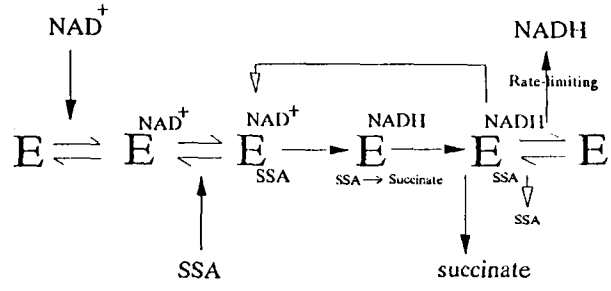


Fig. 9. Fluorescence emission spectra of (1) NADH (10 nM)+enzyme (10 nM), (2) NADH (10 nM)+enzyme (10 nM)+SSA (40 μM) and (3) NADH (10 nM)+enzyme (10 nM)+SSA (100 μM) at pH 8.4, exitation wavelength 340 nm.

centration, the slopes were replotted against NADH at fixed succinic semialdehyde concentrations. The K_i slope of NADH to NAD^+ was 20 μM at all succinic semialdehyde concentrations (Fig. 3). Cleland (1970) has suggested that $K_{islope}=K_{iq}(1+B/K_{ib})$ in the rapid random mechanism but $K_{islope}=K_{iq}$ in the ordered mechanism [B (or b) is the second substrate i.e. aldehyde and Q(or q) is the second product.]. To clarify the mechanism, the effects of the dead-end inhibitor, p-hydroxybenzaldehyde was studied. We produced a double reciprocal plot of the enzyme versus succinic semialdehyde at different fixed p-hydroxybenzaldehyde concentrations at constant NAD^+ concentration. The inhibition by p-hydroxybenzaldehyde was competitive with respect to succinic semialdehyde and a K_i value of 2.3 μM was determined (Fig. 4, 5). A double-reciprocal plot versus NAD^+ at different fixed p-hydroxybenzaldehyde at constant succinic semialdehyde concentration was also obtained. The inhibition by p-hydroxybenzaldehyde was uncompetitive with respect to NAD^+ and the K_i value was 5 μM (Fig. 6, 7).

Initial-rate studies showed that the mitochondrial matrix SSADH (Fig. 8) and cytosolic SSADH was inhibited by high concentrations of succinic semialdehyde. The strong binding of NADH to rat brain mitochondrial matrix SSADH can be demonstrated by fluroescence measurements conducted at 0.1 M sodium pyrophosphate (pH 8.4). As shown in Fig. 9, the addition of NADH to the enzyme was accompanied by an enhancement of fluroescence emitted by NADH over the spectral range 400 to 500 nm. In addition, a blue shift in the emission spectrum of bound NADH as compared to free NADH in solution and an enhancement of relative flouscence were easily detected at an enzyme concentration of 10 nM, succinic semialdehyde (100 μM) and NADH (10 nM). Thus the reaction catalyzed by SSADH can be represented by the following scheme.



References

Blackwell, L. F., Bennett, A. F. and Buckley, P. D. (1983) *Biochemistry* **22**, 3784.

Bradbury, S. L. and Jakoby, W. B. (1971) *J. Biol. Chem.* **246**, 6929.

Churchich, J. and Blaner, W. S. (1979) *J. Biol. Chem.* **254**, 1794.

Cleland, W. W. (1963) *Biochim. Biophys. Acta.* **67**, 104.

Cleland, W. W. (1970) in *The Enzymes* (Boyer, P. D., ed), 3rd., Vol. 2, pp. 1-65, Academic Press, New York.

Danh, H. D., Benedetti, M. S. and Dostert, P. (1983) *J. Neurochem.* **41**, 618.

Dickinson, F. M. and Haywood, G. W. (1987) *Biochem. J.* **24**, 377.

Duncan, R. J. S. and Tipton, K. F. (1971a) *Eur. J. Biochem.* **22**, 257.

Duncan, R. J. S. and Tipton, K. F. (1971b) *Eur. J. Biochem.* **22**, 538.

Duncan, R. J. S. (1979) *Biochem. J.* **183**, 459.

Erwin, V. G. and Deitrich, R. A. (1966) *J. Biol. Chem.* **241**, 3533.

Feldman, R. I. and Weiner, H. (1972) *J. Biol. Chem.* **247**, 267.

Hjelle, J. T., Pertersen, D. R. and Hjelle, J. J. (1983) *J. Pharmacol. Exp. Ther.* **224**, 699.

Joo, C. N. and Kim, H. K. (1991) *Korean Biochem. J.* **24**, 271.

Kammeraat, C. and Veldstra, H. (1968) *Biochim. Biophys. Acta.* **131**, 390.

Kanjenvk, K. and Schwartz, S. (1966) *Federation Proc.* **25**,

627.
Koivula, T., Turner, A. J. and Koivusalo, M. (1981) *J. Nerochem.* **36**, 1893.
Laemmli, U. K. (1970) *Nature* **227**, 680.
Lowry, O. M., Rosenburogh, N. J., Foor, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 205.
Pitts, F. N. and Quick, C. (1965) *J. Neurochem.* **12**, 893.
Rivett, A. J. and Tipton, K. F. (1981) *Eur. J. Biochem.* **117**, 187.
Ryzlak, M. T. and Pietruszko, R. (1988) *Arch. Biochem. Biophys* **266**, 386.
Sidhu, R. S. and Blair, A. H. (1975) *J. Biol. Chem.* **250**, 7899.
Sims, K. L. and Davis, G. A. (1973) *Eur. J. Biochem.* **35**, 450.
Takahashi, K. and Weiner, H. (1980) *J. Biol. Chem.* **255**, 8206.
Takeuchi, A. and Takeuchi, N. (1965) *J. Physiol.* **177**, 225.
Ting, H. H. and Crabble, M. J. C. (1983) *Biochem. J.* **215**, 351.
Tottmar, S. O. C., Petersson, H. and Kiessling, K. H. (1973) *Biochem. J.* **135**, 577.
Weiner, H. and Ardelt, B. (1984) *J. Neurochem.* **42**, 109.