Characterization of succinic semialdehyde dehydrogenase from Aspergillus niger

Santosh Kumar, Sunil Kumar & Narayan S. Punekar*
Metabolism and Enzymology Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India.

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The catabolism of fungal 4-aminobutyrate (GABA) occurs via succinic semialdehyde (SSA). Succinic semialdehyde dehydrogenase (SSADH) from the acidogenic fungus Aspergillus niger was purified from GABA grown mycelia to the highest specific activity of 277 nmol min⁻¹ mg⁻¹, using phenyl Sepharose and DEAE Sephacel chromatography. The purified enzyme was specific for its substrates SSA and NAD⁺. The substrate inhibition observed with SSA was uncompetitive with respect to NAD⁺. While product inhibition by succinate was not observed, NADH inhibited the enzyme competitively with respect to NAD⁺ and noncompetitively with respect to SSA. Dead-end inhibition by AMP and p-hydroxybenzaldehyde (pHB) was analyzed. The pHB inhibition was competitive with SSA and uncompetitive with NAD⁺; AMP competed with NAD⁺. Consistent with the kinetic data, a sequential, ordered Bi Bi mechanism is proposed for this enzyme.

Keywords: Bi Bi mechanism, Enzyme kinetics, GABA metabolism

Besides being an important neurotransmitter of mammalian nervous system, 4-aminobutyrate (GABA) is a non-protein amino acid ubiquitous to fungi. GABA is implicated in the fungal nitrogen and energy metabolism, sporulation, differentiation and development. Fungal GABA arises largely from L-glutamate decarboxylation whereas catabolism of agmatine and 4-guanidinobutyrate could provide additional routes for its formation. Regardless of its source, fungal GABA is catabolized through two steps involving GABA transaminase and succinic semialdehyde dehydrogenase (SSADH). Of these, SSADH catalyzes an essentially irreversible step and is the least studied. The GABA shunt consisting of glutamate decarboxylase, GABA transaminase and succinic semialdehyde (SSADH) offers a variation to the classical Krebs cycle. These three enzymes from Aspergillus niger, a prolific citric acid producer, have been demonstrated under different metabolic states. A higher flux through GABA shunt was detected during acidogenic growth of A. niger. GABA shunt, and by implication SSADH, is suggested to play a role in the alternate version of Krebs cycle in Cyanobacteria. In this Krebs cycle variant, 2-oxoglutarate dehydrogenase complex is replaced with two enzymes (2-oxoglutarate decarboxylase and SSADH) that convert 2-oxoglutarate to succinate via SSA. SSADH is also responsible for the thermotolerance in Saccharomyces cerevisiae. SSADH deficiency leads to hyperGABAergic seizures in humans.

Succinic semialdehyde (SSA) is a metabolite unique to the GABA catabolic pathway. The SSADH activity is demonstrated across bacteria, fungi, mammals, and plants. In most cases the enzyme is specific to SSA with high affinity for this substrate; substrate inhibition at higher SSA concentrations is also common. None of the SSADHs studied thus far catalyze the reverse reaction – reduction of succinate to SSA. The elucidation of its kinetic mechanism is further complicated by the absence of product inhibition by succinate. Dead-end inhibitors like p-hydroxybenzaldehyde (pHB) and N-formylglycine (which is isosteric with SSA) are often used for this purpose. The enzymes from potato tuber and from rat brain follow a sequential ordered Bi Bi mechanism wherein NAD⁺ is the first substrate to bind and NADH is the last product to dissociate. However, a sequential random Bi Bi mechanism is reported for SSADH from pig brain.

In order to elaborate its role in A. niger metabolism, we have followed the induction of SSADH activity under different growth conditions. The molecular and kinetic properties of the purified SSADH from GABA grown mycelia are reported.
Materials and Methods

Organism and growth conditions—Aspergillus niger NCIM 565 was obtained from National Collection of Industrial Microorganisms at NCL, Pune, India. A. niger was grown at 30 °C for 30 h, in one liter flasks containing 100 ml minimal medium as surface cultures. Wherever appropriate, ammonium nitrate was replaced by GABA, L-arginine, L-proline, L-glutamate or L-ornithine (all at 50 mM) as sole nitrogen source. In separate shake culture experiments, A. niger was also grown at 30 °C for 24 h in one liter flasks containing 200 ml minimal medium with agmatine (14.0 mM) or 4-guanidinobutyrate (18.7 mM) as sole nitrogen source. The mycelia were harvested by filtration, washed with distilled water and blotted between folds of filter paper. The cells were stored at −20 °C and used within a week.

Enzyme assay and activity staining—The A. niger SSADH activity was determined by monitoring NADH formation at 340 nm as reported before. For inhibition study, the enzyme in sodium pyrophosphate buffer (pH 9.0) was first incubated for 5 min with individual inhibitors and then with NAD⁺ for further 3 min. The reaction was initiated by addition of SSA (100 μM, final concentration), unless otherwise mentioned. The kinetic data were analyzed using the initial linear portion of the progress curve. One SSADH activity unit corresponds to the amount of enzyme required to form one nmol of NADH per min in the standard assay. Specific SSADH activity is expressed as U mg⁻¹ protein. The Δ¹-pyrroline-5-carboxylate dehydrogenase (P5CDH) was assayed essentially similar to that of SSADH except that 2 mM Δ¹-pyrroline-5-carboxylate replaced SSA in the reaction.

A. niger SSADH activity on native polyacrylamide slab gel electrophoresis (PAGE) gels was visualized by the nitro blue tetrazolium method. For this, the enzyme sample was resolved on native PAGE (see below) but at 4 °C to prevent thermal inactivation. The gel was washed with double distilled water and then incubated in sodium pyrophosphate buffer (pH 8.0) for 15 min. Individual sample lanes were sliced out and used for activity staining. The reaction mixture (total volume of 10 ml in a Petri plate) for in-gel staining of SSADH activity contained 100 mM sodium pyrophosphate buffer (pH 8.0), 100 μM SSA, 5.0 mM NAD⁺, 2.0 mg nitro blue tetrazolium chloride and 1.0 mg phenazine methosulphate. A control without substrate (SSA) was also included. Gels were incubated at 37 °C for 2 h until formazan bands were visible. Subsequently, the gel slices were washed with water, and then with 7.5% acetic acid, to clear the background.

Results

Induction of A. niger SSADH activity—Considerable SSADH activity was observed in the crude extracts of A. niger when the fungus was grown as surface cultures on GABA (Fig. 1). The levels of this enzyme were not significant when grown on other nitrogen sources like ammonium nitrate, glutamate, proline and arginine. Another enzyme acting on semialdehydes namely, Δ¹-pyrroline-5-carboxylate dehydrogenase (P5CDH) was also monitored under these conditions. The P5CDH activity was present only in proline and arginine grown mycelia. Fungal GABA could have alternate metabolic origins including from polyamines. SSADH activity was detected in A. niger mycelia grown on agmatine and 4-guanidinobutyrate. Specific

Purification—SSADH activity from A. niger mycelia was extracted as described before. The active ammonium sulfate fraction (between 30-60 % saturation) of the crude enzyme extract was loaded on to a pre-equilibrated phenyl-Sepharose column (bed volume 10 ml), at a flow rate of 10 ml h⁻¹. After washing the column with the same buffer, SSADH was eluted (4.0 ml fractions) using a simultaneous gradient of 0-25% glycerol and 0.5-0.0 M ammonium sulfate (total volume of 100 ml). The fractions were assayed for enzyme activity; the SSADH peak fractions were pooled and loaded on to a DEAE-Sephacel (bed volume 5.0 ml) column. This column was pre-equilibrated and developed with 100 mM sodium pyrophosphate buffer at pH 8.0 (also containing 14 mM 2-mercaptoethanol and 20% glycerol) at a flow rate of 10 ml h⁻¹. Unbound active fractions were pooled and assayed for SSADH activity and protein content. This final enzyme preparation was directly used for kinetic studies within 24 h.

General protein techniques—Polyacrylamide slab gel electrophoresis (PAGE) was performed according to Davis and proteins were visualized through Coomassie Blue R250 staining. The native molecular weight of SSADH was determined by gel filtration on a calibrated Sephacryl S-200 column with Blue Dextran to determine the void volume. Protein was estimated according to Bradford’s method using bovine serum albumin as reference.

Statistical analysis—The enzyme kinetic results are typical reproductions of at least three independent experiments. All kinetic data were processed using the software SigmaPlot (Version 9.0); a correlation coefficient of 0.95 or higher was always obtained for these data. The nature of inhibition was evaluated by fitting the data to appropriate kinetic equations.
activity of this enzyme in the crude extracts from GABA (31.2 U mg\(^{-1}\)) and 4-guanidinobutyrate (28.4 U mg\(^{-1}\)) grown mycelia was comparable.

**Purification and characterization of A. niger SSADH**—Extraction of SSADH activity from A. niger mycelia was best achieved in a buffer containing Triton X-100 (0.1%) suggesting its mitochondrial location. The SSADH activity was best measured in sodium pyrophosphate buffer (pH 8.0) while reduced activity was observed when Tris-HCl buffer was used. The enzyme activity was unstable and no activity could be detected in buffers devoid of 2-mercaptoethanol; all purification buffers therefore contained 14 mM 2-mercaptoethanol.

Majority of SSADH activity from A. niger mycelial extracts was recovered in the 30-60% (NH\(_4\))\(_2\)SO\(_4\) fraction. This enriched enzyme was further purified to near homogeneity in two chromatography steps: (i) hydrophobic interaction chromatography on phenyl-Sepharose CL-4B; and (ii) negative binding with DEAE-Sephacel matrix. The optimized sequence of purification steps is summarized in Table 1. A total of 9-fold purification of SSADH (specific activity, 277 U mg\(^{-1}\)) with a 13% yield was achieved.

Two distinct peaks of SSADH activity were resolved upon phenyl-Sepharose chromatography. Besides SSADH activity, the first peak (Fig. 2A) fractions also showed activity with other aldehyde substrates namely, acetaldehyde and propionaldehyde (data not shown). The second sharper peak, eluted at 0.0 M (NH\(_4\))\(_2\)SO\(_4\) and 25% glycerol, was specific to SSA. Only these fractions corresponding to specific SSADH activity (Fig. 2B) were pooled and subjected to DEAE-Sephacel step. Negative binding on DEAE-Sephacel eliminated a few contaminant proteins but without any further loss in SSADH activity. Enzyme fractions were analyzed on native PAGE after every step of purification; enriched SSADH enzyme protein was also detected on native PAGE gel by activity staining with SSA as substrate (Fig. 3). Neither the

<table>
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<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U mg(^{-1}))</th>
<th>Yield (%)</th>
<th>Fold</th>
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<tr>
<td>Crude extract</td>
<td>167.4</td>
<td>5226</td>
<td>31.2</td>
<td>100.0</td>
<td>1.0</td>
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<tr>
<td>30-60% (NH(_4))(_2)SO(_4) fraction</td>
<td>71.3</td>
<td>4859</td>
<td>68.1</td>
<td>93.0</td>
<td>2.2</td>
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<tr>
<td>Phenyl-Sepharose</td>
<td>8.3</td>
<td>864</td>
<td>116.1</td>
<td>18.5</td>
<td>4.1</td>
</tr>
<tr>
<td>DEAE-Sephacel (unbound)</td>
<td>2.6</td>
<td>716</td>
<td>277.6</td>
<td>13.1</td>
<td>8.9</td>
</tr>
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</table>

Fig. 1—Comparison of SSADH and P5CDH levels in A. niger mycelial extracts grown on various nitrogen sources.

Fig. 2—Elution profile of A. niger SSADH from the phenyl-Sepharose column. Column run details are as described in Materials and Methods. Peak A fractions displayed aldehyde dehydrogenase activity with acetaldehyde, propionaldehyde as well as SSA. The activity associated with peak B was specific to SSA alone.

Fig. 3—Native PAGE analysis and activity staining of purified A. niger SSADH. (A) The protein profile after different purification steps: Lane 1, 30-60% ammonium sulfate precipitate; Lane 2, pooled fraction from phenyl-sepharose column; Lane 3, unbound fraction from DEAE Sephacel column. (B) SSADH activity staining: The enzyme after the DEAE-Sephacel step was electrophoresed at 4 °C. Individual lanes were cut and incubated with (Lane 2) or without (Lane 3) SSA. Lane 1 corresponds to Coomassie blue stained protein gel of the same sample. The band corresponding to SSADH is marked by arrow.
SSADH activity band nor any other bands were stained when acetaldehyde and propionaldehyde were used as substrates (not shown). In all the kinetic studies A. niger SSADH from the final DEAE-Sephacel step was used.

A. niger SSADH was labile and hence, the purified enzyme (after the final step) was stored at $-20^\circ C$ in 20% glycerol; this preparation lost about 50% activity in five days. The enzyme exhibited a pH optimum in the alkaline range (pH 9-10) and an optimum temperature between 30 and 35 $^\circ$C. The A. niger enzyme was specific for NAD$^+$ and was not active with NADP$^+$ as a cofactor. A single peak of SSADH activity, corresponding to molecular mass of 160 kDa, was observed on Sephacryl S-200 column and on SDS-PAGE it gave a major band of 44 kDa (not shown), suggesting that the enzyme is possibly a tetramer of identical subunits.

**Substrate saturation and initial velocity study**—The SSA saturation of A. niger SSADH did not follow the typical hyperbolic kinetics. Instead, substrate inhibition was indicated as the initial velocity declined at higher SSA concentrations (Fig. 4A). This is also evident from the curvature near $1/v$ axis of the Lineweaver-Burk plot (Fig. 4A, inset). On the other hand, NAD$^+$ saturation followed a typical Michaelian behavior. Kinetic mechanism of A. niger SSADH was examined in some detail through initial velocity studies with both the substrates, SSA and NAD$^+$. Double reciprocal plots of $1/v$ versus $1/[SSA]$, at different fixed concentrations of NAD$^+$, gave a series of straight lines intersecting above the x-axis (Fig. 5A). A similar pattern was also obtained when $1/v$ versus $1/[NAD^+]$ data was plotted at various fixed concentrations of SSA (Fig. 5B). The following kinetic parameters were extracted from this analysis: $V_{max} = 336$ U mg$^{-1}$; $K_{NAD} = 3.1$ mM; $K_{SSA} = 28$ $\mu$M; $K_{i(NAD)} = 2.3$ mM.

**Substrate, product and dead-end inhibitions**—A. niger SSADH was inhibited by SSA above 200 $\mu$M (Fig. 4A) which was further evaluated by Dixon

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**Fig. 4**—Substrate inhibition of A. niger SSADH. (A) Effect of increasing SSA concentrations on A. niger SSADH activity. Inset shows the double reciprocal plot of the same data. (B) Dixon plot for the substrate inhibition of SSADH at different fixed concentrations of NAD$^+$.

**Fig. 5**—Initial velocity study of A. niger SSADH. (A) SSA saturation at different fixed concentrations of NAD$^+$ and (B) NAD$^+$ saturation at different fixed concentrations of SSA.
analysis. A set of parallel lines were obtained indicating that substrate inhibition by SSA was uncompetitive with respect to NAD$^+$; a $K_{i[SSA]}$ of 2.7 mM was calculated from the replot (Fig. 4B). Of the two products, only NADH showed significant product inhibition while succinate (even at 10 mM) did not inhibit the *A. niger* SSADH. Observed NADH inhibition was noncompetitive with respect to SSA (at 1.5 mM NAD$^+$, Fig. 6A) and was competitive with respect to NAD$^+$ (at 20 μM SSA, Fig. 6B). Both the NADH inhibitions were linear (not shown) and the extracted kinetic inhibition constants are listed in Table 2.

*p*-Hydroxybenzaldehyde (pHB) is an excellent dead-end inhibitor of SSADH from many sources. The *A. niger* SSADH was also susceptible to inhibition by pHB. The pHB inhibition was linear and competitive with respect to SSA (Fig. 7A) whereas it showed uncompetitive inhibition with respect to NAD$^+$.

**Table 2—Kinetic constants obtained for different *A. niger* SSADH inhibitors**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate varied</th>
<th>Inhibition type $^a$</th>
<th>$K_{in}$ (mM)</th>
<th>$K_{ii}$ (mM)</th>
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<tr>
<td>SSA (Substrate)</td>
<td>NAD$^+$</td>
<td>UC</td>
<td>2.71</td>
<td>-</td>
</tr>
<tr>
<td>NADH (Product)</td>
<td>NAD$^+$</td>
<td>C</td>
<td>0.55</td>
<td>-</td>
</tr>
<tr>
<td>NADH (Product)</td>
<td>SSA</td>
<td>NC</td>
<td>0.44</td>
<td>0.88</td>
</tr>
<tr>
<td>5’AMP (Dead end)</td>
<td>NAD$^+$</td>
<td>C</td>
<td>22.95</td>
<td>-</td>
</tr>
<tr>
<td>5’AMP (Dead end)</td>
<td>SSA</td>
<td>NC</td>
<td>31.76</td>
<td>45.72</td>
</tr>
<tr>
<td>pHB (Dead end)</td>
<td>SSA</td>
<td>C</td>
<td>3.37  $^b$</td>
<td>-</td>
</tr>
<tr>
<td>pHB (Dead end)</td>
<td>NAD$^+$</td>
<td>UC</td>
<td>-</td>
<td>3.90 $^b$</td>
</tr>
</tbody>
</table>

$^a$All inhibitions were linear. C, Competitive; NC, Noncompetitive; UC, Uncompetitive; $^b$in μM
AMP was a relatively poor dead-end inhibitor of *A. niger* SSADH. The AMP inhibition was competitive with NAD$^+$ and noncompetitive with respect to SSA (not shown). The inhibition constants for the two dead-end inhibitors were determined from the slope/intercept replots and are summarized in Table 2.

**Discussion**

GABA is catabolized in two steps involving GABA transaminase and SSADH in *A. niger*. SSADH catalyzes an essentially irreversible step that allows the carbon flux to enter Krebs cycle. The enzyme therefore assumes significance in the acidogenic metabolism of this fungus.

*A. niger* SSADH activity was specific for SSA as its substrate and was induced in GABA grown mycelia. SSADH activity was also detected in the cell extracts of *A. niger* grown on 4-guanidinobutyrate (and agmatine). Considering the alternate routes for the origin of GABA (Fig. 8), it is possible that 4-guanidinobutyrate (and agmatine) is metabolized via GABA (and hence through SSADH). A specific 4-guanidinobutyrase, hydrolyzing 4-guanidinobutyrate to GABA, is reported from this fungus. *A. niger* also displays a non-specific aldehyde dehydrogenase which could be resolved from SSADH on phenyl-Sepharose column (Fig. 2). Since it was induced only on arginine and proline (Fig. 1), the observed P5CDH activity is distinct from SSADH.

GABA being a neurotransmitter, its metabolism in the central nervous system is important. Accordingly, efforts to purify SSADH are mostly confined to the enzyme from mammalian brain. This is the first report on the enrichment of SSADH from a fungus. That the final preparation was indeed a specific SSADH was ascertained by enzyme assays and activity staining. The phenyl-Sepharose column efficiently resolved the non-specific aldehyde dehydrogenase component (Fig. 2) of the crude *A. niger* mycelial extracts. For this reason, the observed fold purification for SSADH (Table 1) is most likely an underestimate. Commonly observed characteristics of SSADH from various sources include: a) facile purification on a hydrophobic interaction column; b) an optimum pH between 8.5 and 10; c) requirement of high concentration of 2-mercaptoethanol for activity; d) catalysis of an essentially irreversible oxidation of SSA; e) substrate inhibition with SSA; and f) inability to use NADP$^+$ as a cofactor. The *A. niger* SSADH is no exception on all these counts. There are two types of SSADHs reported in the literature; they either contain one cysteine or two cysteines in the catalytic loop. The catalytic loop Cys-SH is required for SSADH activity. The nucleophilic cysteine of the ‘2-Cys-SSADHs’ remains protected under oxidizing conditions by forming a disulfide with the second cysteine.

The irreversible nature of *A. niger* SSADH catalysis and absence of succinate (one of the products) inhibition, limited its steady state kinetic investigation. Expectedly, no evidence for the formation of an enzyme-NADH-succinate complex was found with pig brain SSADH. The kinetic mechanism of *A. niger* SSADH was examined using initial velocity studies with both the substrates and by experiments involving inhibition by substrate, product and dead-end inhibitors (Table 2). A sequential addition of the substrates was indicated by intersecting patterns observed in the initial velocity studies. The uncompetitive inhibition of *A. niger* SSADH by SSA with respect to NAD$^+$ is more in agreement with a compulsory order mechanism, where NADH is the last product to dissociate from the enzyme. Linear inhibition by SSA indicates the binding of a single molecule of SSA to the E-NADH form of the enzyme. While NAD$^+$ and NADH compete for the same form of the enzyme, the non-competitive inhibition by NADH with respect to SSA
suggests that SSA and NADH bind to different enzyme forms. Uncompetitive inhibition of A. niger SSADH by pHB with respect to NAD$^+$ (Fig. 7) is the key result supporting the compulsory order mechanism; if the mechanism was sequential random then the pHB inhibition should have been noncompetitive. This conclusion is further supported through inhibition data with yet another dead-end inhibitor N-formylglycine$^{17}$. The kinetic data gathered so far is consistent with a compulsory ordered Bi Bi mechanism for A. niger SSADH, in which NAD$^+$ binds before SSA and NADH is the last product to dissociate from the enzyme. It is however not possible to exclude a Theorell-Chance mechanism at this point. The proposed mechanism for A. niger SSADH also differs from the ordered Ter-Bi mechanism postulated for the rat brain enzyme$^{26}$. These authors also report an unusually high $K_{M}$ for SSA and no substrate inhibition below 1.0 mM. The noncompetitive inhibition by pHB was interpreted in terms of a random mechanism for the pig brain enzyme$^{22}$. However, the results presented for A. niger SSADH here and those obtained for other SSADHs$^{19,24}$ are in perfect agreement with a sequential ordered Bi Bi mechanism.

The irreversibility of A. niger SSADH may be of some physiological relevance since SSA is rapidly and irreversibly converted into succinate. High specificity and affinity for SSA may further ensure its rapid and efficient conversion. The observed substrate inhibition of SSADH may be an in vitro phenomenon. Substrate inhibitions are usually not important if [S] is kept relatively low, below the physiological levels. The evolutionary process normally eliminates dead-end substrate combination that leads to substrate inhibition. However, if physiological [SSA] does not reach inhibitory levels (as in the case of aldehyde substrates that are toxic to the cell!) then dead-end combinations do persist. This often manifests as substrate inhibition in kinetic studies, particularly in the non-physiological direction.

**Acknowledgment**

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**Notes:**

1. While this paper was under review, characterization of the recombinant SSADH from *Saccharomyces cerevisiae* was reported$^{31}$. This enzyme is active with NAD$^+$ or NADP$^+$ and based on limited kinetic analysis, a ping-pong mechanism was proposed. The mechanism for *A. niger* SSADH proposed by us and those obtained for other SSADHs$^{19,24}$ are in perfect agreement with a sequential ordered Bi Bi mechanism. However, it is not possible to exclude a Theorell-Chance mechanism at this point.

2. *In silico* analysis of *A. niger* SSADH (GenBank Id: XP_001401629.2) suggests that it has two cysteines in the catalytic loop. But this needs to be confirmed experimentally.

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