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INHIBITION OF SUCCINIC SEMIALDEHYDE DEHYDROGENASE BY N-FORMYLGLYCINE

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N-formylglycine was developed as a dead-end inhibitor of the succinic semialdehyde dehydrogenase reaction. At 4 mM, it inhibited *Aspergillus niger* succinic semialdehyde dehydrogenase by 40%. N-formylglycine is a reversible, complete inhibitor; the inhibition is competitive with succinic semialdehyde and uncompetitive with respect to NAD^+ and the K_i values are 4.9 and 10.4 mM respectively. Potato succinic semialdehyde dehydrogenase is also inhibited by N-formylglycine to a similar extent, the nature of the inhibition being identical to that observed with the *A. niger* enzyme.

Keywords: Succinic semialdehyde dehydrogenase; Dead-end inhibition; N-formylglycine, 4-hydroxybenzaldehyde; *Aspergillus niger*

Abbreviations: GABA, 4-aminobutyrate; SSADH, succinic semialdehyde dehydrogenase (EC 1.2.1.16); SSA, succinic semialdehyde; PHB, 4-hydroxybenzaldehyde; NFG, N-formylglycine

INTRODUCTION

In the course of our study on the 4-aminobutyrate (GABA) metabolism in acidogenic *Aspergillus niger*, succinic semialdehyde dehydrogenase (SSADH) was purified and characterized.[1,2] Kinetically the *A. niger* SSADH reaction was, (i) irreversible and (ii) not inhibited by succinate, one of its products. These are features shared by all the SSADHs reported so far.[3–5] For such systems dead-end inhibitors serve as excellent tools to arrive at the steady-state kinetic mechanism.[6,7] Using 4-hydroxy benzaldehyde (PHB), a potent

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dead-end inhibitor of SSADH, and from other studies, the plant and the rat brain enzymes were assigned an Ordered Bi Bi mechanism.[3,5] A similar approach however led to the postulation of a Random Bi Bi kinetic mechanism for the pig brain enzyme.[4] Under such circumstances additional dead-end inhibitors become useful in the kinetic study. In an effort to clarify the *A. niger* SSADH kinetic mechanism other inhibitors (apart from PHB) were sought. We describe here the discovery of N-formylglycine (NFG) as one such molecule and report our results here.

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MATERIAL AND METHODS

Reagents and Enzyme Sources

Aspergillus niger (NCL 565) was obtained from NCIM at NCL, Pune, India. GABA, NAD^+, SSA, NFG, PHB, EDTA, Triton X-100 and 2-mercaptoethanol were purchased from Sigma Chemical Co., St. Louis., USA. Other chemicals (SRL), media components (HiMedia) and buffer reagents (Glaxo, Merck) were purchased locally and were of highest purity and analytical grade.

Enzyme Preparation

Aspergillus niger was grown in minimal media[8] with GABA as the sole source of nitrogen for 30 h at 30°C. All the operations of extraction and purification were carried out at 0–4°C. *A. niger* mycelia (20 g wet weight) were extracted in a pre-cooled pestle and mortar with an equal amount of acid-washed sand. Extraction buffer contained 100 mM sodium pyrophosphate buffer (pH 9.0), 1 mM EDTA, 0.1% Triton X-100 and 14 mM 2-mercaptoethanol. Extracts were then filtered through four layers of cheese cloth. Crude extracts were centrifuged and the supernatant was brought to 30% saturation with respect to ammonium sulfate. The protein fraction precipitating between 30–60% saturation of ammonium sulfate contained the enzyme activity and was resuspended in 20 ml of buffer containing 100 mM sodium pyrophosphate (pH 8.0) and 14 mM 2-mercaptoethanol. This enriched SSADH was further purified using phenyl-Sepharose and DEAE-Sephacel chromatographies (to be published elsewhere).

The SSADH from potatoes was extracted and purified by the method of SatyaNarayan and Nair.[9] The enzyme eluting from the final step of the AMP-Sepharose column was used in these studies.

Protein determinations were done according to Bradford[10] using bovine serum albumin as the standard.

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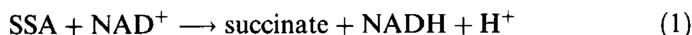
Assay of SSADH Activity

SSADH catalyzes the reaction:



$$SSA + NAD^+ \longrightarrow succinate + NADH + H^+ \quad (1)$$

The enzyme activity was monitored continuously at 340 nm and 25°C in a Shimadzu UV 160A double beam recording spectrophotometer. Routinely, the reaction mixtures in 1.0 ml contained: 100 mM sodium pyrophosphate buffer, pH 9.0, 14 ml 2-mercaptoethanol, 0.5 mM NAD^+ and sufficient enzyme to give an absorbance change of 0.1–0.2 units per min. Considering its higher K_m value, the standard assay for *A. niger* SSADH contained 5.0 mM NAD^+. After a 3 min equilibration, the reaction was initiated by the addition of succinic semialdehyde (SSA) (100 μM, final concentration) to the experimental cuvette. Any variation in the composition of the assay mixture is appropriately described in figure legends.

One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 nmol of NADH/min at 25°C.

All compounds, tested for inhibition, were dissolved in 100 mM sodium pyrophosphate buffer (pH 9.0) and it was ensured that the assay mixture after addition of all the components had a pH of 9.0. Inhibitors were individually incubated with the enzyme in the above mentioned buffer for 5 min at 25°C before initiating the reaction by SSA addition.

Other Methods

Data points for the Lineweaver–Burk plots were fitted by least-square linear regression analyses. Kinetic parameters were determined from the slope and/or intercept replots. Fractional inhibition data were fitted to an exponential decay curve. The software package GRAPRER, Version 1.75 of Golden Software Inc., was used for the statistical evaluation and curve fitting. The data points shown in the figures are representative of at least two independent experiments. Molecular modeling iterations were conducted through an energy minimization software – DTMM, Version 1.1 of Application Techniques Inc.

RESULTS AND DISCUSSION

Arriving at a particular steady-state kinetic mechanism for the SSADH reaction is not easy because succinate as a product does not inhibit this enzyme (Table I).[3–5] Dead-end inhibitors, forming abortive E·I complexes,

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TABLE I Inhibition of SSADH

Compound	*Inhibition* (%)[a]	
	A. niger SSADH	*Potato SSADH*
NFG, 4 mM	37	37
PHB, 20 μM	75	68
4-Hydroxybenzoate, 2 mM	10	14
Glycine, 10 mM	—[b]	—
Formic acid, 2 mM	—	15
Succinic acid, 5 mM	—	—

[a] 100% activity corresponds to 15 units of enzyme in a standard assay but containing 0.5 mM NAD$^+$. The 30–60% ammonium sulfate fraction served as the enzyme source in both cases. For details, see Material and Methods. [b] Inhibition was ≤5%; i.e., less than the experimental error for the assay.

are powerful tools in choosing between Random and Ordered mechanisms.[6,7] Reactivity of the substrate carbonyl group is attenuated when a more electronegative atom is next to it. For example, oxalylglycine is isosteric with 2-ketoglutarate but will not undergo the reactions of 2-ketoglutarate.[7,11] On these grounds NFG was designed as a possible dead-end inhibitor of *A. niger* SSADH. A molecular modeling conducted on a DTMM package also suggested its steric similarity with SSA. NFG is isosteric with SSA but since it is an amide and not aldehyde, it does not undergo the enzymic oxidation like SSA.

Initial screening revealed that NFG inhibits the activity of *A. niger* SSADH. Since, the inhibitory effect of free formate or glycine was negligible (Table I) it was concluded that the observed SSADH inhibition is specifically due to NFG. Succinate as a product did not inhibit the *A. niger* enzyme while PHB turned out to be a potent dead-end inhibitor. Like the *A. niger* SSADH, the potato enzyme is inhibited by NFG and more strongly by PHB. A number of aliphatic carboxylic acids and aldehydes when tested did not inhibit the fungal enzyme (not shown).

The inhibition of SSADH by NFG was further characterized. Fractional inhibition analysis on the *A. niger* and the potato enzymes is shown in Figure 1. For both sources of the enzyme NFG is a complete inhibitor as the $1/i$ vs. $1/[I]$ plots (insets in Figure 1) intersected the $1/i$ axis at one. Double reciprocal plots with SSA as the varied substrate and NFG as the inhibitor show an intersecting pattern where the y-axis intercepts did not differ significantly (Figure 2). This is consistent with a competition between NFG and SSA, for binding the *A. niger* enzyme. With NAD$^+$ as the varied substrate (Figure 2), the lines are parallel indicating that the inhibition is uncompetitive. From analogous results shown in Figure 3, it could be deduced that

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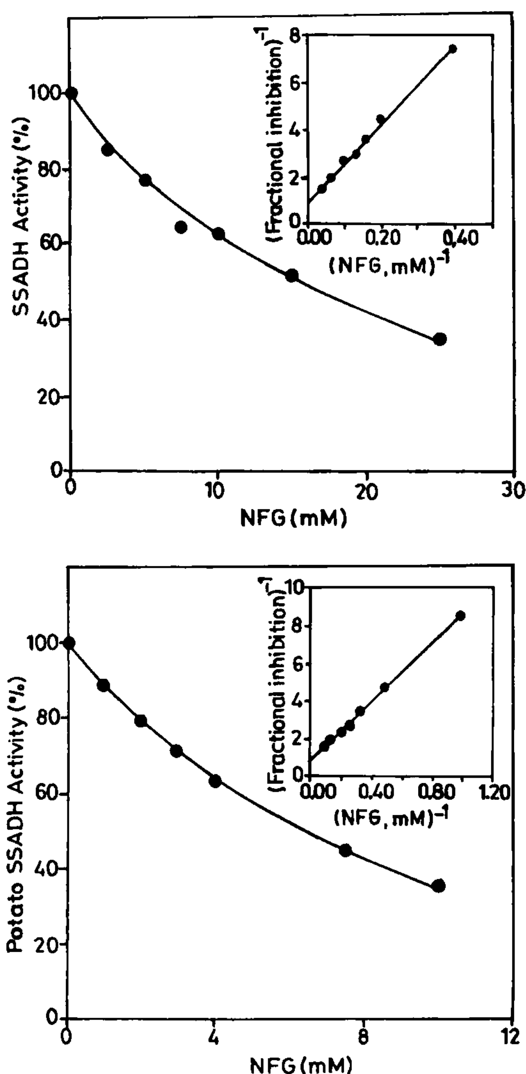

FIGURE 1 Inhibition *of A. niger* SSADH (Top Panel) and potato SSADH (Bottom Panel) by NFG. The concentration of SSA used in the standard assay (Material and Methods) was 100 μM. Insets show a plot of $1/i$ versus 1/[NFG] where fractional inhibition, "i" corresponds to $(v_0 - v_{NFG})/v_0$.

NFG inhibits the plant SSADH competitively with SSA and uncompetitively with NAD^+. Taken together, the NFG inhibition results are consistent with an Ordered Bi Bi mechanism for *A. niger* SSADH.[2]

Although the patterns of their inhibition of SSADH are identical, NFG inhibition is nowhere as potent as that of PHB. The K_i values for NFG are

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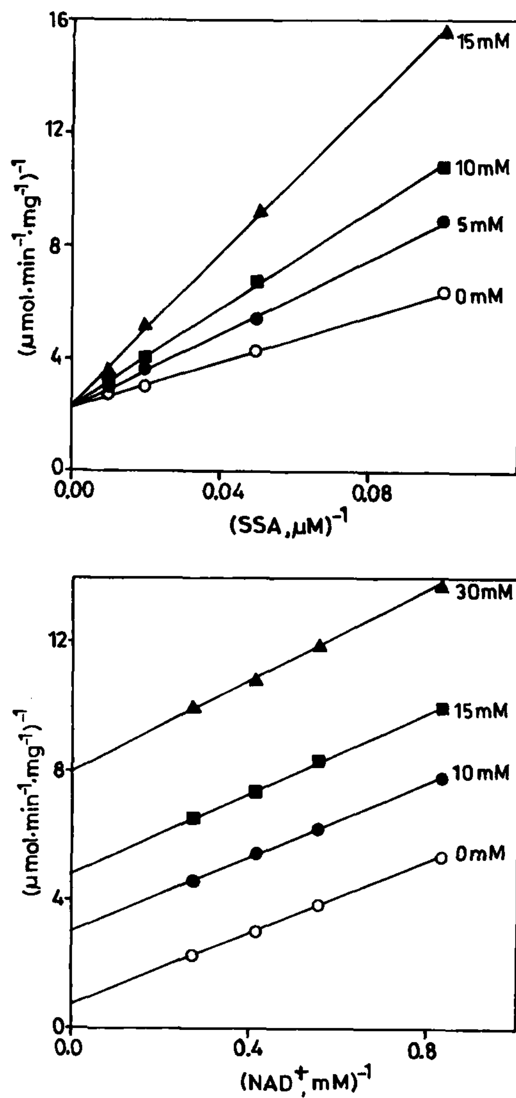

FIGURE 2 Kinetics of *A. niger* SSADH inhibition by NFG. Top Panel: SSA saturation at different fixed concentrations of NFG and at 1.5 mM of NAD^+ in the standard assay. A K_{NFG} of 4.9 mM was calculated from the slope replot. Bottom Panel: NAD^+ saturation at different fixed concentrations of NFG and at 20 μM of SSA in the standard assay. A K_{NFG} of 10.4 mM was calculated from the intercept replot.

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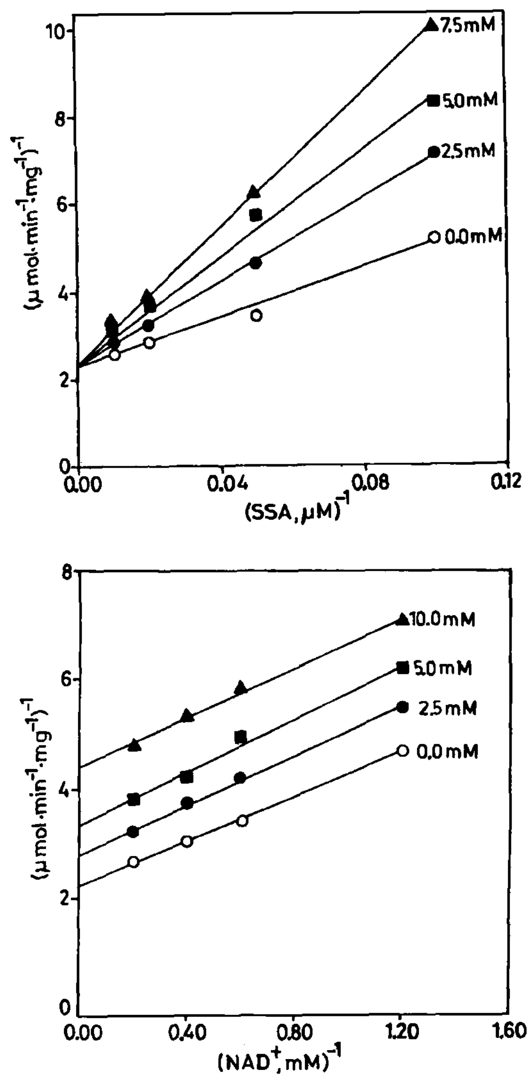

FIGURE 3 Kinetics of potato SSADH inhibition by NFG. Top Panel: SSA saturation at different fixed concentrations of NFG and at 0.1 mM of NAD^+ in the standard assay. A K_{NFG} of 3.1 mM was calculated from the slope replot. Bottom Panel: NAD^+ saturation at different fixed concentrations of NFG and at 10 μM of SSA in the standard assay. A K_{NFG} of 6.5 mM was calculated from the intercept replot.

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in the lower millimolar range (Figures 2 and 3) while those for PHB are in the micromolar range (PHB data for *A. niger* SSADH are not shown).[5] In spite of this NFG turned out to be an excellent tool for evaluating the steady-state kinetic mechanism of SSADH. From the present study on the plant and fungal enzymes it follows that NFG could possibly serve as a dead-end inhibitor of SSADHs in general.

Acknowledgements

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