Succinic Semialdehyde Dehydrogenase from a Pseudomonas Species

I. PURIFICATION AND CHEMICAL PROPERTIES*

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SUMMARY

An NAD-specific succinic semialdehyde dehydrogenase from a Pseudomonas species has been purified. The enzyme has been shown to have low activity toward aminoaldehydes (3-aminopropanal or 4-aminobutanal) as substrate. It contains 24 sulfhydryls per molecule (164,000 daltons). The kinetic constants and the pH profiles with different substrates are documented.

Work in this laboratory, in part reported earlier (1, 2), suggested that a Pseudomonas species grown on polyamines as the sole source of carbon and nitrogen contains not only NAD-specific dehydrogenases for succinic semialdehyde, 3-aminopropanal, and 4-aminobutanal, but also hybrids of these enzymes. In this paper we report the purification and chemical properties of an NAD-specific succinic semialdehyde dehydrogenase (EC 1.2.1.9, succinic semialdehyde oxidoreductase) with inherent low activity toward 3-aminopropanal. We will also demonstrate the presence of several NAD-specific dehydrogenases with different relative activities toward succinic semialdehyde and 3-aminopropanal as substrates. In the following paper we shall present the results of physicochemical and immunochemical studies which support the hypothesis of hybrid enzymes.

EXPERIMENTAL PROCEDURE

3-Aminopropanal and succinic semialdehyde were synthesized as described previously (1, 2). All other reagents were purchased commercially. The phosphate buffer used throughout was prepared by mixing 0.5 M solutions of NaH₂PO₄ and K₂HPO₄ in the appropriate ratio to obtain pH 7.0 (unless otherwise stated) and then diluted to the required concentrations. All buffer solutions contained 5 mM mercaptoethanol unless stated otherwise. DNase type I, RNase type A, and 5,5'-dithio(2-nitrobenzoic acid) were purchased from Sigma.

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Conditions for Cell Growth—Cells were grown at 30° in a 100-liter New Brunswick fermentor in a salt medium described previously (1). 4-Aminobutyrate (0.2%) was used as the sole carbon and nitrogen source. After the cells had reached the desired optical density (at the end of the log phase) they were chilled to 10° and harvested with a Sharples refrigerated centrifuge. Approximately 250 g of cells, wet weight per run, were frozen and kept at −20° until used.

Enzyme Assay—Enzymatic activity was measured at pH 8.5 in a 1.0-ml reaction mixture containing 105 mM K₂HPO₄, 5.0 mM succinic semialdehyde, 4-aminobutanal, or 3-aminopropanal, 0.5 mM NAD, and 5.0 mM mercaptoethanol. The reaction was started by the addition of the enzyme, and the formation of NADH was followed at 340 nm with a Gilson recording spectrophotometer.

Acrylamide Electrophoresis—Acrylamide electrophoresis was performed by a modification of the method of Epstein in gels (7%) 6 cm long (3). Protein bands were located with Coomassie brilliant blue, and enzymatic activity was determined essentially as described by Davis (4).

Before applying the protein samples, pre-electrophoresis was conducted for 100 min. Electrophoreses were then run for 30 min at 400 volts.

Determination of Protein Concentration—During the first steps of purification, protein concentration was determined by the biuret method (5). At later steps, protein was determined by the absorption at 280 nm with a value of E₅₃₂₀ = 10 (6). Values determined by this method agreed well when compared with the biuret method.

Determination of Free Sulfhydryl Groups—Free sulfhydryl groups were determined by two different methods, p-HMB titration according to Boyer (7) and 5,5'-dithio(2-nitrobenzoic acid) titration according to Ellman (8).

Concentration of Protein Fractions—Dilute protein fractions from chromatography were concentrated by absorption, after dialysis against 10 mM phosphatebuffer, pH 7.0, on a small column of DEAE-cellulose and elution with the same buffer containing 0.5 M NaCl. Protein fractions so concentrated were kept in the cold room until used.

Determination of pH Optima—The pH optima of the enzyme (toward succinic semialdehyde and 3-aminopropanal) were deter-
mined in two buffer systems. Between pH 7.0 and 9.0 a 0.1 M sodium phosphate buffer was used, whereas between pH 9.0 and 11.0 a 0.1 M carbonate-bicarbonate buffer system was used. The pH of the reaction mixture was checked before and after reaction to ensure the correct pH of the solutions.

RESULTS

Purification of Succinic Semialdehyde Dehydrogenase—Approximately 450 g of cells grown in the presence of 4-aminobutyrate were suspended and thawed in 1000 ml of 10 mM phosphate buffer (pH 7.0). The cold cell suspension was mixed with 1200 ml of glass beads (3M Co., size 120), and the whole slurry was placed in a colloid mill (Gifford-Wood). The cells were broken for 60 min at 5°C, collected in a beaker, and allowed to settle at room temperature for 30 min. The supernatant was then decanted. The remaining glass beads were washed three times with 250 ml each of buffer, and the washings mixed with the rest of the supernatant and treated for 45 min at room temperature with 1 mg each of DNase and RNase per liter of solution. After DNase and RNase treatment, the solution was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant obtained (crude extract) was centrifuged again in a Spinco model L2 ultracentrifuge using the type 19 rotor at 18,500 rpm for 3 hours at 0°C. The supernatant (soluble fraction) was dialyzed against several changes of 10 mM phosphate buffer.

The dialyzed solution was then applied to a column of DEAE-cellulose (5 x 100 cm) previously equilibrated with the same buffer. The column was first washed with the same buffer (6 liters), and the wash which had no enzymatic activity was discarded. The column was then eluted successively with the same buffer, which was also 0.1 M with respect to NaCl (5 liters), and with the same buffer containing 0.5 M NaCl (5 liters). The fractions collected were assayed for protein and activities toward succinic semialdehyde and 3-aminopropanal as substrates (Fig. 1). The fractions that contained activity were pooled (Fractions A and B in Fig. 1). Fraction B was frozen under liquid N2 for storage. Fraction A was dialyzed against several changes of cold 10 mM phosphate buffer and then applied to a column of DEAE-Sephadex A-50 (5 x 100 cm), washed with 2 liters of the same buffer, and then eluted with the same buffer with a convex gradient of NaCl (9) (5 liters of phosphate buffer as starting buffer and 7 liters of phosphate buffer containing 0.4 M NaCl as limiting buffer). Fractions were collected and protein and activity (toward succinic semialdehyde and 3-aminopropanal) were monitored. Three different fractions containing activity were pooled (Fractions AI, AII, and AIII in Fig. 2). Fractions AI and AII were frozen under liquid N2 for storage. Fraction AIII was purified further by rechromatography on a DEAE-Sephadex A-50 column (4 x 50 cm) that was developed with a linear gradient of NaCl between 0 to 0.4 M in 10 mM phosphate buffer (Fig. 3).

The active fractions collected after rechromatography were pooled and dialyzed against 10 mM phosphate buffer and concentrated (see under "Experimental Procedure"). The concentrated protein solution (41 ml) was dialyzed against 10 mM buffer containing 0.1 M NaCl. This solution was rechromatographed (in 10-m1 aliquots) through a Sephadex G-200 column (2 x 100 cm) that was pre-equilibrated with the same buffer. The collected fractions were assayed for protein and activity (see Fig. 4). Further and final purification was obtained by concentrating the pools obtained from several gel filtration runs and rechromatographing on the same Sephadex G-200 column. Table I shows the summary of a typical purification.

Criteria of Homogeneity—Acrylamide gel electrophoresis of the different fractions obtained after each purification step was run and the gels stained for protein and activity. The results are shown in Fig. 5. The final purified preparation shows, at pH 7.3 or 9.0, one major protein band and a minor protein band, both of which were active toward both succinic semialdehyde and

![Fig. 1. DEAE-cellulose chromatography of soluble fraction. Soluble fraction (2850 ml) containing 11.5 mg of protein per ml, 3.96 units of succinic semialdehyde dehydrogenase per ml, and 0.836 unit of 3-aminopropanal dehydrogenase per ml was applied to the column. The vertical arrows indicate the point at which the buffer changes were made. Fractions (20 ml) were collected at 4°C. Tubes 150 to 220 and 325 to 365 were pooled to give Fractions A and B, respectively. Fraction A was dialyzed and used immediately. Fraction B was frozen under liquid N2 for storage. Fraction A was dialyzed against several changes of cold 10 mM phosphate buffer and then applied to a column of DEAE-Sephadex A-50 (5 x 100 cm), washed with 2 liters of the same buffer, and then eluted with the same buffer with a convex gradient of NaCl (9) (5 liters of phosphate buffer as starting buffer and 7 liters of phosphate buffer containing 0.4 M NaCl as limiting buffer). Fractions were collected and protein and activity (toward succinic semialdehyde and 3-aminopropanal) were monitored. Three different fractions containing activity were pooled (Fractions AI, AII, and AIII in Fig. 2). Fractions AI and AII were frozen under liquid N2 for storage. Fraction AIII was purified further by rechromatography on a DEAE-Sephadex A-50 column (4 x 50 cm) that was developed with a linear gradient of NaCl between 0 to 0.4 M in 10 mM phosphate buffer (Fig. 3). The active fractions collected after rechromatography were pooled and dialyzed against 10 mM phosphate buffer and concentrated (see under "Experimental Procedure"). The concentrated protein solution (41 ml) was dialyzed against 10 mM buffer containing 0.1 M NaCl. This solution was rechromatographed (in 10-m1 aliquots) through a Sephadex G-200 column (2 x 100 cm) that was pre-equilibrated with the same buffer. The collected fractions were assayed for protein and activity (see Fig. 4). Further and final purification was obtained by concentrating the pools obtained from several gel filtration runs and rechromatographing on the same Sephadex G-200 column. Table I shows the summary of a typical purification.

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![Fig. 2. Separation of Fraction A into three fractions on DEAE-Sephadex A-50. Fraction A (1520 ml) from the previous step, containing 7.45 units of succinic semialdehyde dehydrogenase per ml and 6.64 units of 3-aminopropanal dehydrogenase per ml was applied to a column of DEAE-Sephadex A-50 (5 x 100 cm). The vertical arrow indicates the point at which the gradient was started. Fractions (20 ml) were collected in the cold, and protein (—) and enzyme activities (sucinic semialdehyde dehydrogenase, O—O; 3-aminopropanal dehydrogenase, Δ—Δ) were determined and plotted as in Fig. 1. Tubes 140 to 160, 161 to 180, and 200 to 230 were collected to give Fractions AI, AII, and AIII, respectively. Fractions AI and AII were frozen under liquid N2. Fraction AIII was used immediately. □—□, α-ketoglutarate-4-aminobutyrate transaminase, each unit representing 1 amole of product per min per ml of fraction.]
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**Table I**

| Step                      | Volume | Total protein | Total units | Recovery | Specific activity | Ratio of SNA to 3APA |
|---------------------------|--------|---------------|-------------|----------|-------------------|---------------------|
| Crude extract             | 3,000  | 58,500        | 12,400      | 100      | 0.226             | 4.38                |
| Soluble                   | 2,850  | 31,500        | 11,300      | 90       | 0.335             | 4.75                |
| DEAE-cellulose            | 1,520  | 5,410         | 11,320      | 90       | 2.065             | 11.6                |
| DEAESephadex A-50 (I)     | 620    | 745           | 7,630       | 67       | 10.25             | 59.0                |
| DEAESephadex A-50 (II)    | 300    | 222           | 3,080       | 25       | 13.50             | 67.0                |
| DEAESephadex A-50 (III)   | 40     | 100           | 2,570       | 21       | 26.00             | 63.0                |
| DEAESephadex G-200 (I)    | 20.1   | 16.5          | 548         | 17.8     | 32.20             | 68.0                |
| DEAESephadex G-200 (II)   |        |               |             |          |                   |                     |

* A unit is the amount of enzyme that reduces 1 µmole of NAD per min with succinic semialdehyde as substrate.

Specific activity is defined as units of enzyme activity per mg of protein (divide values in fourth column by those in third column).

Relative activities with succinic semialdehyde (SSA) and 3-aminopropanal (3APA) as substrate. Due to the somewhat labile nature of the enzyme, this final ratio varied from preparation to preparation between 65 and 200.

* Only 4020 units (388 mg) applied to this column.

* Only 640 units (24.9 mg) applied to this column.

* This purified enzyme has low activity toward 4-aminobutanal (one-half of that toward 3-aminopropanal).

**pH Optimum**—Fig. 6 illustrates the activities toward succinic semialdehyde and 3-aminopropanal at different pH. Maximal activity is observed with both substrates at pH 10.5. The ratio of activities with the two substrates is constant up to pH 9.0.
Fig. 6. pH versus dehydrogenase activities of purified succinic semialdehyde dehydrogenase. Succinic semialdehyde dehydrogenase (●—●) and 3-aminopropanal dehydrogenase (▲—▲) activities are plotted against pH. The units are in micromoles of NADH per min per 0.2 mg of purified enzyme. The ratios of succinic semialdehyde dehydrogenase to 3-aminopropanal dehydrogenase at different pH are shown (m—a). The effect of pH on $V_{\text{max}}$ is illustrated on the inset. ●—●, pH 8.5 and ▲—▲, pH 10.0. The units are $V$, micromoles of NADH per min per ml of enzyme and $S$ (3-aminopropanal), millimolar.

At higher pH, the ratio of activities toward succinic semialdehyde and 3-aminopropanal diminishes from approximately 200 to a minimum of about 25 at pH 10.5.

In order to investigate whether the unprotonated form of 3-aminopropanal is or is not the active species of this substrate, a study of the change in velocity with changing substrate concentration was conducted at pH 8.5 and pH 10.0. The results of such an experiment, plotted as $1/v$ versus $1/S$, are shown on the inset of Fig. 6.

**Table II**

| Variable substrate | Fixed substrate | $K_m$ | $V_{\text{max}}$ |
|--------------------|-----------------|-------|----------------|
| Succinic semialdehyde | NAD | 4.0 | 290 |
| 3-Aminopropanal | NAD | 1.1 | 0.2 |
| NAD | Succinic semialdehyde | 2.1 | 250 |
| 3-Aminopropanal | NAD | 2.2 | 0.2 |

Fig. 7. Graphic determination of kinetic constants. Activities were determined in 0.15 M pyrophosphate buffer at pH 8.5. A, the units are $V$, micromole of NADH per min per ml of enzyme and S (succinic semialdehyde), millimolar. The different concentrations of NAD are ○—○, 0.5 mM; △—△, 0.25 mM; and □—□, 0.125 mM. B, the units are $V$, micromoles of NADH per min per ml of enzyme and S (3-aminopropanal), millimolar. The different concentrations of NAD are ○—○, 0.5 mM; △—△, 0.25 mM; and □—□, 0.125 mM. C, the units are $V$, micromole of NADH per min per ml of enzyme and S (NAD), millimolar. The different concentrations of succinic semialdehyde are ○—○, 1.12 mM; △—△, 0.56 mM; □—□, 0.25 mM; ●—●, 0.28 mM; and ▲—▲, 0.32 mM. D, the units are $V$, micromoles of NADH per min per ml of enzyme and S (NAD), millimolar. The different concentrations of 3-aminopropanal are ○—○, 1.21 mM; △—△, 0.56 mM; and □—□, 0.28 mM.

Fig. 8. Effect of $p$-HMB on succinic semialdehyde dehydrogenase. $p$-HMB (50 μl, 2 mM) was added to 1 ml of a solution of succinic semialdehyde dehydrogenase (0.133 mg per ml of protein) in phosphate buffer, pH 7.0, and incubated at room temperature. At the times indicated, 0.1-ml aliquots were removed and activity toward succinic semialdehyde in the presence (○—○) or the absence (●—●) of mercaptoethanol and toward 3-aminopropanal in the presence (▲—▲) or the absence (△—△) of mercaptoethanol was determined. Mercaptoethanol concentration was 50 mM.

This requirement for high concentrations of sulphydryl donors prompted us to investigate the effect of sulphydryl reagents on the enzyme. Fig. 8 shows the effect of $p$-HMB on the two dehydrogenase activities. When the enzyme was treated with $p$-HMB for short durations and assayed in the presence of mercaptoethanol, there was a partial recovery of the activity.
ever, after 1 hour of treatment, the effect of p-HMB is completely irreversible, indicating that some sulfhydryl groups are required for structural integrity of the enzyme (12).

To determine the number of free cysteine residues present on the enzyme a titration with p-HMB according to Boyer was carried out (7). Titration by this method indicated the presence of 23 moles of free sulphydryls per mole of enzyme (mol wt 164,000). This value was confirmed by titration with 5,5’-dithio(2-nitrobenzoic acid), which gave a value of 24 moles of —SH per mole of enzyme.

**DISCUSSION**

We have previously reported that a *Pseudomonas* species grown on various polyamines (putrescine, spermidine, or bisaminopropylamine) contains a constitutive NADP-specific succinic semialdehyde dehydrogenase and many NAD-specific dehydrogenases which are active toward 4-aminobutanal, 3-aminopropanal, or succinic semialdehyde (or all three) and which can be separated by column chromatography or gel electrophoresis (or both) (1, 2). It was suspected that some of these enzyme fractions may be hybrid enzymes consisting of subunits of different dehydrogenases (2). Further fractionation of these fractions led us to believe that hybridization may occur between subunits of succinic semialdehyde dehydrogenase, 3-aminopropanal dehydrogenase, and 4-aminobutanal dehydrogenase (see accompanying paper (10)). As reported earlier, cells grown on 4-aminobutyrate have low levels of aminoaldehyde dehydrogenase(s) and a very high level of succinic semialdehyde dehydrogenase (1). These cells were therefore used to purify the NAD-specific succinic semialdehyde dehydrogenase. Results of column fractionation and gel electrophoresis reported in this paper show that from extracts of these cells at least four distinct dehydrogenase fractions can be obtained, having different relative activities toward succinic semialdehyde or 3-aminopropanal as substrate (Table I). Fraction B can be further fractionated into several fractions with different relative succinic semialdehyde to 3-aminopropanal dehydrogenase activity ratios.

The fraction with the highest succinic semialdehyde to 3-aminopropanal dehydrogenase ratio (Fraction AII) has been purified to homogeneity and found to possess low 3-aminopropanal dehydrogenase activity (Table I). The constancy of the succinic semialdehyde to 3-aminopropanal ratio during the final stages of purification and the results of gel electrophoresis clearly show that this enzyme possesses both activities. This is also supported by the pH profile of this enzyme.

Studies on the activities of the enzyme at different pH showed that there is a sharp rise of activity as the pH is increased from 9.1 to 9.5. Although the activities toward both aldehyde substrates are increased, the increments are not proportional. At higher pH, the ratio of the dehydrogenase activity toward 3-aminopropanal and succinic semialdehyde is increased. These changes of activities with increasing pH appear to be the result of two factors. First, the enzyme undergoes structural changes, probably involving dissociation into subunits (10). Second, one of the substrates, 3-aminopropanal, changes from a charged ammonium ion to an uncharged amine. Since the normal substrate of the enzyme is succinic semialdehyde, a negatively charged carboxylate ion, it is reasonable to expect the unnatural substrate, 3-aminopropanal, to be a better substrate in the uncharged form than in the positively charged form. That the unchanged form of 3-aminopropanal is indeed the active species is proven by the fact that a pH change between 8.5 and 10.0 has a marked effect on velocity, while the value of the *V*ₘₐₓ remained unchanged. These results establish the purity of the succinic semialdehyde dehydrogenase which possesses inherent low activity toward 3-aminopropanal and is not contaminated by aminoaldehyde dehydrogenase(s). This conclusion is crucial for the immunochromical studies described in the following paper (10).

It should be mentioned that the enzymatic activities at high pH were obtained by adding small aliquots of enzyme at pH 7 to buffered reaction mixtures at high pH and measuring the initial rates of reaction. If the enzyme is kept at pH > 9, it is unstable and irreversibly dissociates into subunits (10) with loss of activity.

The results presented here and in earlier papers (1, 2) are in agreement with the earlier reports by Jacoby’s laboratory (13-15) and by Nakamura (16) that certain *Pseudomonas* contain NADP-linked aminoaldehyde dehydrogenase and both NAD- and NADP-specific succinic semialdehyde dehydrogenases. The presence of multiple dehydrogenases with variable aldehyde substrate specificity is, however, unique to this organism. Since this has been discussed in earlier papers (1, 2), it will not be repeated here.

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2 Unpublished results.
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