SEPARATION AND PROPERTIES OF DPN AND TPN-LINKED SUCCINIC SEMIALDEHYDE DEHYDROGENASES FROM *PSEUDOMONAS AERUGINOSA*

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SUMMARY

Pseudomonas aeruginosa oxidizes succinic semialdehyde to succinate.

From a soluble extract of the cells, DPN and TPN-linked succinic semi aldehyde dehydrogenases were separated, partially purified, and their properties described.

INTRODUCTION

Recently JAKOBY AND SCOTT[1] reported that the partially purified SSA dehydrogenase from *Pseudomonas fluorescens* requires pyridine nucleotide and that TPN was 8.2 times more active than DPN. The present studies with *Ps. aeruginosa* show that there are two different SSA dehydrogenases, one DPN and one TPN-linked. The separation, purification and properties of both enzymes are described.

MATERIALS AND ANALYTICAL METHODS

Materials

The following chemicals were obtained from commercial sources: DPN, TPN (Sigma, Pabst), DPNH, TPNH, ATP, ADP, AMP, and CoA (Sigma) and EGTA was kindly supplied by Dr. C. N. REILLEY. SSA was obtained by the hydrolysis of formyl succinic acid diethylester before every experiment by the modified method of BESSMAN *et al.*[2]. Formyl succinic acid diethylester was synthesized by the method of CARRIERE[3]. Malonic semialdehyde was synthesized by the hydrolysis of potassium β-diethoxy propionate by the modified method of BESSMAN *et al.*[2] for SSA. Potassium β-diethoxy propionate was synthesized by the method of WOHL AND EMMERICH[4]. Calcium phosphate gel, aged at least two months before using, was prepared by the method of KEILIN AND HARTREE[5].

Abbreviations: SSA, succinic semialdehyde; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; CoA, coenzyme A; EGTA, ethylene glycol bis-(β-aminoethyl ether)-N,N'-tetraacetic acid; GABA, γ-amino butyric acid; EDTA, ethylene diamine tetraacetic acid; Tris, tris-(hydroxymethyl)aminomethane chloride. PCMB, *p*-chloromercuribenzoate.

Analytical determinations

Protein was estimated by the method of LOWRY *et al.*[6] and the method of WARBURG AND CHRISTIAN[7] was used for the enzyme after the protamine treatment. SSA was determined by the method of BESSMAN *et al.*[2] and chromatographically by using the solvent system of FLAVIN AND OCHOA[8]. MICHAELIS constants were determined according to the method of LINEWEAVER AND BURK[9]. Acetyl and succinyl phosphate were determined by the hydroxamate method of LIPMAN AND BURK[10]. DPN and TPN were assayed by the method of COLOWICK *et al.*[11].

EXPERIMENTAL

Preparation of the enzymes

Ps. aeruginosa utilized GABA as a sole source of carbon and nitrogen. From a growing culture, SSA and succinate were identified by chromatography using the solvent system of FLAVIN AND OCHOA[8]. The cells also utilized arginine, putrescine and glutamate as a sole source of carbon and nitrogen; and β-alanine and malonate as a source of carbon. Ethylene diamine, propylene diamine, cadaverine, acetate, propionate, asparatate and ornithine were not utilized for growth.

The cells were grown aerobically in the following medium: dibasic potassium phosphate 0.15 %; monobasic potassium phosphate 0.05 %; $MgSO_4$ 0.01 %; GABA 0.5 % and yeast extract 0.1 %. GABA was sterilized by filtration through a Hercules Filter L-3 and added to the previously autoclaved inorganic medium. Cultures were harvested after 24 to 48 h with shaking at 37°, the cells were separated by centrifugation and washed twice with 0.01 *M* potassium phosphate buffer pH 7.2 containing 5 m*M* of mercaptoethanol.

Enzymic activity was measured by observing the rate of DPNH and TPNH formation spectrophotometrically at 340 mμ and the molecular extinction coefficient of $6.22 \cdot 10^{-6}$ cm^2/mole for DPNH and TPNH was employed[12]. The 0.5-ml reaction mixture for the measurement of activity contained SSA 0.2 μM, TPN or DPN 0.5 μM, $MgCl_2$ 0.5 μM, tris-(hydroxymethyl)aminomethane chloride at pH 8.7, 20 μM and the enzyme which contained about 0.5 μM of mercaptoethanol. The reaction was followed at 25° after the addition of 0.2 μM of SSA. A unit of enzyme activity was defined as the amount catalyzing the formation of 1 mμM of DPNH or TPNH per minute in this standard reaction mixture. Specific activity was defined as the number of units of activity/mg enzyme protein.

Unless otherwise noted, all buffers used for the preparation were 0.01 *M* of potassium phosphate buffer pH 7.2 which contained 5 m*M* of mercaptoethanol. Centrifugations were carried out at 14,000 $\times$ *g* for 30 min and all procedures were conducted in a cold room at approximately 4°. Suspensions of washed cells which contained 1 g of wet weight/10 ml of buffer, were sonicated in a 9-kc Raytheon oscillator under cooling for 10 min. The cellular debris was removed by centrifugation at 14,000 $\times$ *g* for 1 h. The supernatant solution usually contained a small amount of pyridine nucleotide oxidases, which interfered with the determination of SSA dehydrogenases. 50 mg of protamine sulfate (2 % solution) was then added per gram of protein. The precipitate collected by centrifugation was discarded and the supernatant was dialyzed against buffer for 15 h.

TPN-linked enzyme

To 118 ml of the protamine supernatant, 17 g solid ammonium sulfate was added (0.225 saturation). The precipitate was removed by centrifugation and discarded. A precipitate was formed by the further addition of 17 g of ammonium sulfate to the supernatant solution (0.225 to 0.450 saturation). This precipitate was centrifuged and dissolved in 82 ml of buffer and refractionated by the addition of 13 g of solid ammonium sulfate. The small precipitate obtained was removed by centrifugation. To the clear supernatant 13 g of ammonium sulfate was added. This precipitate was centrifuged, redissolved in 70 ml of buffer and dialyzed 15 h against buffer.

To 70 ml of this preparation, 2 mg (dry wt.) of calcium phosphate gel/mg of protein was added. The gel was evenly suspended and gently stirred for 30 min and then centrifuged at 7,000 $\times$ *g*. If enzyme activity still remained in the supernatant, more calcium phosphate gel was added to the solution. The combined gel fractions containing the enzyme were eluted twice with 20 ml of 0.1 *M* of potassium phosphate buffer pH 7.2 and the eluate was dialyzed against buffer for 15 h. To 40 ml of the gel eluate 6.3 g of ammonium sulfate was added and the small precipitate was removed by centrifugation. The precipitate obtained by the further addition of another 6.3 g of ammonium sulfate was dissolved in 40 ml of buffer and dialyzed 15 h against buffer.

This fraction was then dialyzed against buffer containing $1 \cdot 10^{-3}$ *M* of EDTA for 15 h and then dialyzed against EDTA-free buffer for another 15 h. Twelve ml of this preparation contained 18 mg of protein, and had 273 units of total activity or 15.3 of specific activity. TPN was about 19 times more active than DPN. It was not possible to get a preparation completely free of the DPN enzyme.

DPN-linked enzyme

To the 119 ml of the supernatant obtained after the precipitation of TPN enzyme (step 3), 24.8 g of ammonium sulfate was added (0.450 to 0.750 saturation). The precipitate was collected by centrifugation and dissolved in 70 ml of buffer. The solution was refractionated by the addition of ammonium sulfate to collect the precipitate formed between 0.450 and 0.750 saturation and this was dissolved in 44 ml of the buffer, and dialyzed 15 h against buffer. 207 mg of the gel (dry wt.) was added to 44 ml of this fraction. The suspension was gently stirred for 30 min and then centrifuged. 37 ml of the supernatant was dialyzed 15 h against buffer. To 37 ml of the gel supernatant, 11.7 g of ammonium sulfate was added and the resultant precipitate was removed by centrifugation. To the supernatant obtained 7.8 g of ammonium sulfate was added. The precipitate formed was collected by centrifugation, dissolved in 12 ml of buffer and dialyzed 15 h against buffer containing $1 \cdot 10^{-3}$ *M* of EDTA and then against EDTA-free buffer for 15 h. It was then immersed in a water-bath at 50° for 10 min. The heated preparation was cooled to —20° immediately after the heating. After thawing, the cloudy precipitate was removed by centrifugation. The clear supernatant contained only the DPN-linked enzyme. Table I summarizes these procedures.

Properties of the enzymes

Specificity: Both the purified enzymes had no activity with formaldehyde, acetaldehyde, propionaldehyde, isovaleraldehyde, benzaldehyde, anisaldehyde, glyceraldehyde and glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate dehydro-

TABLE I

PURIFICATION OF TWO SUCCINIC SEMIALDEHYDE DEHYDROGENASES FROM *Pseudomonas aeruginosa*

The reaction mixture for DPN-linked enzyme system contained 0.2 μM of SSA, 0.5 μM of DPN, 0.5 μM of $MgCl_2$, 20 μM of Tris buffer pH 8.7. For TPN-linked enzyme system it contained 0.2 μM of SSA, 0.5 μM of TPN, 1.0 μM of $MgCl_2$, 20 μM of Tris buffer pH 8.6. Final volume 0.5 ml; Temperature, 25.5°.

Step	Procedure	Total volume (mg)	Total protein (mg)	Total activity units	Specific activity units/mg	Activity ratio TPN/DPN
	TPN-linked enzyme					
1	Crude extract	110	1,097	5,450	4.96	0.74
2	Protamine	118	542	6,020	11.1	0.51
3	1st Ammonium sulfate	82	288	2,250	7.81	4.9
4	2nd Ammonium sulfate	70	173	1,700	9.83	7.1
5	Calcium phosphate gel eluate	41	52	172	3.31	8.4
6	3rd Ammonium sulfate	13	25	208	8.25	12.0
7	EDTA treatment	12	18	273	15.3	19.0
	DPN-linked enzyme					
1	Crude extract	110	1,097	7,320	6.67	1.3
2	Protamine	118	542	11,850	20.93	2.0
3	1st Ammonium sulfate	70	252	7,650	30.18	3.5
4	2nd Ammonium sulfate	44	207	6,400	31.90	4.2
5	Calcium phosphate gel negative	37	69	674	9.64	9.4
6	3rd Ammonium sulfate	12	27	560	20,37	13
7	EDTA treatment	11	22	1,010	46.70	35
8	Heat treatment	10	9.4	744	78.80	∞

genase was reported to oxidize SSA by HARTING AND VELICK[13]. However, the TPN-linked enzyme had activity with malonic semialdehyde, though SSA was 3.5 times more active. The DPN-linked enzyme did not oxidize malonic semialdehyde, and could not use TPN. The TPN-linked enzyme still reduced DPN, though its activity was only 5–15 % of that of TPN.

Stability: Both the crude extracts, after storage at —20° for a week, showed about 80 % loss of their original activities. After purification, the loss was 15 to 20 % in a month. Throughout the purification procedure and storage, the presence of 5 mM of mercaptoethanol was essential for stability. The enzymes were rapidly inactivated at pH values below 6.5 and above 8.5.

Effect of pH: The DPN-linked enzyme had a sharp pH optimum at about pH 8.7, which was independent of the three buffers used. The maximum velocity was greatest in Tris buffer, less in bicarbonate and veronal. The TPN-linked enzyme, had an optimum at about pH 8.6 in veronal, Tris and bicarbonate buffers. The velocities in the last two buffers were lower than in veronal. The pH curves are given in Fig. 1. Potassium phosphate buffer inhibited both enzymes.

Stoichiometry: With 4 ml of the reaction mixture which contained 2.0 μmoles SSA, 4 μmoles DPN, 4 μmoles $MgCl_2$, 80 μmoles Tris buffer pH 8.7 and 740 μg of DPN-linked enzyme (step 8, activation ratio ∞), 100 μmoles of SSA were oxidized for each 115 mμmoles of DPNH formed in the first 2 min and 142 mμmoles of SSA were oxidized per 150 mμmoles of DPNH formed in 5 min. Thus for each mole of DPNH formed, one mole of SSA was oxidized.

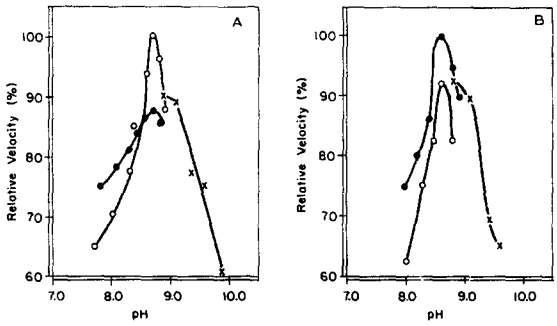

Fig. 1. Effect of pH on the initial velocities of DPN and TPN-linked enzymes. The reaction mixture for the DPN-linked enzyme system contained 0.2 μM of SSA, 0.5 μM of DPN, 0.5 μM of $MgCl_2$, 20 μM of buffer and 140 μg of enzyme (step 8). Final volume, 0.5 ml; temperature, 25.5°. For the TPN-linked enzyme system, the components are the same as in the DPN system, except of TPN, 1.0 μM of $MgCl_2$ and 220 μg of TPN enzyme (step 7, activity ratio 19.0). O—O, Tris buffer; ●—●, veronal buffer; ×—×, carbonate buffer. A, DPN-linked enzyme; B, TPN-linked enzyme.

Effect of enzyme concentration: For the DPN-linked enzyme, the initial velocity was directly proportional between 50 and 150 μg of protein and for the TPN-linked enzyme between 100 and 200 μg of protein.

Effect of substrate concentration: MICHAELIS constants for SSA and DPN or TPN were determined over SSA concentrations ranging from $5 \cdot 10^{-5} M$ to $4 \cdot 10^{-4} M$ and pyridine nucleotide concentrations from $3 \cdot 10^{-4} M$ to $8 \cdot 10^{-3} M$. The results are shown in Fig. 2.

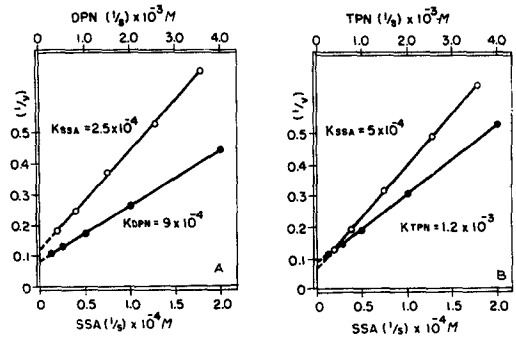

Fig. 2. The effects of succinic semialdehyde and DPN or TPN concentrations on the initial velocity. The reaction mixtures were the same as given in Table I.

Effect of metal ions: $MgCl_2$ increased the reaction velocities of both enzymes. $1 \cdot 10^{-3}$ and $2 \cdot 10^{-3} M$ of $MgCl_2$ resulted in 148 and 150 % increases in the initial velocities of the DPN-linked enzyme system, and 69 and 143 % increases in that of the TPN-linked enzyme system. As shown in Fig. 3, for each initial concentration of DPN a maximal velocity was reached when the $MgCl_2$ concentration was equal to that of DPN, and for the TPN enzyme a maximal velocity was reached when the $MgCl_2$ concentration was twice that of TPN. Without $MgCl_2$ the activity was 43 % of that of the complete system for the DPN-linked enzyme and 41 % for the TPN-linked enzyme. This difference in the molecular ratio of $MgCl_2$ to pyridine nucleotides suggests that the Mg-pyridine nucleotide complexes are substrates for the SSA dehydrogenases.

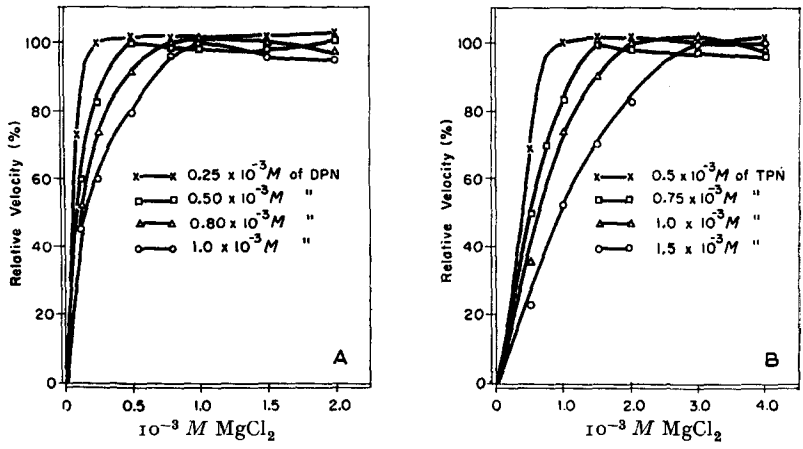

Fig. 3. The effect of $MgCl_2$ on the initial velocity in the presence of various concentrations of DPN or TPN. The reaction mixtures were the same as given in Table I except for $MgCl_2$.

Similar molecular complexes have been proposed by KUBY *et al.*[14] for the relation of ATP and $MgCl_2$ in ATP-creatine transphosphorylase.

Both calcium and manganese increased the rate, but to a lesser extent than magnesium. Other divalent ions were inactive or inhibitory (the pH was lowered to 7.2 to prevent precipitation). Inhibition by EDTA, which occurred in the earlier steps of purification and in the absence of $MgCl_2$, was partially overcome by the addition of $MgCl_2$, $CaCl_2$ or $MnCl_2$ in this order, as shown in Fig. 4. However, there was no

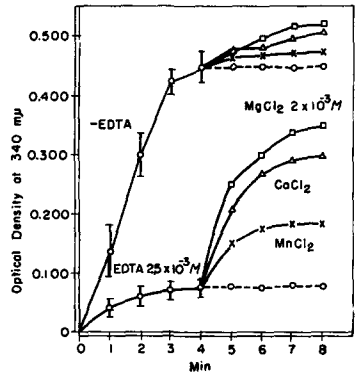

Fig. 4. The effect of $MgCl_2$, $CaCl_2$ or $MnCl_2$ on the inhibition by EDTA. The reaction mixture contained 0.2 μM SSA, 0.5 μM DPN, 20 μM Tris buffer pH 7.2 and 270 μg DPN-linked enzyme of step 5 (activity ratio 9.9). Addition of the metal ion after 4 min.

difference in the recovery rates from the EGTA inhibition with $MgCl_2$ or $CaCl_2$, although SCHMIDT AND REILLEY[15] showed that the stability-constant of EGTA-Ca was 10.7 and that of EGTA-Mg 5.4. 8-hydroxy quinoline and *O*-phenanthroline each at $1 \cdot 10^{-3}$ *M* showed 80 and 30 % respectively. There was no quantitative or qualitative difference between DPN and TPN-linked enzymes in their reactions to the addition of the other metal ions or various inhibitors.

Effect of sulfhydryl compounds: During the purification procedures of both enzymes, there were great losses in enzyme activities unless mercaptoethanol was added. *p*-chloromercuribenzoate, added in a final concentration of $1 \cdot 10^{-5}$ *M* to the enzymes containing about $1 \cdot 10^{-3}$ *M* of mercaptoethanol, caused an inhibition of 93 %. The addition of glutathione, $1 \cdot 10^{-3}$ *M*, reactivated them. But when the enzymes were preincubated with SSA, the same concentration of PCMB showed no inhibition. Mercuric chloride and cupric chloride both at $1 \cdot 10^{-4}$ *M* resulted in 80 to 90 % inhibition. Sodium arsenite, which was reported by JAKOBY[16] as a selective reagent for free sulfhydryl groups, was not as effective as the heavy metals or PCMB. These results indicate that the sulfhydryl group is one of the essential components in the enzyme protein for the oxidation of SSA to succinate.

Effect of ATP or CoA: No stimulatory effects were found upon the addition of ATP up to $1 \cdot 10^{-2}$ *M*. With or without ATP, CoA was not required even after the treatment of both enzymes with Dowex-1 resin for the removal of any CoA by the method of STADTMAN *et al.*[17]. There was no evidence for the formation of succinyl CoA, malonyl CoA, acetyl CoA, succinyl phosphate, malonyl phosphate and acetyl phosphate.

Reversibility. In the presence of succinate neither DPNH nor TPNH was oxidized in the presence or absence of $1 \cdot 10^{-3}$ *M* of semicarbazide, added to trap the semialdehyde which might be formed. Semicarbazide in this concentration did not inhibit the reactions.

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REFERENCES

1 W. B. JAKOBY AND E. M. SCOTT, *J. Biol. Chem.*, 234 (1959) 937.
2 S. P. BESSMAN, J. ROSSEN AND E. C. LAYNE, *J. Biol. Chem.*, 201 (1953) 385.
3 E. CARRIERE, *Ann. Chim.*, 17 (1920–22) 38.
4 A. WOHL AND W. EMMERICH, *Ber.*, 33 (1900) 2760.
5 D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc. (London) B*, 124 (1938) 397.
6 O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
7 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941–42) 384.
8 M. FLAVIN AND S. OCHOA, *J. Biol. Chem.*, 229 (1957) 965.
9 H. LINEWEAVER AND H. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
10 F. LIPMANN AND L. C. TURTLE, *J. Biol. Chem.*, 159 (1945) 21.
11 S. P. COLOWICK, N. O. KAPLAN AND M. M. CIOTTI, *J. Biol. Chem.*, 191 (1951) 447.
12 B. L. HORECKER AND A. KORNBERG, *J. Biol. Chem.*, 175 (1948) 385.
13 J. HARTING AND S. F. VELICK, *J. Biol. Chem.*, 207 (1954) 857.
14 S. A. KUBY, L. NODA AND H. A. HARDY, *J. Biol. Chem.*, 210 (1954) 65.
15 R. W. SCHMIDT AND C. W. REILLEY, *Anal. Chem.*, 29 (1957) 264.
16 W. B. JAKOBY, *J. Biol. Chem.*, 232 (1958) 75.
17 E. D. STADTMAN, G. D. NOVELLI AND F. LIPMANN, *J. Biol. Chem.*, 191 (1951) 365.