TARTARIC ACID METABOLISM II. CRYSTALLINE PROTEIN CONVERTING MESO-TARTRATE AND DIHYDROXYFUMARATE TO GLYCERATE

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The metabolism of the D, L, and meso isomers of tartaric acid occurs by pathways which include dehydration to oxalacetate by bacterial enzymes (1-3) and a DPN-linked oxidation to dihydroxyfumaric acid by plant and animal extracts (4,5). Dagley and Trudgill (6) have proposed a nonoxidative decarboxylation of L(+) and meso-tartaric acids to glyceric acid by extracts of Pseudomonas acidovorans in the presence of DPN and a divalent metal; net reduction of DPN was not observed.

The present report concerns an investigation of the system from P. acidovorans which revealed the presence of 3 enzymatic activities (Reactions 1-3).

1. Meso-tartaric acid + $DPN^+ \rightleftarrows$ dihydroxyfumaric acid + DPNH + H^+
2. Dihydroxyfumaric acid + DPNH + $H^+ \rightarrow$ glyceric acid + CO_2 + DPN^+
3. L(+) or meso-tartaric acid + $DPN^+ \underset{}{\overset{Mn + RSH}{\rightleftharpoons}}$ dihydroxyfumaric acid + DPNH + H^+

Purification has resulted in the crystallization of protein responsible for Reactions 1 and 2 and the separation of material active only in Reaction 3.

Assay. Tartaric dehydrogenase. Reaction 1 was observed at 340 mμ for 1.5 min. at 25° in a total volume of 1 ml containing an appropriate dilution of enzyme and the following, in μmoles: Tris chloride, pH 8.4, 100; meso-tartaric acid, 10; DPN, 1. Reaction 3 was followed with L(+)-tartrate in an identical manner except that the assay solution was supplemented with 0.4 μmole of $MnCl_2$ and 1 μmole of mercaptoethanol. Reductive decarboxylase. Reaction 2 was observed at 340 mμ under the same conditions except that the assay system contained the following in μmoles: potassium phosphate at pH 6.5, 200; DPNH, 0.2; dihydroxyfumaric acid, dissolved in water within 10 min. of use, 5. One unit of each

enzyme activity is defined as the formation of 1 μmole of product per minute.

Enzyme Purification. P. acidovorans (Pseudomonas A (7)) was obtained from Dr. U. Bachrach and was grown in basal medium (6) with 0.1% L(+)-tartrate as the sole carbon source. Extracts were prepared by sonic oscillation at 10 kc of a 1:4 (w/v) suspension of cells in 0.02 M potassium phosphate, pH 7.2, for 10 min. After centrifugation at 18,000 x g for 30 min., the extract was adjusted to pH 4.5 with 0.1 N HCl and the resultant precipitate was discarded. The supernatant solution was adjusted to pH 7.0, and protamine sulfate (1 mg/ml of extract) was added. Ammonium sulfate was added to the resulting supernatant fluid and the fraction precipitating between 57% and 75% of saturation, i.e., after the sequential addition of 40 and 13 g of salt per 100 ml, was dissolved in 0.04 M Tris at pH 7.4. After dialysis against 200 volumes of the same buffer, protein was adsorbed onto aluminum hydroxide gel ("Amphogel," washed so as to

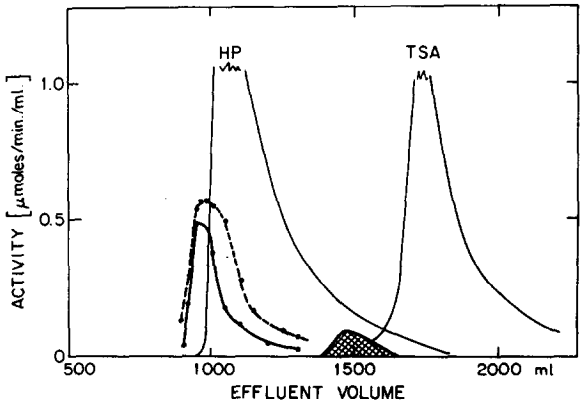

Fig. 1. Chromatography of P. acidovorans preparation on DEAE-cellulose, measuring the following enzymatic activities: oxidation of DPNH with dihydroxyfumarate (open circles); reduction of DPN with meso-tartrate (closed circles); reduction of DPN with L(+) or meso-tartrate in the presence of 0.5 mM $MnCl_2$ and 5 mM mercaptoethanol (cross-hatched); oxidation of DPNH with hydroxypyruvate (H-P); oxidation of DPNH with tartronic semialdehyde (TSA). The preparation applied to the column differed from that described in the text in that a broader ammonium sulfate fraction (30 to 65%) was taken and no acid step was used. Elution from the column was as described except for the use of 1.5 L volumes in both reservoir and mixing flask.

remove the peppermint flavor) using approximately 25 mg of gel per mg of protein. The gel was eluted twice with 4 volumes of 0.1 M Tris, pH 7.4, and then twice with 0.1 M potassium phosphate at pH 7.5. The phosphate washes were pooled, dialyzed against 200 volumes of 0.04 M Tris, pH 7.4, for 18 hours, and applied to a DEAE-cellulose column (4 x 33 cm) previously equilibrated with the same buffer. Activity for Reactions 1 and 2 was eluted with a linear gradient using a reservoir of 2000 ml of 0.5 M NaCl in 0.04 M Tris, pH 7.4, and a mixing flask containing 2000 ml of the buffer. Fractions collected between 800 ml and 1000 ml of eluate volume (cf. Fig. 1) were treated by addition of ammonium sulfate to 75% of saturation (53 g/100 ml). The resulting precipitate was collected by centrifugation and suspended in 60% saturated ammonium sulfate prepared by dilution of an ammonium sulfate solution saturated at 0^{o} using approximately 1 ml per 2.5 mg of protein. After stirring at approximately 0^{o} for 15 min., the preparation was centrifuged and the precipitate was suspended in 55% saturated ammonium sulfate solution in a similar manner. Both supernatant fluids were set aside at 23^{o} to allow crystallization and were harvested after 4 days. The 60% salt fraction, yielding 65% of the crystalline material, was recrystallized in the same manner and harvested after 6 days.

The results of the purification procedure are summarized in Table I from which it is evident that the ratio of enzymatic activity of Reaction 1 to 2 is reasonably constant throughout purification. L(+)-Tartaric acid dehydrogenase (Reaction 3) is removed by acid treatment and salt fractionation. Although L(+)-tartaric acid dehydrogenase chromatographs as a separate peak on DEAE-cellulose and could be easily separated in this way (Fig. 1), the acid step was introduced in order to remove hydroxypyruvate reductase. The crystalline material was free from hydroxypyruvate reductase, tartronic semialdehyde reductase, and L(+)-tartaric dehydrogenase.

No difference in the concentration of this system was noted when the organism was grown with meso-tartaric acid as the sole carbon source.

Homogeneity. The evidence for both catalytic activities, i.e., Reactions 1

TABLE I

Summary of the purification procedure

Fraction	Vol.	Protein	Reaction 1		Reaction 2		Ratio* 2:1
			Total activity	Specific activity	Total activity	Specific activity	
	ml	mg	units	units/mg	units	units/mg	
1 Extract	290	9740	185	0.019	350	0.036	1.89
2 Acid treatment	380	8360	135	0.016	274	0.033	2.06
3 Ammonium sulfate	80	800	110	0.106	211	0.205	1.93
4 Gel	180	360	113	0.31	223	0.62	1.97
5 DEAE-cellulose	200	56	45	0.80	84	1.50	1.86
6 Crystallization	3.5	9.5	9.4	0.99	17.8	1.87	1.89
Supernate	13.0	21.5	18.3	0.86	32.2	1.50	1.76
7 Recrystallization	2.4	5.2	5.4	1.05	12.3	2.36	2.26

* Ratio of the specific activities of the two reactions.

and 2, residing in one protein entity rests on the constant ratio of the two activities throughout purification and crystallization as well as in the homogeneity of the protein component which was isolated. Thus, a single, coincident protein and activity peak was obtained when crystalline material was subjected to gel filtration on a P-200 column (2 x 100 cm); the ratio of the two activities in each tube containing protein was equal and was identical to that obtained throughout fractionation. This material revealed a single protein component in the ultracentrifuge with an s_{25w} of 4.79S. Using a synthetic boundary cell, an s_{25w} of 4.62S was calculated from the distribution of the two activities; the ratio of the activity of Reaction 2 to 1 was 1.9 on each side of the partition. On disc gel electrophoresis, a single band of protein was observed which contained both activities.

Products. Incubation of crystalline protein with DPN and meso-tartrate resulted in the formation of glyceric acid which was identified by its elution pattern from Dowex-1-formate (8) and by its characteristic colored product

(max., 695 mμ) upon heating with chromatropic acid (9). Some free dihydroxyfumarate formed in the course of this reaction was determined spectrophotometrically at 292 mμ. Upon incubation of the enzyme with dihydroxyfumarate and DPNH, glycerate was identified as a product in the same manner as outlined above.

Conclusions. It would appear that both meso-tartaric acid dehydrogenase and dihydroxyfumaric acid reductive decarboxylase activity reside in a single protein or protein complex. In the presence of substrate and the appropriate form of pyridine nucleotide, no cofactors are required. A separable enzyme, L(+)-tartaric acid dehydrogenase, catalyzes the formation of dihydroxyfumaric acid from either L(+) or meso-tartrate only in the presence of a divalent metal and a mercaptan. The absence of hydroxypyruvate reductase and tartronic semialdehyde reductase activities in the crystalline preparation indicates that neither hydroxypyruvate nor tartronic semialdehyde are free intermediates in the conversion of dihydroxyfumarate to glycerate.

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