Studies on Glucarate Catabolism: The Oxodeoxyglucarate Aldolase Activity of Glucarate Hydro-lyase from *Pseudomonas acidovorans*

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Glucarate hydro-lyase was isolated and purified to near homogeneity from cells of *Pseudomonas acidovorans* grown on glucarate. By using gel filtration and ion-exchange chromatography, it was shown that the oxodeoxyglucarate aldolase activity observed in such extracts is associated with the glucarate hydro-lyase protein.

Blumenthal & Fish (1963) first reported the induced catabolism of D-glucarate by a strain of *Escherichia coli* and demonstrated the involvement of an Mg^{2+} -dependent hydro-lyase and aldolase. The hydro-lyase catalyses the conversion of glucarate, $CO_2H-[CHOH]_4-CO_2H$, into oxodeoxyglucarate, $CO_2H-CO-CH_2-[CHOH]_2-CO_2H$, and the aldolase the conversion of the latter into pyruvate and tartronatesemialdehyde, $CO_2H-CO-CH_3 + CHO-CHOH-CO_2H$. Subsequent studies by Trudgill & Windus (1966) have shown that this pathway is common to all glucarate-metabolizing members of the Enterobacter family and that a second pathway, utilizing glucarate hydro-lyase (EC 4.2.1.40) and oxodeoxyglucarate hydro-lyase (decarboxylating) (EC 4.2.1.41), elucidated by Jeffcoat *et al.* (1969a), is common to all members of the Pseudomonad family. This last enzyme converts oxodeoxyglucarate into CO_2 and 2-oxoglutarate semialdehyde, $CHO-CH_2-CH_2-CO-CO_2H$. Both families of bacteria therefore utilize a glucarate hydro-lyase in the initial step of glucarate catabolism and it was decided to compare the properties of these two proteins.

Attempted purification of glucarate hydro-lyase free of oxodeoxyglucarate aldolase (EC 4.1.2.20) from *Klebsiella aerogenes* N.C.I.B. 418 (R. Jeffcoat, unpublished work) by using heat treatment, $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography, gel filtration and polyacrylamide-gel electrophoresis was unable to resolve the two proteins completely. The overall similarity in their chemical and physical properties suggested a common evolutionary origin (Jeffcoat & Dagley, 1973) and the possibility of common mechanistic features in their catalytic activity. It was therefore necessary to obtain homogeneous preparations of the two proteins and assay them both for aldolase and hydro-lyase activity to test the hypothesis. To eliminate the possible contamination of the hydro-lyase by aldolase enzyme,

glucarate hydro-lyase was isolated from *Pseudomonas acidovorans*, which does not synthesize a separate aldolase.

Experimental

Maintenance and growth of the organism. *Pseudomonas acidovorans* N.C.I.B. 10013 was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, U.K. The organism was maintained and grown under conditions identical with those described by Jeffcoat (1972). Protein was determined with reference to bovine serum albumin (BDH Biochemicals, Poole, Dorset, U.K.) from a calibration curve prepared by the method of Lowry *et al.* (1951). One unit of enzyme activity is defined as the amount of enzyme transforming $1 \mu\text{mol}$ of substrate/min at 30°C .

Assay of the enzyme activity. Glucarate hydro-lyase from *Ps. acidovorans* was assayed as described by Jeffcoat (1972). 4-Deoxy-5-oxoglucarate aldolase activity was measured by a coupled assay with 10 units of pig heart lactate dehydrogenase [Boehringer Corp. (London) Ltd., London W.5, U.K.], $0.5 \mu\text{mol}$ of NADH, $1 \mu\text{mol}$ of oxodeoxyglucarate, $50 \mu\text{mol}$ of $MgSO_4$, $100 \mu\text{mol}$ of potassium phosphate buffer, pH 7.5, and 0–2 units of oxodeoxyglucarate aldolase. The qualitative determination of the aldolase was most conveniently carried out by incubating $10 \mu\text{mol}$ of oxodeoxyglucarate in the absence of the coupled assay system and stopping the reaction by the addition of 10 ml of 5 mM-2,4-dinitrophenylhydrazine in 2M-HCl. After incubation at 30°C for 60 min, the insoluble 2,4-dinitrophenylhydrazone was separated by centrifugation in a bench centrifuge and dissolved in 0.3% (w/v) NaOH in 90% (v/v) ethanol. The purple solution, with λ_{max} 572 nm, was typical of the derivative produced by incubating 2,4-dinitrophenylhydrazine with tartronic semialdehyde, a product of the aldol fission of oxodeoxyglucarate (Dagley *et al.*, 1961).

Purification of the enzyme. Glucarate hydro-lyase purified from *Ps. acidovorans* as described by

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Jeffcoat (1972) was further purified by gel filtration on a column (100 cm $\times$ 2 cm) of Bio-Gel P-200 (50–100 mesh), equilibrated with 0.05 M-potassium phosphate buffer–0.1 M-NaCl, pH 7.5. These conditions were also used for gel filtration on Sephadex G-100.

Preparation of enzyme substrates. Potassium hydrogen glucarate and oxodeoxyglucarate were prepared as described by Jeffcoat (1972).

Results

Oxodeoxyglucarate aldolase activity of glucarate hydro-lyase. Glucarate hydro-lyase from *Ps. acidovorans*

Fig. 1. Oxodeoxyglucarate aldolase activity of glucarate hydro-lyase from *Ps. acidovorans*

Each incubation contained, in a total volume of 3 ml, 1 unit of glucarate hydro-lyase, 2 μ mol of oxodeoxyglucarate, 100 μ mol of potassium phosphate, pH 7.5, 50 μ mol of $MgSO_4$, approx. 0.5 μ mol of NADH and 10 units of lactate dehydrogenase (○). In system (●) the $MgSO_4$ was replaced by 20 μ mol of EDTA and in system (Δ) *p*-chloromercuribenzenesulphonate was added to a final concentration of 1 mM. Lactate dehydrogenase was not added to system (Δ) until 7 h after the start of the incubation (arrow 1). After incubation for 8 h, 7.8 units of oxodeoxyglucarate aldolase was added to system (Δ) (arrow 2).

vorans was purified as described and incubated with oxodeoxyglucarate under the conditions shown in Fig. 1. By using a coupled assay with pig heart lactate dehydrogenase and NADH, a linear decrease in the E_{340} over the 6 h incubation period was shown. In the absence of lactate dehydrogenase or glucarate hydro-lyase there was no detectable oxidation of the NADH, as was also true when *p*-chloromercuribenzenesulphonate was added to a final concentration of 1 mM. EDTA (50 mM) caused 87% inhibition of the reaction rate. *p*-Chloromercuribenzenesulphonate inhibited the reaction; it has been shown to have no effect on oxodeoxyglucarate aldolase activity, when isolated from *Klebsiella aerogenes* (R. Jeffcoat, unpublished work), and this was confirmed by the addition of 7.8 units of the aldolase to the system that had been incubated for 8 h with *p*-chloromercuribenzenesulphonate. The rapid oxidation of the NADH clearly indicated that *p*-chloromercuribenzenesulphonate inhibits neither oxodeoxyglucarate aldolase nor lactate dehydrogenase under the conditions of the assay. Further, it strongly suggests that the low aldolase activity of the hydro-lyase preparation is not due to adsorption of small amounts of oxodeoxyglucarate aldolase on the glucarate hydro-lyase protein.

Identification of the products of the incubation of glucarate hydro-lyase and oxodeoxyglucarate. Glucarate hydro-lyase (3 units) was incubated at 30°C with 100 μ mol of potassium phosphate buffer, pH 7.5, 50 μ mol of $MgSO_4$, 5 μ mol of oxodeoxyglucarate, 10 units of penicillin G and 1 mg of streptomycin in a total volume of 3 ml. After 24 h, 10 ml of 5 mM-2,4-dinitrophenylhydrazine in 2 M-HCl was added and the mixture was incubated for 1 h at 30°C. The insoluble derivatives were treated as described under 'Experimental'. The visible spectrum from 400 to 700 nm was identical with that obtained for the products from an incubation mixture containing oxodeoxyglucarate and the aldolase from *Klebsiella aerogenes*, and was indicative of the formation of tartronate semialdehyde (Dagley *et al.*, 1961). The soluble acidic 2,4-dinitrophenylhydrazones were extracted as described by Jeffcoat *et al.* (1969a) and co-chromatographed with authentic pyruvate 2,4-dinitrophenylhydrazone when subjected to ascending paper chromatography in the solvent system butan-1-ol-ethanol-0.5 M- NH_3 (7:1:2, by vol.; El Hawary & Thompson, 1953). A further portion of the soluble derivatives, after reduction with platinum oxide and H_2 (Chamberlain & Dagley, 1968), gave a substance that migrated with alanine when subjected to paper electrophoresis at pH 1.9 and 120 V/cm for 20 min. Under similar conditions, the reduced 2,4-dinitrophenylhydrazone of oxodeoxyglucaric acid migrated with aspartic acid. The amino acids were detected by warming the paper after dipping it in a solution of 100 parts of 1% (w/v)

ninhydrin in acetone and 15 parts of a solution containing 1 g of cadmium acetate dihydrate in 100 ml of water and 50 ml of acetic acid (Offord, 1969).

Evidence for one protein having glucarate hydro-lyase and oxodeoxyglucarate aldolase activity. (1) Table 1 summarizes the ratios of the hydro-lyase and aldolase activities in extracts which were eluted from a Sephadex G-100 column and a DEAE-cellulose column under the conditions described under 'Experimental'. The ratios were calculated from the activities of the fractions throughout the peaks eluted from the two columns. The results indicate that the aldolase activity always lies between 0.3 and 0.4% of the hydro-lyase activity.

(2) Enzyme samples were subjected to disc electrophoresis at pH 9.5 (Davis, 1964) and the gels were sliced longitudinally with a razor blade. One half of the gel was incubated with 10 μ mol of glucarate for 10 min as in the qualitative assay for the aldolase and then transferred to 5 mM 2,4-dinitrophenylhydrazine in 2 M-HCl. The appearance of a yellow band located the site of the glucarate hydro-lyase. The sections, usually 1–2 mm wide, corresponding to the hydro-lyase band from the non-stained half of four such gels were incubated with glucarate for 24 h at 30°C and the supernatant was then qualitatively assayed for tartronate semialdehyde. A purple solution with $\lambda_{\max}$ 572 nm was again indicative that the gel slices contained enzyme material capable of catalysing the conversion of glucarate into tartronate semialdehyde. Such activity could not be demonstrated in slices of gel that failed to show glucarate hydro-lyase activity.

Table 1. Ratios of oxodeoxyglucarate aldolase and glucarate hydro-lyase activities in purified extracts from *Pseudomonas acidovorans*

Specific activities are expressed as μ mol of substrate utilized or product formed/min per mg at 20°C, with an $E_{280}^{1\%}$ arbitrarily chosen as 10 for the estimation of the protein concentration.

Purification step	Specific activity of glucarate hydro-lyase	100 $\times$ Specific activity of aldolase Specific activity of hydro-lyase
Sephadex G-100	4.75	0.26
	7.85	0.27
	8.85	0.26
	10.00	0.27
	7.95	0.28
	5.15	0.26
DEAE-cellulose	7.2	0.34
	7.9	0.33
	7.9	0.34
	7.9	0.27

Discussion

Morse & Horecker (1968) proposed that in Mg^{2+} -dependent aldolases, of which 4-deoxy-5-oxoglucarate aldolase is an example, the metal ion may facilitate the reaction by polarizing the carbonyl group and facilitating transition to an intermediate enolate ion. Meloche & Wood (1964) have proposed a similar mechanism for hydro-lyases involving electrophilic sites on the enzyme which are responsible for the polarization of the carbonyl oxygen and which leads to the loss of a proton from the adjacent carbon atom. It has been suggested by Jeffcoat (1972) that one of the electrophilic sites in glucarate hydro-lyase is an Mg^{2+} ion. The role of Mg^{2+} in aldol and dehydration reactions appears therefore to be one of polarization of a carbonyl group which facilitates the loss of a proton and stabilization of the intermediate, possibly through a ring structure. Although the mechanism of hydro-lyases has been studied much less than that of aldolases, it is already clear that similarities exist; like aldolases they can be divided into metal-ion-dependent (Blumenthal & Fish, 1963; Jeffcoat *et al.*, 1969a; Jeffcoat, 1972) and Schiff-base (Jeffcoat *et al.*, 1969b; Portsmouth *et al.*, 1967) types. Such similarities between the two types of enzyme prompted investigation of whether hydro-lyases can catalyse aldol fissions.

Preparations of the hydro-lyase had a constant ratio of aldolase to hydro-lyase activity at two stages of the purification procedure. After polyacrylamide-gel electrophoresis, the final preparation gave one band having both hydro-lyase and aldolase activities when assayed in the gel. When the hydro-lyase activity was inhibited with *p*-chloromercuribenzenesulphonate the aldolase activity was also lost, although *p*-chloromercuribenzenesulphonate does not inhibit the oxodeoxyglucarate aldolase from *K. aerogenes*. All this is taken as strong evidence that the purified hydro-lyase has intrinsic oxodeoxyglucarate aldolase activity.

Extracts of oxodeoxyglucarate aldolase from *K. aerogenes*, when tested for hydro-lyase activity, were unable to catalyse the dehydration of glucarate. The differing sensitivities of the aldolase and hydro-lyase aldolase activity towards *p*-chloromercuribenzenesulphonate probably do not reflect a different mechanism of action of the two proteins, since it has been demonstrated that *p*-chloromercuribenzenesulphonate modifies amino acids either involved in the binding of glucarate to the hydro-lyase or responsible in some way for maintaining the structure of the active centre but not involved in the catalytic mechanism (Jeffcoat, 1972).

The results of the present paper extend the known similarities between the hydro-lyases and aldolases. Rutter (1964, 1965) from his study of aldolases has proposed that enzymes of a similar mechanism may

have a common genetic origin, but further detailed studies on the structure of these two enzymes will be needed to determine whether or not they are so related.

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