Studies on the Subunit Structure of 4-Deoxy-5-oxoglucurate Hydro-lyase (Decarboxylating) from *Pseudomonas acidovorans*

By ROGER JEFFCOAT*

Department of Biochemistry, University of Leeds, 9 Hyde Terrace, Leeds LS2 9LS, U.K.

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1. Homogeneous preparations of D-4-deoxy-5-oxoglutarate hydro-lyase (decarboxylating) (EC 4.2.1.41) were analysed in the ultracentrifuge by the high-speed sedimentation-equilibrium method of Yphantis (1964). The molecular weight in 0.1 M-potassium phosphate buffer, pH 7.2, in 6 M-guanidine hydrochloride and in 0.1 M- β -mercaptoethanol in 6 M-guanidine hydrochloride was 113 000, 56 000 and 30 400 respectively. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate indicated a minimum molecular weight of 30 500. 2. Measurement of the thiol content of the enzyme, before and after reduction with NaBH₄ or dithiothreitol under denaturing conditions, indicated the presence of eight thiol residues and two interchain disulphide bridges/enzyme molecule. 3. Amino acid analysis showed that the intact enzyme contains a total of approximately 100 arginine and lysine residues, but digestion of the enzyme with trypsin yielded about 49 peptides staining with ninhydrin in a peptide 'map'. 4. With the knowledge that the enzyme contains only two substrate-binding sites, it is suggested that the enzyme probably consists of four polypeptide chains arranged in an $\alpha_2\beta_2$ conformation.

The work of Rutter (1964, 1965) on the distribution and properties of fructose 1,6-diphosphate aldolases has enabled him to establish two broad classes of this enzyme. Class 1 aldolases are tetrameric and characterized by the formation of a Schiff base in the enzyme-substrate complex, whereas Class 2 enzymes are dimeric and are strongly dependent on bivalent metal ions. Until recently little evidence was available to suggest that any other enzyme could be classified in this way, but there is now increasing support that such similarities also exist between hydro-lyases isolated from different sources. This stems from the isolation of Mg²⁺-dependent hydro-lyases (Blumenthal & Fish, 1963; Jeffcoat, 1972) and Schiff-base-forming hydro-lyases (Portsmouth *et al.*, 1967; Jeffcoat *et al.*, 1969). Closer links between aldolases and hydro-lyases are now emerging, since preliminary observations have suggested that metal-ion-dependent aldolases (Morse & Horecker, 1968) and Mg²⁺-dependent hydro-lyases (Jeffcoat, 1972) utilize the formation of a metal-containing ring structure in the enzyme-substrate complex, and Jeffcoat (1974) has demonstrated the Mg²⁺-dependent aldol cleavage of 4-deoxy-5-oxoglucurate by glucarate hydro-lyase. Comparisons of the subunit structure of aldolases and glucarate hydro-lyase have not been possible, since homogeneous preparations of glucarate hydro-lyase are not available.

* Present address: Biosciences Division, Unilever Research, Colworth House, Sharnbrook, Bedford MK44 1LQ, U.K.

The present paper reports the subunit studies carried out on the Schiff-base-forming hydro-lyase, 4-deoxy-5-oxoglucurate hydro-lyase (decarboxylating) (EC 4.2.1.41). The preparation of homogeneous samples of this enzyme was first reported by Jeffcoat *et al.* (1969) and is unusual in that it has the capacity both to decarboxylate and to dehydrate its substrate, 4-deoxy-5-oxoglucurate. The reaction occurs via the formation of an enzyme-substrate-Schiff-base complex (Hassall *et al.*, 1969) to yield 2,5-dioxovalerate, CO₂ and water without the release of detectable amounts of any intermediate.

Experimental

Pseudomonas acidovorans NCIB 10013 was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, U.K. and 4-deoxy-5-oxoglucurate hydro-lyase (decarboxylating) was purified as previously described (Jeffcoat *et al.*, 1969). Homogeneity was based on a single peak in the analytical ultracentrifuge and single bands on polyacrylamide gels and immunoelectrophoresis (Jeffcoat *et al.*, 1969). The latter was carried out in 1.5% (w/v) agar with 7 V/cm for 45 min at 4°C. The electrophoretic buffer was 0.1 M-veronal adjusted to pH 8.2 with NaOH and a single precipitin arc was located after staining with a solution containing 1 g of AmidoBlack, 54 ml of acetic acid, 7.2 g of sodium acetate and 100 ml of glycerol in a final volume of 1 litre of distilled water. Protein concentrations were calculated from the value of 8.3 for the $E_{1\text{cm}}^{1\%}$ at 280 nm

as determined for the enzyme by Hassall *et al.* (1969).

Amino acid analyses were determined after hydrolysis of the enzyme in constant-boiling HCl in sealed, evacuated tubes at 110°C. Various times between 24 and 96 h were used to enable corrections to be made for the destruction of amino acid residues. During the hydrolyses valine and isoleucine are slowly released and it was therefore necessary to correct the observed values by extrapolation to a maximum value. In most cases this was the value obtained from the 96 h incubation. Similarly the observed values for serine and threonine had to be corrected by extrapolation to zero-time values to compensate for the breakdown of these residues during hydrolysis. Cysteine was determined by performic acid oxidation by the method of Hirs (1956) and tryptophan by the spectrophotometric method of Goodwin & Morton (1946).

Thiol groups and disulphide bridges were determined by using Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)], in the presence of 8 M-urea before and after reduction with NaBH₄ by the method of Cavallini *et al.* (1966). After reduction of the protein, NaBH₄ was removed by acidification with dilute HCl and by the addition of 2 ml of acetone to the 3 ml incubation system.

Reduction and carboxymethylation was carried out in the dark in a double-arm Warburg flask which had been flushed with N₂. The incubation mixture contained 4 mg of enzyme, 150 µmol of EDTA, and 150 µmol of Tris (adjusted to pH 8.6 with HCl) dissolved in a final volume of 3 ml of 8 M-urea. The incubations were carried out at 30°C for 4 h in the presence and absence of a 400-fold excess of dithiothreitol. After this time a 2.6-fold molar excess of iodo[2-¹⁴C]acetate (0.15 µCi/µmol) over the dithiothreitol was added to both systems and incubated for a further 90 min. The specific radioactivity of the iodoacetate was calculated by diluting the radioactive material (stated to be 32 µCi/µmol), obtained from The Radiochemical Centre, Amersham, Bucks., U.K., with unlabelled iodoacetate, which had been recrystallized from diethyl ether. A 100 µl sample was then counted in 15 ml of the scintillation fluid given below.

Protein was isolated by gel filtration on a column (20 cm × 2 cm) of Sephadex G-25 equilibrated with

0.05 M-potassium phosphate adjusted to pH 7.2 with KOH. The protein eluted from the column was determined with reference to a calibration curve prepared by the method of Lowry *et al.* (1951), with 4-deoxy-5-oxoglucuronate hydro-lyase as the protein source. Radioactivity measurements were made by counting 100 µl samples for a total of 1 × 10⁴ counts and recording the time in a Beckman multi-channel liquid-scintillation system LS-200B in the solvent system of 5 g of 2,5-diphenyloxazole and 100 g of naphthalene in 1 litre of scintillation-grade dioxan, which can tolerate a maximum of 10% (v/v) of its own volume without significant quenching.

Molecular-weight determinations were performed in the Beckman model E analytical ultracentrifuge by the high-speed sedimentation-equilibrium method of Yphantis (1964). The studies were carried out at 20°C by using approx. 3 mm solution columns in double-sector cells equipped with sapphire windows. In all cases, the attainment of equilibrium was determined directly by measuring fringe shifts at given radial positions on successive photographs, and water blanks were run to correct for any window distortion. Partial specific volumes were calculated from the amino acid composition of the enzyme (Table 2) and the value of 0.73 cm³/g was used for the molecular-weight determinations in all solvents. The limitations of this approach are discussed in the Results section. Minimum molecular weights in the presence of sodium dodecyl sulphate were determined by the procedure described by Weber & Osborn (1969).

Results and Discussion

Molecular-weight determinations

Homogeneous preparations of 4-deoxy-5-oxoglucuronate hydro-lyase were subjected to sedimentation-equilibrium analyses in the analytical ultracentrifuge under the conditions shown in Table 1. The logarithm of fringe displacement was plotted against the square of the radial distance and the results for each system are recorded in Fig. 1. From these data, the molecular weight for the native enzyme in 0.1 M-potassium phosphate buffer, pH 7.2, was calculated to be 113 000. In the presence of the

Table 1. Summary of the molecular-weight determinations of 4-deoxy-5-oxoglucuronate hydro-lyase

Procedure	Solvent	Mol. wt.
Sedimentation equilibrium	0.15 M-KCl-0.05 M-KH ₂ PO ₄ adjusted to pH 7.2 with KOH	113 000
	6 M-Guanidine hydrochloride (unbuffered)	56 000
	6 M-Guanidine hydrochloride-0.1 M-β-mercaptoethanol (unbuffered)	30 400
Gel electrophoresis	1 g of sodium dodecyl sulphate, 3.9 g of NaH ₂ PO ₄ ·H ₂ O and 19.3 g of Na ₂ HPO ₄ ·7H ₂ O per litre of water	30 500

Fig. 1. High-speed sedimentation-equilibrium data for a 0.03% solution of enzyme

Centrifugation was carried out in (a) 0.15M-KCl, 0.05M-potassium phosphate buffer, pH 7.2, at 21736 rev./min; (b) 6M-guanidine hydrochloride at 39420 rev./min; (c) 0.1 M-β-mercaptoethanol in 6M-guanidine hydrochloride, at 39420 rev./min. The data are plotted as logarithm of fringe displacement against the square of the radial distance in cm.

Table 2. Amino acid composition of 4-deoxy-5-oxoglucarate hydro-lyase (decarboxylating) from *Pseudomonas acidovorans*

For details of corrections made for the destruction or slow release of residues during hydrolysis see the Experimental section.

Amino acid residue	Composition (residues/molecule of enzyme mol.wt. 113000)
Aspartate	80.9
Threonine	52.1
Serine	46.4
Glutamate	85.7
Proline	44.4
Glycine	89.8
Alanine	136.2
Valine	70.3
Methionine	28.5
Isoleucine	53.6
Leucine	100.0
Tyrosine	38.8
Phenylalanine	45.2
Lysine	55.3
Histidine	18.0
Arginine	41.4
Cysteic acid	12.3
Tryptophan	15.5

denaturing solvent, 6M-guanidine hydrochloride, this value for the molecular weight is halved to 56000, which suggests that the active form of the enzyme is a dimeric species. However, if a reducing agent is added to the guanidine hydrochloride, the molecular weight is approximately halved yet again, to 30400. This value for the minimum molecular weight was also obtained when the enzyme was preincubated with sodium dodecyl sulphate and β-mercaptoethanol before electrophoresis on polyacrylamide gels in the presence of the detergent.

As already mentioned these molecular-weight determinations were calculated from a value of 0.73 cm³/g for the partial specific volume ($\bar{v}$), but the validity of such assumptions has been questioned and discussed at length by Reisler & Eisenberg (1969). Hade & Tanford (1967) underline the need for a more-detailed investigation into the value of $\bar{v}$ in such solvents as guanidine hydrochloride. In their paper they state, however, that the partial specific volume in guanidine hydrochloride will not in general differ significantly from its value in dilute salt solutions. Further, calculations of the apparent specific volume taking into account preferential binding of solvent components (Marler *et al.*, 1964; Small & Lamm, 1966; Kielley & Harrington, 1960) have shown that the partial specific volume is between 0.01 and 0.02 cm³/g higher than the corrected value. The maximum error introduced into the molecular-weight calculations presented in the present paper by failing to make the above corrections would not significantly alter the conclusions made from these calculations. It is therefore considered that the ultracentrifugation data taken in conjunction with the gel-electrophoresis data are consistent with the tetrameric nature of the enzyme, in which two polypeptide chains of molecular weight 30000 are linked by disulphide bridges into a 56000-mol.wt. unit, which in turn dimerizes into a 113000-mol.wt. species, the active enzyme.

Amino acid composition of the enzyme

This is given in Table 2.

Determination of the number of cysteine and cystine residues

The cysteine content of the enzyme was determined by using carboxymethylation or reaction with 5,5'-dithiobis-(2-nitrobenzoic acid), before and after

Table 3. *Determination of the thiol content of the enzyme before and after reduction*

Incorporation was determined from the known specific radioactivity of the iodoacetate and a molecular weight of 113000 for the enzyme.

Assay procedure	Thiol content (μmol of cysteine/ μmol of enzyme)	
	Before reduction	After reduction
1. Ellman's reagent (reduction with NaBH_4)	7.9	12.0
2. Carboxymethylation (reduction with dithiothreitol)	8.4	12.0

reduction, and the results are summarized in Table 3. From these results it was concluded that the enzyme contains eight thiol groups and a further four residues bound in the form of two disulphide bridges. In view of the large excess of dithiothreitol used in these experiments to ensure complete reduction of the disulphide bridges, the possibility exists that the subsequent addition of a large quantity of iodoacetate might result in the carboxymethylation of amino acid residues other than cysteine. This possibility is unlikely, since in control experiments in which the protein was carboxymethylated with unlabelled iodoacetate, isolated by gel filtration, reduced with dithiothreitol and finally carboxymethylated with iodo[2- ^{14}C]acetate, only 4 mol of labelled material was incorporated/mol of enzyme mol.wt. 113000. Further, the incorporation of iodoacetate into fully reduced enzyme is in good agreement with the total cysteine estimated as cysteic acid by acid hydrolysis and amino acid analysis (Table 2). Since it has been demonstrated above that the enzyme exists as a dimer, it is likely that each 56000-molecular-weight unit contains only one disulphide bridge and that this is responsible for the binding of the two 30000-molecular-weight polypeptide chains.

Tryptic digestion and 'fingerprinting'

Previous observations by Hassall *et al.* (1969) have shown that the enzyme binds only two substrate molecules/molecule of enzyme (mol.wt. 113000). In view of this result it is possible that the four polypeptide chains comprising the native enzyme are not identical but consist of two different types of polypeptide chain of equal molecular weight. To gain support for this hypothesis tryptic digestion followed by 'fingerprinting' was carried out. The

Fig. 2. *Diagrammatic representation of the 'fingerprinting' of the tryptic peptides of D-4-deoxy-5-oxoglucuronate hydro-lyase*

Chromatography was carried out first in 3-methylbutan-1-ol-pyridine-water (6:6:7, by vol.) followed by electrophoresis for 100 min at pH 3.5 and 45 V/cm in pyridine-acetic acid-water (1:10:89, by vol.)

protein was first oxidized with performic acid by the method of Hirs (1956) and dissolved in 0.5% ammonium bicarbonate, pH 8.5, before being incubated at 37°C for 4 h with 2 mg of 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one (TPCK)-treated trypsin for every 1 mg of enzyme protein to be digested. The reaction was stopped by adjusting the pH to approx. 3 with dilute HCl followed by freeze-drying. From the results of the amino acid analysis shown in Table 2, it would be expected that a single polypeptide chain containing a total of 96 arginine and lysine residues would yield 97 peptides after tryptic digestion. Similarly, two identical chains would yield 49 peptides and four identical chains 25 peptides. The tryptic 'map' produced after performic acid oxidation of the enzyme, followed by tryptic digestion at pH 8.5, is shown in Fig. 2. The details of the chromatography and electrophoresis are given in the diagram, which shows the presence of between 45 and 50 ninhydrin-positive regions, with no ninhydrin-positive material detectable at the origin. (The regions marked with a broken line gave only a weak ninhydrin reaction.) Although analysis of peptide 'maps' composed of large numbers of peptides is often difficult and inconclusive it is felt

that this particular 'map' is informative in that it sets out to distinguish between the presence of 25, 49 and 97 peptides from the parent enzyme. The possibility of lack of detection of poorly stained material only adds greater support to the proposed heterogeneity of the polypeptide chains, unless one is prepared to suggest that 50 such peptides go undetected and thus infer that the enzyme is composed of a single polypeptide chain, mol.wt. 113000, with two active centres. The results of the 'fingerprinting' would seem much more likely to be consistent with the hypothesis that the four polypeptide chains are not identical but arranged in an $\alpha_2\beta_2$, or perhaps more accurately $(\alpha\beta)_2$, conformation.

Evidence for the heterogeneity of the polypeptide chains of fructose 1,6-diphosphate aldolase is now well established (for references see Lai & Horecker, 1972). However, it has been suggested that the only difference in the primary sequence of the two chains (Lai *et al.*, 1970) is that residue 361 in the α chain is an asparagine and in the β chain an aspartate residue. Clearly the situation in the 4-deoxy-5-oxoglutarate hydro-lyase is more complicated, since not only are the chains held together in part by covalent forces but the primary structure of the chains must differ greatly to yield the observed number of tryptic peptides. Any similarities therefore that might exist between aldolases and hydro-lyases, in terms of enzyme mechanism as suggested by Jeffcoat *et al.* (1969), do not extend as far as the nature of the heterogeneity of the polypeptide chains that form the active enzyme.

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