Isolation and characterization of glyoxylate dehydrogenase from the fungus *Sclerotium rolfii*

Anthony J. BALMFORTH and Alan THOMSON

Department of Chemistry, University of Lancaster, Lancaster LA1 4YA, U.K.

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Glyoxylate dehydrogenase (glyoxylate:NAD⁺ oxidoreductase) was purified 600-fold in three steps from crude extracts of the fungus *Sclerotium rolfii* (*Corticium rolfii* Curzi). Two of the purification steps involved dye-affinity chromatography. The enzyme is a tetramer of M_r 250 000, with identical subunits of M_r 57 000. Inhibition studies suggest that there is one essential thiol group per active site.

The enzyme glyoxylate dehydrogenase (glyoxylate:NAD⁺ oxidoreductase) was discovered by Maxwell & Bateman (1968) in cell-free extracts from *Sclerotium rolfii* (*Corticium rolfii* Curzi). The enzyme catalyses the reaction:

which appears to be the major pathway for oxalate production in this fungus. Activity believed to be due to a similar enzyme has also been observed in extracts from *Fomes annosus* (Huttermann *et al.*, 1980) and from *Candida tropicalis* (Kachhy *et al.*, 1972). Production of the enzyme in *S. rolfii* is induced by pH values greater than 3.5, and the oxalic acid produced by its action increases the acidity of the medium to the optimum growth area for the fungus, about pH 2.5.

Two other pathways have been found which involve nicotinamide coenzymes in the oxidation of glyoxylate to oxalate. Glyoxylate dehydrogenase (acylating) (EC 1.2.1.17) is an NADP⁺-dependent enzyme from *Pseudomonas oxalaticus* which produces oxalyl-CoA as the initial product (Quayle & Taylor, 1961). It has also been found that lactate dehydrogenase from rabbit muscle catalyses disproportionation of glyoxylate to glycollate and oxalate, the former being then oxidized back to glyoxylate by the enzyme (Duncan & Tipton, 1969).

Glyoxylate dehydrogenase from *S. rolfii* has been studied in crude extracts, but has not hitherto been isolated; we report here the purification and some properties of the enzyme. We have also verified that it is a distinct species and that the activity is not a side reaction catalysed by some other enzyme.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and methods

Materials

Ultrogel AcA-34 was obtained from LKB Instruments, South Croydon, Surrey, U.K.; Sepharose 4B was from Pharmacia, Hounslow, Middx., U.K.; Reactive Blue 2 on cross-linked agarose was from Sigma Chemical Co., Poole, Dorset, U.K. Procion Red P-4B on Sepharose 4B was prepared by the method of Lowe *et al.* (1980), by using Procion Red P-4B kindly given by Imperial Chemical Industries, Blackley, Manchester, U.K. Other standard chemicals were suitable grades from Sigma or BDH Chemicals, Poole, Dorset, U.K.

Basal salts medium was prepared by adding to 1 litre of distilled water 1.0 g of NH₄NO₃, 697 mg of K₂HPO₄, 181 mg of MgSO₄, 149 mg of KCl, 26 mg of Fe(NO₃)₃, 6.2 mg of MnSO₄, 5.5 mg of ZnSO₄ and 1 mg of thiamin.

Enzyme assays

Glyoxylate dehydrogenase activity was determined by following the reduction of NAD⁺ at 340 nm with a Pye-Unicam SP.8-100 Ultraviolet Spectrometer equipped with a cell-temperature controller set at 30°C. The reaction mixture contained glycine/NaOH buffer, pH 9.2 ($I = 0.05$ M), 0.1 ml of 10 mM-NAD⁺, 0.1 ml of 40 mM-sodium glyoxylate and the enzyme preparation in a final volume of 3 ml. The reaction was initiated by the addition of sodium glyoxylate. The concentration of NADH formed was calculated by assuming a molar absorption coefficient of $6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 340 nm. One unit of activity is defined as the amount of enzyme required to reduce 1 μ mol of NAD⁺/min under assay

conditions. Specific activity is expressed as the number of units of enzyme activity per mg of protein. Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin that had been kept over anhydrous silica gel as standard.

Buffer preparations

All solutions, including buffers, normally contained 5 mM-2-mercaptoethanol to stabilize enzyme activity. Buffers were prepared according to the tables of Long (1961), with the exception of 0.1 M-phosphate buffer, pH 7.2, which was prepared by titrating a 0.1 M solution of KH_2PO_4 with a 0.1 M solution of Na_2HPO_4 , and Tris/acetate buffers, which were prepared by titrating a Tris solution with acetic acid and making up to the given concentration of Tris.

Cell growth

S. rolfssii (CMC 137510) was maintained on 2% agar plates containing basal salts medium plus 0.5% glucose. Such plates produce large quantities of sclerotia in 3 weeks incubation at 25°C. Starter cultures (200 ml) were grown in basal salts medium at pH 3.5 containing 0.055 M-glucose and 0.055 M-citrate. They were each inoculated with 40–60 sclerotia from agar plates and grown at 30°C in shake culture for 6–7 days.

A Gallenkamp modular fermenter was set up with the following accessories: pH-titration unit (set up for addition of 4 M-NaOH and 4 M-HCl), temperature-control unit, aeration unit, condenser/air outlet, medium-inlet/harvest-outlet tube and an oxygen inlet. A 20-litre fermenter vessel was used, and some modifications to the original vessel head and stirrer were necessary to allow vessels of this size to be used successfully. The fermentation vessel contained 18 litres of basal salts medium with added yeast extract (20 g) and glucose (400 g). The sterilized culture medium was adjusted to pH 3 before the inoculum (three starter cultures) was added. The culture was incubated at 30°C to promote rapid growth and maintained at pH 3 for 6 days. On day 6 the pH-titrator was adjusted to maintain pH 5, thereby stimulating the production of glyoxylate dehydrogenase. Culture medium (2 litres) was exchanged for glucose solution (400 g in 2 litres of water) at this stage to satisfy the increasing nutrient requirement of the culture. Enzyme production was increased if oxygen rather than air was blown through the mixture during this induction phase. The culture was harvested on day 7.

Purification of glyoxylate dehydrogenase

All work was performed at 0–4°C. In a typical experiment mycelium was separated from culture

medium (18 litres) by filtration, by using a wine-straining bag. It was washed twice with deionized water, strained, weighed (400 g) and resuspended in 0.1 M-phosphate buffer, pH 7.2 (1:2, w/v), by using a homogenizer. The mycelium was disrupted by using three 1 min pulses of ultrasound, separated by cooling periods, in an MSE 150 ultrasonic disintegrator set at maximum output. The resulting suspension was centrifuged at 30000g for 30 min. The supernatant (1080 ml) was adjusted to 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ by addition of a saturated solution of the salt buffered to pH 7.2, and the mixture was centrifuged at 30000g for 30 min. The precipitate was discarded, and the supernatant adjusted to 50% saturation of $(\text{NH}_4)_2\text{SO}_4$ and centrifuged as above. The precipitate was washed with 50%-satd. $(\text{NH}_4)_2\text{SO}_4$, pH 7.2, and collected by centrifugation as above. It could be stored in 70%-satd. $(\text{NH}_4)_2\text{SO}_4$ at pH 7.2 and 4°C for several weeks without apparent loss of activity.

The precipitate was dissolved in 50 mM-Tris/acetate buffer, pH 7.0 (100 ml), and dialysed overnight against two changes of deionized water (each 20 litres). Then 500 mM-Tris/acetate buffer, pH 7.0, containing 10% (w/v) streptomycin sulphate was added dropwise to the diffusate to produce a solution containing 1% (w/v) streptomycin sulphate. The mixture was centrifuged at 30000g for 30 min at 20°C and the supernatant (210 ml) diluted with 50 mM-Tris/acetate buffer, pH 7.0 (1:1, v/v). This solution was applied to a Procion Red P-4B-Sephacrose 4B column (17.5 cm × 2.6 cm), which had been previously equilibrated with 50 mM-Tris/acetate buffer, pH 7.0. The column was washed with the equilibration buffer (120 ml) until the A_{280} decreased to less than 0.01. The flow direction was reversed before application of a linear gradient of KCl (0–1 M, total volume 220 ml) in equilibration buffer (Fig. 1). The pooled active fractions (nos. 67–75; total vol. 112 ml) from this column were applied to a Reactive Blue 2-cross-linked agarose column (5.5 cm × 1.0 cm), which had been previously equilibrated with 50 mM-Tris/acetate buffer, pH 7.0. The column was washed with 50 mM-Tris/acetate buffer, pH 8.5. The flow rate was reversed before application of a pulse of 0.5 mM-NAD⁺ in this pH 8.5 buffer (Fig. 2). Fractions containing enzyme activity (nos. 3–8; total vol. 10.8 ml) were pooled.

Polyacrylamide-disc-gel electrophoresis

Gels containing 5% (w/v) acrylamide at pH 8.5 were prepared and stained for protein with Coomassie Blue R-250 by methods based on the work of Gabriel (1971a,b). Glyoxylate dehydrogenase activity was located on polyacrylamide gels, with glyoxylate as substrate, by the method of Turner & Hopkinson (1979).

Fig. 1. Affinity chromatography on Procion Red P-4B-Sepharose 4B

For experimental details see the text. Fractions (10 ml) were assayed for activity (—○—), and A_{280} was continuously monitored (—). The arrow indicates flow reversal and commencement of KCl gradient.

Fig. 2. Affinity chromatography on Reactive Blue 2 cross-linked agarose

For experimental details see the text. Fraction numbering commences at application of the NAD^+ pulse. Fractions (1.8 ml) were assayed for activity (—○—), and protein (—●—) was measured by the method of Bradford (1976).

SDS/polyacrylamide-gel electrophoresis

Gels containing 10% acrylamide and 0.1% SDS at pH 7.0 were prepared by the procedure of Weber & Osborn (1969). A Sigma Dalton Mark VI molecular-weight kit was used for protein standards.

Analytical isoelectric focusing

Isoelectric focusing was performed at 10°C with an LKB Ampholine PAG-plates kit, pH 3.5–9.5, by following the manufacturer's instructions. The pH gradient was determined by using a modified form of that described by Ledback & Wrigley

(1976). Gel discs (1 cm diam.) were cut out along the gradient length and placed in tubes containing deionized water (2 ml). The tubes were kept overnight at room temperature, shaken vigorously, cooled to 10°C, and the pH was measured directly in the tubes.

Determination of M_r by gel filtration

An Ultrogel AcA34 column (82 cm × 1.6 cm) was equilibrated with phosphate buffer, pH 7.0 ($I = 0.05\text{M}$) and calibrated with pyruvate kinase (M_r 235 000), aldolase (158 000) and lactate dehydrogenase (150 000). The calibration proteins were detected by their A_{280} , and the glyoxylate dehydrogenase by assay of its activity.

Measurements of activity

All studies of enzyme activity were made at 30°C. K_m values for the two substrates were determined in pH 7.2 phosphate buffer ($I = 0.05\text{M}$) with 0.05–2.0 mM concentrations of each. Glyoxylate dehydrogenase stability and activity were measured over the pH range 4.5–11.0, in the following buffer systems: pH 4.5–6.0, acetate buffer; pH 6.0–7.6, phosphate buffer; pH 7.5–8.6, *N*-glycylglycine/NaOH buffer; and pH 8.7–11.0, glycine/NaOH buffer. All buffers were of equivalent ionic strength ($I = 0.05\text{M}$). In stability studies, solutions of enzyme in the buffers were stored at 4°C and assayed at suitable intervals. Studies of activity versus pH were made by following the procedure of the assay, but with final concentrations of 1 mM-glyoxylate and 1 mM- NAD^+ in the appropriate buffer. Tests of potential activators and inhibitors were made in pH 7.2 phosphate buffer ($I = 0.05\text{M}$) with 0.15 mM-glyoxylate and 0.15 mM- NAD^+ . Tests of potential substrates were made in the same buffer by using 1 mM- NAD^+ .

Results and discussion

S. rofsii produces a polysaccharide during fermentation whose presence produced high viscosity in all the extracts before the first affinity chromatography. Because of this, the sample applied to the Procion Red column had to be substantially diluted to avoid compression of the gel. The fractions containing glyoxylate dehydrogenase were eluted from both affinity columns by reversing the flow, because this gave the active material in the smallest possible volume of solution. The distorted peak shape in the final Reactive Blue column chromatography (Fig. 2) arises through use of this technique and also through the use of an NAD^+ pulse to initiate elution; there was no difference in activity or electrophoretic behaviour between samples taken from the earlier and later parts of the peak. Binding of the enzyme to the Reactive

Table 1. Summary of purification of glyoxylate dehydrogenase from *S. rolfii* mycelium

	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell-free extract	1080	120	1205	0.1	100	1
(NH ₄) ₂ SO ₄ fractionation followed by streptomycin ppt.	210	107	204	0.5	89	5
Procion Red P-4B affinity chromatography	113	109	17.4	6.3	91	63
Reactive Blue 2 cross-linked Agarose affinity chromatography	10.8	96	1.58	60.8	80	608

Fig. 3. Analytical isoelectric focusing of glyoxylate dehydrogenase preparations

Samples were focused for 90 min at 10°C on LKB pH 3.5–9.5 Multiphor gel. Sample A is that described in the text; sample B had been subject to a further chromatographic treatment. The continuous line indicates pH measured as described in the text.

Blue column was highly pH-dependent; at pH 7.0 the enzyme could not be eluted by concentrations up to 1M-KCl and/or 10mM-NAD⁺, whereas at pH 8.5 elution commenced at concentrations of NAD⁺ below 0.2mM in the absence of added salt. A summary of the complete purification scheme is given in Table 1.

Purity was checked by both electrophoresis and electrofocusing. The enzyme, which was essentially immobile at pH 8.5 in gels containing more than 7% acrylamide, gave a single protein band on electrophoresis at pH 8.5 in 5% acrylamide which was identical in position with the single band that appeared when gels were stained for glyoxylate dehydrogenase activity.

Analytical isoelectric focusing showed one major band corresponding to pI 5.9 and two minor bands, pI 5.7 and 5.8 (Fig. 3), and this pattern was found in all samples tested, including samples that had been subjected to further ion-exchange or gel chromatography. The enzyme was totally inactivated during electrofocusing, so it was not possible to determine whether one or all of these bands represented the enzyme. It is possible that this multi-

plicity is due to the presence of isoenzymes, but it seems more likely to us that the multiple bands are an artefact of the electrofocusing technique. This type of behaviour has been reported by Illingworth (1972), who found that pure yeast isocitrate dehydrogenase gave multiple bands, which he ascribed to complexation of the protein with Ampholines in the electrofocusing medium.

The *M_r* of the enzyme was determined by gel filtration on Ultrogel AcA34 as 250000 ± 20000, where it was eluted shortly before pyruvate kinase (*M_r* 235000). Because of the paucity of readily available standard proteins in this *M_r* region, the *M_r* value given must be regarded as somewhat tentative, even though results for the three standard proteins used were on a precise straight line when plotted as log(*M_r*) versus elution volume. The *M_r* determined by SDS/polyacrylamide-gel electrophoresis was 57000 ± 3000. We conclude that glyoxylate dehydrogenase is a tetramer of four identical subunits. It is therefore similar to other NAD⁺-dependent dehydrogenases, e.g. horse liver aldehyde dehydrogenase, which has *M_r* 230000 and subunit *M_r* 53000 (Eckfeldt *et al.*, 1976).

Preparations of the enzyme at any stage after the initial (NH₄)₂SO₄ precipitation lost no significant activity on storage for 10 days at pH 6–7 at 4°C either as precipitate or in solution, provided that 5mM-2-mercaptoethanol was present. At pH 5 or pH 9 50% of the activity remained after 10 days, and this could be increased to 75% by the addition of 10% (v/v) glycerol. Removal of the thiol from solutions by gel filtration resulted in an irreversible loss of activity, with half-life around 4h at room temperature. It was not possible to freeze-dry the enzyme and retain activity.

The kinetic analysis was based on a two-substrate ternary-complex mechanism. This yielded at pH 7.2 and 30°C a *K_m* value of 0.12mM for glyoxylate and 0.18mM for NAD⁺; the former value is in good agreement with that found by Maxwell & Bateman (1968). A pH/activity profile (Fig. 4) indicated maximal activity at pH 9.0. The observed activity is buffer-dependent, because of anionic inhibition (see below), but activity appears

Fig. 4. pH/activity profile on glyoxylate dehydrogenase. Glyoxylate dehydrogenase was assayed at 30°C with 1 mM-NAD⁺ and 1 mM-glyoxylate in phosphate buffer (○), glycylglycine/NaOH buffer (△) and glycine/NaOH buffer, (●), all at $I = 0.05M$.

to be dependent on the basic form of a group with pK_a approx. 8.2. The decline in activity above pH 9.5 is due to instability of the enzyme.

Because of the glyoxylate dehydrogenase-like activity exhibited by lactate dehydrogenase, we have examined a wide range of metabolites as substrates or effectors of the enzyme, in order to check that the activity is not a secondary activity of some more common enzyme. The following compounds were found to be ineffective as substrates, activators or inhibitors of the enzyme at concentrations of 1 mM in the presence of NAD⁺ or NADH as appropriate: acetaldehyde, acetyl-CoA, citrate, ethanol, formaldehyde, fumarate, glyceraldehyde, glycollate, isocitrate, α -oxoglutarate, lactate, malate, oxalate, oxaloacetate, oxalosuccinate, pyruvate, succinate, succinyl-CoA, cyclic AMP, ADP, ATP and CoA. We therefore conclude that this is an enzyme of unique activity.

Like many dehydrogenases, the enzyme is inhibited by anions, the inhibition increasing according to the chaotropic series (Table 2). Maxwell & Bateman (1968) suggested that the enzyme was a metalloenzyme, on the basis of inhibition by azide and cyanide, but it is possible that the inhibition by these ions was a simple anionic inhibition. The activity of a glyoxylate dehydrogenase solution from which the mercaptoethanol had been removed by gel filtration was totally inhibited by silver or mercury(II) ions at 1 mM concentration, and partially by cadmium and copper(II) ions, but was unaffected by a range of other metal ions at the

Table 2. Effect of univalent anions and cations on glyoxylate dehydrogenase activity

Anions were made up as 0.1 M sodium salts directly in assay buffer. Residual activity was measured after 5 min incubation of enzyme in salt-containing buffer.

Added anion	Residual activity (%)
None	100
Fluoride	100
Acetate	98
Chloride	49
Bromide	31
Nitrate	16
Iodide	15
Perchlorate	10

same concentration. Treatment of the enzyme solutions with 1 μ M-iodoacetamide in the absence of mercaptoethanol at 20°C resulted in total inactivation in a reaction which showed excellent first-order kinetics with a half-life of 22 min. These results strongly suggest that the enzyme has a single essential thiol group per active subunit. It is probably this group that has pK_a 8.2.

The results that we have obtained suggest that glyoxylate dehydrogenase is a typical NAD⁺-dependent dehydrogenase with a distinct specificity towards glyoxylate. The presence of the essential thiol group suggests that the mechanism will be similar to that proposed for glyceraldehyde phosphate dehydrogenase (Harrigan & Trentham, 1973), involving oxidation of a thiohemiacetal intermediate to a thiol ester, followed by hydrolysis.

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