The Purification and Properties of Malonic Semialdehyde Oxidative Decarboxylase from *Prototheca zopfii*

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1. An enzyme, which in the presence of NAD⁺ and CoA oxidizes malonic semialdehyde to acetyl-CoA, has been purified from an extract of the colourless alga *Prototheca zopfii*. 2. The purified enzyme has optimum pH 7.5, is specific for NAD⁺ and requires a thiol compound for maximum activity. 3. The enzyme is inhibited by arsenite, *N*-ethylmaleimide and urea. 4. The results are discussed in relation to those obtained by other workers with a similar bacterial enzyme, and a possible reaction sequence is proposed.

Malonic semialdehyde oxidative decarboxylase [malonate semialdehyde-NAD(P) oxidoreductase (acetylating CoA)] catalyses the reaction:

This reaction has been demonstrated in crude extracts of propionate-grown cells of the colourless alga *Prototheca zopfii*, and implicated in the pathway of propionate assimilation in this organism (Callely & Lloyd, 1964). The stoichiometry of the overall process, the requirement for substrate amounts of CoA and the fact that acetaldehyde is not a free intermediate have been shown on an unpurified enzyme preparation (Callely & Lloyd, 1964). A similar reaction has been reported by Yamada & Jakoby (1960) in *Pseudomonas fluorescens* grown on acetylenemonocarboxylic acid as sole carbon source, and the enzyme was purified 60-fold from this organism. These investigators suggested that this complex reaction is catalysed by a single enzyme, which they classified as an aldehyde dehydrogenase. The enzyme from propionate-grown *Prototheca* was therefore purified, to study its properties and compare it with the bacterial enzyme.

MATERIALS AND METHODS

Materials. Malonic semialdehyde was freshly prepared before use (from the acetal of malonic semialdehyde ethyl ester provided by Professor M. J. Coon) and assayed by the colorimetric method of Robinson & Coon (1963). NAD⁺, NADP⁺, salmine sulphate, urea, sodium arsenite, 2,3-dimercaptopropan-1-ol (BAL) and thiamine pyrophosphate were obtained from British Drug Houses Ltd., Poole, Dorset; CoA was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; ATP was from Sigma Chemical Co.,

St Louis, Mo., U.S.A.; *N*-ethylmaleimide, 2-mercaptoethanol and lipoic acid were from L. Light and Co. Ltd., Colnbrook, Bucks. Lipoyl dehydrogenase was a gift from Professor V. Massey.

Growth of the organism. The organism was grown in 5l. batches on the propionate medium described by Callely & Lloyd (1964), and harvested at 10000 rev./min. (6500g) in the continuous-flow head of an MSE 17 centrifuge.

Protein estimations. Protein concentrations were determined by measuring the extinctions at 260 and 280 mμ (Warburg & Christian, 1941).

Standard assay system. The enzyme was assayed by the rate of increase of extinction at 340 mμ produced by the reduction of NAD⁺ during the reaction. The cuvettes (light-path 1 cm.) contained, in a total volume of 0.45 ml., the following reaction mixture (μmoles): tris buffer, pH 7.5, 25; 2-mercaptoethanol, 2.5; CoA, 0.25; NAD⁺, 0.75; enzyme; malonic semialdehyde, approx. 0.2. The blank contained water in place of malonic semialdehyde, but all the other additions; the reaction was started in the test cuvette by the addition of malonic semialdehyde.

The reduction of NAD⁺ was linear with time over the first 2.5 min. of the reaction, and with protein concentration over the range of enzyme concentrations employed. The reaction was therefore followed by reading extinction values at 30 sec. intervals for 2.5 min. at room temperature (20°) in a Unicam SP.500 spectrophotometer.

Unit of enzyme activity. This is defined as the amount of enzyme producing 1.0 μmole of NADH/min. during the first 2 min. of the reaction.

Buffer solutions. Phosphate buffers were prepared by making up solutions of KH₂PO₄ at the required molarity and adjusting the pH with *N*-NaOH. When tris was used, solutions of the concentration required were adjusted to the pH stated with 2*N*-HCl.

Purification procedure. The following procedure was developed by a series of small-scale tests on the crude extract.

Step 1: Preparation of the extract. Cells (5.0g. wet wt.), which had been stored at -18°, were suspended in 25 ml. of

Table 1. *Purification of malonic semialdehyde oxidative decarboxylase*

Experimental details are given in the Materials and Methods section

	Fraction	Vol. (ml.)	Activity (units/ml.)	Total units	Protein (mg./ml.)	Specific activity	Purifica- tion	Yield (%)
I	Supernatant obtained from crude extract after centrifuging	29	0.72	20.8	24.5	0.0294	—	—
II	Supernatant obtained after removal of salmine sulphate precipitate by centrifuging	36	1.14	41.0	6.12	0.186	6.2×	195
III	Supernatant after removing the precipitate formed on adjusting to pH 5.0	36	1.51	54.4	6.0	0.252	8.45×	260
IV	Eluate from treatment with calcium phosphate gel	9.4	1.04	9.8	1.6	0.65	22×	46
V	Fraction precipitated by 70–90% saturated $(\text{NH}_4)_2\text{SO}_4$	1.8	1.72	3.1	1.01	1.71	57.7×	14.8

50mm-phosphate buffer, pH 7.0, containing 2-mercaptoethanol (5.0mm) (as in all the buffer solutions used in this procedure), and disrupted by passing through a French press. The resulting extract was centrifuged at 15000g for 15 min. at 0° and the supernatant (fraction I) decanted off. All subsequent operations were performed at 2°.

Step 2: Treatment with salmine sulphate. Fraction I was made with 0.1M with respect to $(\text{NH}_4)_2\text{SO}_4$ by adding 3.0ml. of 1.0M- $(\text{NH}_4)_2\text{SO}_4$, and 1% (w/v) salmine sulphate solution was added dropwise until a volume 40% of that of fraction I had been delivered. The resulting suspension was centrifuged and the supernatant (fraction II) retained.

Step 3: Adjustment of pH. Fraction II was adjusted to pH 5.0 with 30% (v/v) acetic acid. A precipitate was removed by centrifuging and the supernatant retained (fraction III).

Step 4: Treatment with calcium phosphate gel. Keilin-Hartree gel, which had been prepared by the method cited by Dixon & Webb (1958), was used as a suspension containing 25.6mg. dry wt. of adsorbent/ml. Sufficient gel was added to fraction III to give a protein/gel ratio 1:0.5; a precipitate was removed by centrifugation and discarded. More gel was added to the supernatant to give a protein/gel ratio 1:0.75. A precipitate was removed as before and gel was added to give the same protein/gel ratio once again. On adding further portions of gel to the supernatant the enzyme was adsorbed (see Table 1). The enzyme was eluted by adding 9.5ml. of 66mm-phosphate buffer, containing $(\text{NH}_4)_2\text{SO}_4$ (20%, w/v), and the gel was removed by centrifuging and discarded. The supernatant (fraction IV) was further purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation.

Step 5: $(\text{NH}_4)_2\text{SO}_4$ Fractionation. Saturated $(\text{NH}_4)_2\text{SO}_4$ solution was employed; the precipitates produced by up to 50% saturation with the salt contained no activity when dissolved in 50mm-phosphate buffer. The 50–70% saturated fraction contained only one-tenth of the total enzyme of fraction IV, but the remaining enzyme was precipitated on adjusting to 90% saturation. This precipitate was taken up in 50mm-phosphate buffer (fraction V). This solution was kept at –18°; it represented a 58-fold purification over the initial soluble fraction of the extract.

RESULTS

Properties of the purified enzyme

Stability. The enzyme, dissolved in 50mm-phosphate buffer, was kept at –18° for 6 weeks with no appreciable loss of activity (Table 2). The bacterial enzyme must be stored in the presence of mercaptoethanol and NAD^+ (Yamada & Jakoby, 1960); such precautions to protect the algal enzyme are not necessary.

Heating at 45° for 5 min. decreased the activity by 75%, and heating at 55° for 5 min. produced complete inactivation (Table 2). The enzyme withstood storage overnight at 2° at pH 5.0.

Optimum pH. Fig. 1 shows the variation of enzyme activity with pH in tris buffer at room temperature. The optimum pH is 7.5.

Effect of substrate concentration on reaction rate. The apparent K_m values for malonic semialdehyde, NAD^+ and CoA were determined by plotting the reciprocals of substrate concentrations (Lineweaver & Burk, 1934) under conditions of limiting substrate concentrations of the component under investigation with the other substrates constant and in excess.

The K_m for malonic semialdehyde was 0.26mm with NAD^+ concentration at 1.6mm and CoA at 0.54mm; 1.0mm-malonic semialdehyde produced a low reaction rate owing to substrate inhibition. The K_m for CoA was 0.13mm, with NAD^+ present at a concentration of 1.6mm and malonic semialdehyde at 0.44mm. The K_m for NAD^+ was 0.34mm with CoA present at a concentration of 0.54mm and malonic semialdehyde at 0.44mm.

Specific requirements. When NADP^+ replaced NAD^+ in the standard assay system with an eight-

Table 2. *Effect of various additions or pretreatment of the enzyme on the rate of NAD⁺ reduction during the oxidative decarboxylation of malonic semialdehyde*

Each cuvette contained 9.5 μ g. of purified enzyme protein. Experimental details are given in the text.

Procedure	NADH produced (m μ moles/min.)
Standard assay	16.4
+ arsenite (1.0 mM)	4.6
+ arsenite (1.0 mM) + 2,3-dimercapto-propanol (1.25 μ moles)	9.3
+ arsenite (1.0 mM) + 2-mercaptoethanol (1.25 μ moles)	6.0
+ <i>N</i> -ethylmaleimide (20 mM)	1.6
+ <i>N</i> -ethylmaleimide (20 mM); enzyme pre-incubated for 20 min. with NAD ⁺	1.5
Enzyme preincubated for 10 min. with urea (2.75 M)	0
Enzyme heated at 45° for 5 min.	4.0
Enzyme heated at 55° for 5 min.	0
Enzyme stored 6 weeks at -18°	16.2
No mercaptoethanol present in assay system	1.7
NAD ⁺ replaced by NADP ⁺ in assay system	1.2

Fig. 1. pH-activity curve for the oxidative decarboxylation of malonic semialdehyde. Standard spectrophotometric assay conditions were used, with 15 μ g. of purified enzyme protein and tris buffer.

fold purified preparation of the enzyme, the reaction proceeded at 7.5% of the rate observed with NAD⁺ (Table 2).

When mercaptoethanol was absent from the standard assay system the reaction proceeded slowly (Table 2).

Effect of inhibitors. Preincubation of the enzyme with 2.75 M-urea for 10 min. produced complete inactivation (Table 2).

Preincubation of the enzyme with 20 mM-*N*-ethylmaleimide decreased the activity to about

Fig. 2. Inhibition of the oxidative decarboxylation of malonic semialdehyde by sodium arsenite. All the cuvettes contained 7 μ g. of purified enzyme protein and the concentration of reactants was the same as in the standard assay procedure described in the text. Final concentrations of the inhibitor were: A, none; B, 0.01 mM; C, 0.05 mM; D, 0.1 mM; E, 0.5 mM; F, 1.0 mM; G, 5.0 mM; H, 10.0 mM.

7.5%, and preincubation with 8.0 mM-NAD⁺ did not protect against inhibition by this reagent at the concentration used (Table 2).

Sodium arsenite was inhibitory in the presence of mercaptoethanol but not in its absence (Table 2). The effect of preincubating a range of concentrations of the inhibitor in the complete assay system is shown in Fig. 2. A 0.1 M concentration of arsenite produced a 50% inhibition of the rate of the reaction. The inhibition was of the 'mixed' type with respect to the aldehyde. The inhibitory effect of arsenite could be partially reversed by the addition of 1.25 μ moles of 2,3-dimercaptopropanol (Table 2). A similar concentration of 2-mercaptoethanol also increased the rate of NAD⁺ reduction, but less markedly.

Dialysis of the enzyme. When a 13-fold-purified preparation of the enzyme was dissolved in 60 mM-phosphate buffer, containing mercaptoethanol (5 mM), and dialysed against 10 vol. of the same buffer at 2° for 2 hr., there was almost complete loss of activity. The addition of NAD⁺ to the dialysing fluid did not prevent this decrease in the activity of the enzyme. On storage at 2°, this dialysed enzyme preparation recovered full activity within 24 hr. The addition of 20 μ l. of the diffusate, which had been concentrated by evaporation to 1.0 ml., did not affect the activity of the freshly dialysed enzyme,

nor did the addition of 1.0 μ mole of ATP or 6 μ g. of a preparation of lipoyl dehydrogenase, purified by Professor V. Massey from pig heart. Magnesium sulphate (1.0 μ mole), thiamine pyrophosphate or lipoic acid, added individually or as mixtures consisting of the different combinations of these cofactors, were also ineffective in restoring the activity. The explanation for this phenomenon has not been found.

DISCUSSION

The properties of the malonic semialdehyde oxidative decarboxylase purified from *Prototheca* are similar to, but not identical with, the bacterial enzyme discovered by Yamada & Jakoby (1960). Although the enzyme from *Prototheca* requires 2-mercaptoethanol in the assay system for maximal activity, it can be stored for 6 weeks at -18° in the absence of this compound without any inactivation. The bacterial enzyme, however, cannot be kept without 2-mercaptoethanol and NAD^+ if activity is to be maintained; if NAD^+ is omitted, activity can be restored by preincubating for 24 hr. with NAD^+ . The optimum pH for the algal enzyme is slightly lower than that of the bacterial enzyme when assayed under identical conditions. The substrate concentrations for NAD^+ and CoA that produce half maximum velocity of the reaction are of the same order for both enzymes, but the K_m for malonic semialdehyde is ten times as great for the enzyme from *Prototheca*. Substrate inhibition by high concentrations of malonic semialdehyde is observed in both cases.

The fact that free acetaldehyde is not an intermediate is demonstrated by the inability of this compound to produce the reduction of NAD^+ in the assay system. Yamada & Jakoby (1960) also have evidence that malonyl-CoA is not an intermediate in the bacterial reaction.

The requirement for 2-mercaptoethanol and also the inhibitory effects of arsenite and *N*-ethylmaleimide suggest that the action of the enzyme from *Prototheca* necessitates thiol groups. It is a characteristic of all the aldehyde dehydrogenases previously studied that the sensitivity to arsenite is manifest only in the presence of exogenous mercaptan and is reversible by dithiols but not by monothiols (Jakoby, 1957, 1958). The mechanism of arsenite inhibition with these enzymes is believed to differ from that involved in the oxidative decarboxylation of pyruvate and α -oxoglutarate (Sanadi, 1963). The aldehyde dehydrogenases are 50% inhibited at concentrations varying from 10 μ M to 1 mM (Jakoby, 1963), whereas lipoic acid-linked complexes are inhibited to this extent in the presence of 1 μ M-arsenite (Gunsalus, 1953).

No evidence has been obtained in the present investigation to suggest that thiamine pyrophos-

phate, lipoic acid or lipoyl dehydrogenase is involved. Inactive freshly dialysed preparations of the enzyme were not stimulated by adding any of these components. Further, the 60-fold-purified preparation is colourless.

Jakoby (1963) has suggested that arsenite inhibits aldehyde dehydrogenases by combining with closely juxtaposed thiol groups. The resultant ring complex (Stocken & Thompson, 1946) would be expected to be more stable with 6- and 5-membered ring structures of lipoic acid and 2,3-dimercaptopropanol respectively, than with the complex resulting from combination with adjacent protein thiol groups, and variations in the positioning of the latter might explain the broad range of concentrations required for inhibition.

The inhibition produced by *N*-ethylmaleimide may reflect a conformational change in tertiary structure rather than a specific reaction with a thiol group at the active site; evidence for this conclusion comes from the observation that preincubation with NAD^+ does not protect the enzyme against the action of this inhibitor. Treatment with urea presumably induces a similar type of non-specific denaturation.

It seems likely that the active entity in the oxidative decarboxylation of malonic semialdehyde involves complexes involving enzyme, NAD^+ , CoA and aldehyde. There is no evidence that more than

Scheme 1. A possible mechanism for the oxidative decarboxylation of malonic semialdehyde.

one enzyme is implicated. A possible reaction sequence is shown in Scheme 1. It involves the initial production of an enzyme-hemimercaptal complex. The next reaction is concerted, with the electrons donated by the decarboxylation providing an extra driving force for the transfer of hydride to NAD^+ (Jencks, 1962). The final stage is the exchange of the acetyl group from the enzyme to CoA. Such a scheme does not necessitate closely opposed thiol groups. Nirenberg & Jakoby (1960) have suggested a transient enzyme-ortho acid complex as an intermediate. In the algal system arsenite is not a strictly competitive inhibitor with regard to the aldehyde, and the inhibition is not so marked in the absence of 2-mercaptoethanol in the assay system; omission of the latter does not completely prevent the oxidation of malonic semialdehyde. These observations may be explained by the production of free thiol groups from protein disulphide bridges in the presence of exogenous mercaptan, one of which becomes available for combination with arsenite, and the other involved in hemimercaptal production (Jakoby, 1963).

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