The Isocitrate Dehydrogenases of *Acinetobacter lwoffii*

SEPARATION AND PROPERTIES OF TWO NICOTINAMIDE–ADENINE DINUCLEOTIDE PHOSPHATE-LINKED ISOENZYMES

By COLIN H. SELF and P. DAVID J. WEITZMAN

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, U.K.

(Received 9 June 1972)

Two isoenzymes of NADP-linked isocitrate dehydrogenase have been identified in *Acinetobacter lwoffii* and have been termed isoenzyme-I and isoenzyme-II. The isoenzymes may be separated by ion-exchange chromatography on DEAE-cellulose, by gel filtration on Sephadex G-200, or by zonal ultracentrifugation in a sucrose gradient. Low concentrations of glyoxylate or pyruvate effect considerable stimulation of the activity of isoenzyme-II. The isoenzymes also differ in pH-dependence of activity, kinetic parameters, stability to heat or urea and molecular size. Whereas isoenzyme-I resembles the NADP-linked isocitrate dehydrogenases from other organisms in having a molecular weight under 100 000, isoenzyme-II is a much larger enzyme (molecular weight around 300 000) resembling the NAD-linked isocitrate dehydrogenases of higher organisms.

We have briefly reported the existence of two isoenzymes of NADP-linked isocitrate dehydrogenase [*threo*-D₅-isocitrate–NADP oxidoreductase (decarboxylating), EC 1.1.1.42] in *Acinetobacter lwoffii* (Self & Weitzman, 1970). The activity of one of these isoenzymes was stimulated by low concentrations of either glyoxylate or pyruvate whereas that of the other isoenzyme was unaffected. In the present paper we describe the separation of the two isoenzymes and some of their molecular and kinetic properties.

Materials and Methods

Chemicals used were analytical grade or the finest grade available. Ammonium sulphate, especially low in content of heavy metals, was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., trisodium DL-isocitrate from Sigma Chemical Co., Kingston, Surrey, U.K., and protamine sulphate from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; DEAE-cellulose was Whatman grade DE-11.

The organism used was *A. lwoffii*, strain 4B (isolated from a laboratory reservoir of distilled water by P.D.J.W.) and cultures were maintained on nutrient–agar or acetate–agar slopes. A culture of *Escherichia coli* B was kindly provided by Professor H. L. Kornberg.

Isocitrate dehydrogenase activity was assayed at 25°C by following the formation of NADPH at 340 nm. Measurements were made with a Unicam SP.800 spectrophotometer fitted with a thermostatically controlled cuvette holder and a Unicam SP.21 external recorder. Unless otherwise stated, assay mixtures consisted of 20 mM-tris–HCl–10 mM–

MgCl₂–1 mM-EDTA–0.2 mM-NADP⁺–4 mM-DL-isocitrate, at pH 8.0. Reactions were initiated by the addition of enzyme to give a final volume of 1.0 ml. The effect of glyoxylate or pyruvate on enzyme activity was tested by including these compounds in the assay mixture before the addition of the enzyme. Any stimulatory effect thereby observed was calculated in terms of 'activation', which we define as the activity measured in the presence of the activator divided by the activity in its absence.

Glutamate dehydrogenase (EC 1.4.1.4) was assayed by following the oxidation of NADPH at 340 nm in the same tris–Mg²⁺–EDTA buffer as used for isocitrate dehydrogenase and in the presence of 0.1 mM-NADPH, 2 mM- α -oxoglutarate and 2 mM-NH₄Cl. Lactate dehydrogenase (EC 1.1.1.27) was assayed in a similar manner in the presence of 0.1 mM-NADH and 2 mM-pyruvate. Isocitrate lyase (EC 4.1.3.1) was assayed spectrophotometrically (Dixon & Kornberg, 1959) at 324 nm in 25 mM-imidazole–HCl, pH 6.8, 5 mM-MgCl₂, 1 mM-EDTA, 4 mM-phenylhydrazine–HCl and 2 mM-DL-isocitrate. Haemoglobin and catalase (EC 1.11.1.6) were both determined by their absorbance at 405 nm.

Concentrations of NADP⁺ were determined by reducing to completion a sample of NADP⁺ with excess of isocitrate and isocitrate dehydrogenase and measuring the change in absorbance at 340 nm; isocitrate was determined similarly by enzymic reaction with excess of NADP⁺. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Cellulose acetate electrophoresis was done with the Millipore Phoroslide apparatus at 100 V for

20min with 100mm-tris-veronal buffer, pH8.6. Isocitrate dehydrogenase bands were made visible by first treating the strips with a solution containing substrates, Nitro Blue Tetrazolium and phenazine methosulphate and then incubating them at 35°C for a few minutes.

Zonal ultracentrifugation was done at 10°C with an MSE B XIV titanium zonal rotor and an MSE Superspeed 65 centrifuge. The rotor was accelerated to 2500rev./min ($g_{av.} = 1000$) and then filled with sucrose solution of linearly increasing concentration from 5 to 20% (w/v) in buffer of composition 5mm-tris-HCl-1mm-EDTA-10mm-MgCl₂, at pH8.0. The rotor was full when sucrose solution emerged from the centre of the rotor core. Enzyme solution (5ml) was introduced to form a layer at the core of the rotor (at the light end of the gradient) and then pushed further into the centrifugal field with 15-20ml of buffer. The feed-head assembly was removed and the rotor was accelerated to 47000rev./min ($g_{av.} = 115000$) and maintained at this speed for the required period. The rotor was then slowed to 2500rev./min ($g_{av.} = 1000$), the feed-head assembly replaced and the contents of the rotor were gently displaced to a fraction collector by pumping in 30% sucrose solution coloured slightly with Blue Dextran. Fractions (20ml) were collected until tubes were seen to contain Blue Dextran.

Results

Identification and separation of the isoenzymes

A. lwoffii was grown aerobically at 30°C in a salts medium (Kornberg *et al.*, 1960) containing 50mm-sodium acetate as sole carbon source and harvested towards the end of exponential growth with a Sharples continuous-flow centrifuge. The cells were washed with water, resuspended in buffer of composition 20mm-tris-HCl-10mm-MgCl₂-1mm-EDTA, at pH8.0, and sonicated in chilled 50ml batches for 4min with an MSE 100W disintegrator at full power. The sonicated extracts were combined and cell debris was removed by centrifugation at 25000g and 4°C for 1h. The isocitrate dehydrogenase activity in this extract was stimulated about 70% by 0.2mm-glyoxylate.

Nucleic acid was removed from this crude extract by precipitation with protamine sulphate. An aqueous solution of protamine sulphate (2%, w/v) was added dropwise to the solution of enzyme with constant stirring until 1.3mg of protamine sulphate had been added per 10mg of protein in the extract. The mixture was stirred for 20min and then centrifuged at 25000g for 5min. If the decanted supernatant solution was turbid, a further 0.2mg of protamine sulphate/10mg of protein was added slowly and the

mixture again stirred for 20min and centrifuged. If necessary the addition of more protamine sulphate followed by centrifugation was repeated until a clear supernatant was obtained.

The effectiveness of (NH₄)₂SO₄ fractionation was next investigated. The supernatant solution obtained above was treated with (NH₄)₂SO₄, 5% fractions being collected between 45% and 75% saturation. It was found that isocitrate dehydrogenase activity was spread over the whole range. However, the degree of activation by glyoxylate decreased in the fractions with higher (NH₄)₂SO₄ saturation. Thus the 45-50%-saturated fraction exhibited an activation of about 2.5 whereas the 65-70% fraction was completely unactivated. These results suggested that more than one form of the enzyme might be present. A sample of the supernatant solution obtained after protamine sulphate treatment was examined by electrophoresis on cellulose acetate. On staining for enzyme, two bands were observed, thereby providing further evidence for multiple forms in these extracts.

The material precipitating between 45% and 75%-saturated (NH₄)₂SO₄ was dissolved in a small volume of buffer and dialysed against 50mm-sodium phosphate-1mm-EDTA buffer, pH8.0. The dialysed enzyme was then examined by the following three techniques.

Ion-exchange chromatography. Ion-exchange chromatography was done on a column of DEAE-cellulose (2.5cm×35cm) equilibrated with 100mm-sodium phosphate, pH8.0, and 1mm-EDTA. The dialysed enzyme was applied to the column, which was then eluted with buffer of linearly increasing phosphate concentration (0.4mm/ml) from 100mm to 250mm-sodium phosphate, 1mm-EDTA being present and the pH being maintained at 8.0 throughout. Fractions (5ml) were collected and assayed for isocitrate dehydrogenase both in the absence and in the presence of 0.2mm-glyoxylate.

Two distinct peaks of enzyme activity were found in the eluted fractions (Fig. 1). The first to be eluted was termed isoenzyme-I and was completely unaffected by glyoxylate. The second component was termed isoenzyme-II and was activated about sixfold by glyoxylate (Fig. 1). An essentially identical activation was produced by pyruvate.

Gel filtration. Gel filtration was performed on a column (2.5cm×35cm) of Sephadex G-200 equilibrated with 20mm-tris-HCl-10mm-MgCl₂-1mm-EDTA buffer, pH8.0. A sample (2ml) of the dialysed enzyme was applied and 2ml fractions were collected and assayed for isocitrate dehydrogenase in the absence and in the presence of 0.2mm-glyoxylate. Again, two distinct peaks of enzyme activity were observed (Fig. 2). Here, however, it was the first peak that showed activation by glyoxylate and which was, presumably, isoenzyme-II. The second peak appeared to be isoenzyme-I and was unaffected by glyoxylate.

Fig. 1. Separation of the isoenzymes by ion-exchange chromatography

Chromatography on DEAE-cellulose was done as described in the text, and 5 ml fractions were collected. ●, Isocitrate dehydrogenase activity (arbitrary units); ○, activation by 0.2 mM-glyoxylate.

Fig. 2. Separation of the isoenzymes by gel filtration

Gel filtration on Sephadex G-200 was done as described in the text and 2 ml fractions were collected. ●, Isocitrate dehydrogenase activity (arbitrary units); ○, activation by 0.2 mM-glyoxylate.

Zonal ultracentrifugation. The difference in molecular sizes of the two isoenzymes indicated by gel-filtration behaviour prompted us to examine the use of zonal ultracentrifugation for the separation of the isoenzymes. Zonal ultracentrifugation was performed for 20 h at 47000 rev./min ($g_{av.} = 115000$) (see the Materials and Methods section). The fractionated contents of the rotor were assayed for enzyme in the absence and in the presence of 0.2 mM-glyoxylate. The results (Fig. 3) show that the isoenzymes are separable by zonal ultracentrifugation. In agreement with the results of gel filtration it is the larger isoenzyme, isoenzyme-II (more rapidly sedimenting), which is activated by glyoxylate.

These results clearly indicate the existence of two distinct forms, or isoenzymes, of isocitrate dehydrogenase in extracts of *A. lwoffii*. We wished, however, to confirm that both isoenzymes occur naturally in the cells and that their appearance was not a consequence of the breakdown of one form to the other. The fact that both crude extracts and partially purified preparations of isoenzyme-II retained their sensitivity to glyoxylate activation over a period of 3 months ruled out the likelihood of a rapid breakdown of isoenzyme-II to isoenzyme-I. Similarly, when samples of isoenzyme-II that had been separated from isoenzyme-I by DEAE-cellulose chromatography or gel filtration were re-subjected to these techniques only isoenzyme-II was found: no 'new' isoenzyme-I could be detected. To discount the appearance of the two forms as a consequence of the

ultrasonication used in the extraction we examined the effects of varying the time of sonication of cell suspensions, and of resonicating a sample of isoenzyme-II, on the glyoxylate activation; no effects were observed. Further, disruption of the organisms with a French pressure cell, rather than by sonication, produced an extract with identical properties and containing both isoenzymes.

It therefore appears most probable that there are two natural isoenzymes of isocitrate dehydrogenase in *A. lwoffii*.

In an attempt to gain some insight into the physiological roles of these isoenzymes we examined *A. lwoffii* grown on different carbon sources. It was found that in extracts of cells grown on nutrient, gluconate, glutamate or succinate, the isocitrate dehydrogenase activity was stimulated by glyoxylate to approximately the same extent as that from acetate-grown cells. This suggests that on all these growth compounds *A. lwoffii* produces a similar mixture of isoenzymes. Aspartate, citrate, glycine and glucose did not support the growth of the organism.

Isocitrate dehydrogenase has been examined in a moderately halophilic strain of *A. lwoffii* and enhanced activity was observed when NaCl was

Fig. 3. Separation of the isoenzymes by zonal ultracentrifugation

Zonal ultracentrifugation was done as described in the Materials and Methods section at 47000 rev./min for 20h and 20ml fractions were collected. ●, Isocitrate dehydrogenase activity (arbitrary units); ○, activation by 0.2M-glyoxylate.

included in the extraction buffer (Vargues & Brisou, 1966). We examined the effect of NaCl on our strain of *A. lwoffii* and found that sonic extracts of cells grown on acetate with 0.15–0.25M-NaCl contained about twice the isocitrate dehydrogenase activity obtained in the absence of NaCl. Further, the inclusion of 0.2M-NaCl during the sonication of cells grown without added NaCl resulted in a 50% increase in the specific activity of the enzyme. However, examination of the degree of activation by glyoxylate indicated that the inclusion of NaCl during growth or in the extraction buffer did not affect the ratio of isoenzymes.

Preparative fractionation of isocitrate dehydrogenases

To study the kinetic behaviour of the isoenzymes it was necessary to have preparations of them separated from each other and also from the interfering enzymes isocitrate lyase and glutamate dehydrogenase. Interference by isocitrate lyase arises both from its competition for isocitrate and from its production of glyoxylate, and glutamate dehydrogenase can affect the measurement of NADPH formation by catalysing the reaction of NADPH with α -oxoglutarate (the other product of the isocitrate dehydrogenase reaction) if NH_4^+ is present (e.g. after precipitation with $(\text{NH}_4)_2\text{SO}_4$ to concentrate the enzymes). Chromatography on DEAE-cellulose offered a means of preparing isoenzyme-I free from isoenzyme-II and from isocitrate lyase and glutamate dehydrogenase, but, as with Sephadex G-200, much of the isoenzyme-II so obtained was contaminated with these two last-named enzymes.

Zonal ultracentrifugation, however, separated both isoenzymes from isocitrate lyase and glutamate dehydrogenase but did not achieve an ideal separation of the isoenzymes from each other. To overcome these difficulties, the following procedure, based on the results described above, was adopted. Sonic extracts were treated with protamine sulphate and then fractionated with $(\text{NH}_4)_2\text{SO}_4$. The 45–75% saturation fraction was resuspended and dialysed against 50mM-sodium phosphate–1mM-EDTA, pH8.0. Chromatography of this material on DEAE-cellulose gave a good preparation of isoenzyme-I. However, for the preparation of isoenzyme-II the dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was subjected to zonal ultracentrifugation and the fractions containing most isoenzyme-II activity were pooled and chromatographed on DEAE-cellulose. The experimental conditions for chromatography and zonal ultracentrifugation were as described above.

It should be emphasized that this procedure was adopted because it successfully separated the isoenzymes from each other, although the yields were poor and only small increases in specific activities were obtained. Attempts to achieve further purification of the isoenzymes have not, so far, been successful.

Properties of the isoenzymes

The kinetic and molecular properties described here emphasize the distinct nature of the isoenzymes. Studies on the activation of isoenzyme-II by glyoxylate and pyruvate and on the interaction of this activation with the regulation of the isoenzyme by adenylates (Parker & Weitzman, 1970) are presented elsewhere (Self *et al.*, 1973).

Both isoenzymes were specific for NADP, no activity being observed with NAD even in the presence of glyoxylate or pyruvate.

pH-dependence. Investigation of the pH-dependences of activity of the isoenzymes showed that isoenzyme-I exhibits an optimum at pH8.5–9 and isoenzyme-II at pH8 (Fig. 4).

Kinetic characteristics. Under normal assay conditions both isoenzymes exhibit somewhat greater activity with Mn^{2+} than with Mg^{2+} . Ratios for activity with Mn^{2+} /activity with Mg^{2+} were 1.2 for isoenzyme-I and 2.1 for isoenzyme-II. Apparent K_m values for isocitrate and NADP^+ have been determined for each isoenzyme in the presence of Mg^{2+} or Mn^{2+} . For these determinations assays were performed in 0.1M-tris-HCl, pH8.0, with 10mM- MgCl_2 or 10mM- MnCl_2 , and 1mM-EDTA. To overcome the problem of the gradual precipitation of manganese hydroxide from buffer solutions containing MnCl_2 , assays with Mn^{2+} as the metal cofactor were done by setting up reaction mixtures in the absence of the metal ion and finally adding MnCl_2 .

(to 10mM) to initiate the reaction. No perceptible precipitation occurred during the period of the assay.

The dependence of activity on isocitrate concentration was determined in the presence of 1mM-NADP⁺ and the dependence on NADP⁺ concentration was examined with 4mM-DL-isocitrate. In all cases normal hyperbolic dependences were observed and double-reciprocal plots were linear. The apparent K_m values are listed in Table 1. The most marked difference between the two isoenzymes lies in their dependence on NADP⁺: in the presence of Mg²⁺, the K_m for NADP⁺ of isoenzyme-II is more than 10 times that of isoenzyme-I.

Inactivation by heat or urea. Isoenzyme-I was less readily inactivated by heat than was isoenzyme-II. Fig. 5 shows the rate of thermal inactivation of the isoenzymes at 43°C. Similarly, isoenzyme-I was more stable to urea treatment. Fig. 6 illustrates the effect of including various concentrations of urea in the assay of the isoenzymes.

Molecular size. The results of gel filtration and zonal ultracentrifugation presented above indicate the larger molecular size of isoenzyme-II than of isoenzyme-I. To measure the approximate molecular weights of the isoenzymes a mixture of the two of them with catalase (mol.wt. 248000), lactate dehydrogenase (mol.wt. 140000) and haemoglobin (mol.wt. 67000) was run on a Sephadex G-200 column in 20mM-tris-HCl-10mM-MgCl₂-1mM-EDTA-0.1M-KCl buffer, pH8.0 (Fig. 7). The positions of the isocitrate dehydrogenase peaks suggest that the molecular weight of isoenzyme-II may be in the region of 300000 and that of isoenzyme-I may be less than 100000.

The molecular sizes of the *A. lwoffii* isoenzymes were compared with that of the isocitrate dehydrogenase of *E. coli*. The latter enzyme shows no activation by glyoxylate or pyruvate and we have reported that zonal ultracentrifugation of extracts of acetate-grown *E. coli* shows only a single peak of

activity (Self & Weitzman, 1970). Fig. 8 further emphasizes the close similarity between the sedimentation properties of *E. coli* isocitrate dehydrogenase and *A. lwoffii* isoenzyme-I in zonal ultracentrifugation experiments. The positions of peak activity in the fractions obtained from the contents of the rotor have been plotted against the time of centrifugation at 47000rev./min. Isoenzymes-I and -II are clearly different, whereas the *E. coli* enzyme closely resembles isoenzyme-I. Confirmation of this resemblance is provided by gel-filtration experiments.

Fig. 4. pH-dependence of activity of the isoenzymes

Activities were measured in buffers of composition 20mM-tris-HCl-10mM-MgCl₂-1mM-EDTA, in the presence of 1mM-NADP⁺ and 4mM-DL-isocitrate, and are expressed in arbitrary units. ●, Isoenzyme-I; ○, isoenzyme-II.

Table 1. Apparent K_m values for isocitrate dehydrogenase isoenzymes

Assays were done in 0.1M-tris-HCl, pH8.0, 1mM-EDTA and either 10mM-MgCl₂ or 10mM-MnCl₂. The dependence of activity on NADP⁺ concentration was determined at 4mM-DL-isocitrate and the dependence on isocitrate at 1mM-NADP⁺.

Isoenzyme	Metal ion	Substrate	Apparent K_m (μ M)
I	Mg ²⁺	NADP ⁺	25
II	Mg ²⁺	NADP ⁺	310
I	Mn ²⁺	NADP ⁺	54
II	Mn ²⁺	NADP ⁺	100
I	Mg ²⁺	<i>threo</i> -D ₅ -Isocitrate	24
II	Mg ²⁺	<i>threo</i> -D ₅ -Isocitrate	17
I	Mn ²⁺	<i>threo</i> -D ₅ -Isocitrate	26
II	Mn ²⁺	<i>threo</i> -D ₅ -Isocitrate	13

Fig. 5. Thermal inactivation of the isoenzymes

Enzyme activities were measured under standard conditions after various times of heating at 43°C in 20mM-tris-HCl-10mM-MgCl₂-1mM-EDTA, pH8.0, and are expressed in arbitrary units. ●, Isoenzyme-I; ○, isoenzyme-II.

Fig. 6. Urea inactivation of the isoenzymes

Activities were measured under standard conditions but with the addition of various concentrations of urea and are expressed in arbitrary units. ●, Isoenzyme-I; ○, isoenzyme-II.

Fig. 7. Molecular sizes of the isoenzymes

The isoenzymes were run on a column of Sephadex G-200 with Blue Dextran and the marker proteins catalase, lactate dehydrogenase and haemoglobin. ●, Isocitrate dehydrogenase activity.

Fig. 8. Similarity of the isoenzyme-I and *E. coli* isocitrate dehydrogenase in zonal ultracentrifugation

Zonal ultracentrifugation was done as described in the Materials and Methods section. The position of peak enzyme activity in the eluted fractions has been plotted against the time of centrifugation at 47000 rev./min ($g_{av.} = 115000$). ●, Isoenzyme-I; ○, isoenzyme-II; □, *E. coli* isocitrate dehydrogenase.

An extract of acetate-grown *E. coli* B, partially purified by protamine sulphate treatment and $(NH_4)_2SO_4$ fractionation, was run on Sephadex G-200 with the marker proteins catalase, lactate dehydrogenase and haemoglobin as described above. The position of the elution peak of the *E. coli* isocitrate dehydrogenase coincided with that of isoenzyme-I.

Occurrence of the isoenzymes in other organisms

The finding of isocitrate dehydrogenase isoenzymes in *A. lwoffii* followed our investigation of the stimulation of the enzyme activity by glyoxylate. We have tested a number of other organisms for a similar response of their isocitrate dehydrogenase activity as an indication of the existence of isoenzymes like those described here (see Table 2). Of the organisms tested, only four showed activation of isocitrate dehydrogenase by glyoxylate, and of these, *Mima polymorpha*, *Moraxella* sp. and *Moraxella*

calcoacetica are probably best placed in the genus *Acinetobacter* (Baumann *et al.*, 1968). However, clearly glyoxylate stimulation of isocitrate dehydrogenase, and therefore, presumably, the existence of isoenzymes, is not restricted to a particular strain of *Acinetobacter* and may well be even more widespread than is indicated by our limited survey.

Discussion

Our initial observation of the stimulation of isocitrate dehydrogenase activity in crude extracts of *A. lwoffii* has led to the discovery of two NADP-linked isoenzymes in this organism. An early step in the attempted purification of the enzyme, by $(NH_4)_2SO_4$ fractionation, gave fractions containing isocitrate dehydrogenase that were differentially stimulated by glyoxylate. This suggested that more than one form of the enzyme exists, a possibility also suggested by the double electrophoretic bands of activity in crude extracts. The results of three fractionation procedures, ion-exchange chromatography, gel filtration and zonal ultracentrifugation, provided evidence confirming the existence of the two isoenzymes. The latter have been shown to differ in molecular size and charge characteristics and in kinetic behaviour, pH optima, stability to heat and urea, and response to glyoxylate and pyruvate. The isoenzymes also differ in their response to adenylate regulation (Parker & Weitzman, 1970).

Both ion-exchange chromatography and gel filtration have been successfully employed for the separation of many isoenzyme systems, the latter technique being appropriate when the isoenzymes are of considerably different molecular size. The use of zonal ultracentrifugation for the separation of isoenzymes is, however, a novel application of this technique, and one likely to prove of value in the large-scale separation of other isoenzyme systems. The success of zonal ultracentrifugation in the present work also indicates its usefulness in the handling of relatively small molecules (mol.wt. ~100000), in addition to its more usual application to much larger particles and organelles.

The molecular weights of mammalian NADP-linked isocitrate dehydrogenases are in the region of 60000 (Moyle & Dixon, 1956; Siebert *et al.*, 1957; Colman, 1968; Magar & Robbins, 1969), whereas the bacterial enzyme has a somewhat higher molecular weight. The enzyme from *Azotobacter vinelandii* has a molecular weight of about 80000 (Chung & Franzen, 1969; Barrera & Jurtshuk, 1970) whereas that from *Bacillus stearothermophilus* has a molecular weight of 92500 (Howard & Becker, 1970). A larger NADP-linked isocitrate dehydrogenase has not been reported. Our results indicate that isoenzyme-I from *A. lwoffii* and the isocitrate dehydrogenase of *E. coli* have molecular weights

Table 2. *Glyoxylate activation of bacterial isocitrate dehydrogenases*

Isocitrate dehydrogenase activities of sonic extracts were measured in 20mM-tris-HCl-10mM-MgCl₂-1mM-EDTA-0.2mM-NADP⁺-4mM-DL-isocitrate buffer, pH8.0, in the absence and in the presence of 0.2mM-glyoxylate.

Organisms showing activation	
<i>Mima polymorpha</i>	A.T.C.C. 9957
<i>Moraxella</i> sp. (<i>Vibrio</i> 0-1)	A.T.C.C. 11171
<i>Moraxella calcoacetica</i>	73*
<i>Pseudomonas aeruginosa</i>	N.C.I.B. 8295
Organisms showing no activation	
<i>Arizona arizonae</i>	N.C.T.C. 8297
<i>Azotobacter vinelandii</i>	N.C.I.B. 8660
<i>Bacillus megaterium</i> *	
<i>Escherichia coli</i> *	
<i>Klebsiella (Aerobacter) aerogenes</i>	N.C.T.C. 10006
<i>Kurthia zopfii</i>	C 5*
<i>Proteus vulgaris</i>	N.C.T.C. 4175
<i>Pseudomonas fluorescens</i>	C.C.E.B. 488; D 1086*; D 1087*
<i>Pseudomonas ovalis</i>	N.C.I.B. 8296
<i>Salmonella anatum</i>	Uetake 293*
<i>Serratia marcescens</i>	N.C.T.C. 10211; D 106*

* From the culture collections of the Medical Research Council Microbial Systematics Research Unit and the Department of Biochemistry, University of Leicester.

similar to these bacterial enzymes, but that isoenzyme-II is a much larger enzyme with a molecular weight of about 300000, i.e. similar to that of NAD-linked isocitrate dehydrogenase from higher organisms (Sanwal & Stachow, 1965; Cox & Davies, 1969; Giorgio *et al.*, 1970; Barnes *et al.*, 1971).

Isoenzymes of NADP-linked isocitrate dehydrogenase have been observed in other organisms. Electrophoretically distinct forms of the enzyme have been found in the mitochondrial and supernatant fractions of a variety of eukaryotic cells (Lowenstein & Smith, 1962; Bell & Baron, 1964; Henderson, 1968). Multiple forms were reported to occur in glucose-grown *E. coli* (Reeves *et al.*, 1968*a,b*) but only one form was detected in electrophoresis (Rowe & Reeves, 1971).

However, no marked difference in molecular size has been found between the isoenzymes from a particular source, nor have they been observed to differ in regulatory response. The *A. lwoffii* isoenzymes differ particularly in these respects and present a system resembling that of eukaryotic cells. These possess both an NAD-linked and an NADP-linked isocitrate dehydrogenase, the former being the larger molecule and subject to adenylate control (Atkinson, 1966; Plaut, 1970).

As with many other isoenzyme systems the physiological significance of the existence of multiple forms is unclear. Variations of the growth medium were investigated but no conditions were found for suppressing or enhancing the production of one of the

isoenzymes. The response of isoenzyme-II to metabolite activation may give this isoenzyme a regulatory role, but it is not known why isoenzyme-I should also be required. Some measure of compartmentation may exist in these cells, and the isoenzymes could then be differentially located. In eukaryotic cells the location of the NAD-linked isocitrate dehydrogenase within the mitochondrion and of the NADP-linked enzyme in the cytosol offers an explanation for the existence of the two forms, yet here, too, the additional presence of an NADP-linked isocitrate dehydrogenase inside the mitochondrion adds further complexity.

We thank Dr. Dorothy Jones for supplying some of the organisms listed in Table 2, and the Medical Research Council for a Studentship to C. H. S. This work was supported, in part, by the Science Research Council (Grant B/SR/5109).

References

- Atkinson, D. E. (1966) *Annu. Rev. Biochem.* **35**, 85
- Barnes, L. D., Kuehn, G. D. & Atkinson, D. E. (1971) *Biochemistry* **10**, 3939
- Barrera, C. R. & Jurtshuk, P. (1970) *Biochim. Biophys. Acta* **220**, 416
- Baumann, P., Doudoroff, M. & Stanier, R. Y. (1968) *J. Bacteriol.* **95**, 1520
- Bell, J. L. & Baron, D. N. (1964) *Biochem. J.* **90**, 8p
- Chung, A. E. & Franzen, J. S. (1969) *Biochemistry* **8**, 3175
- Colman, R. F. (1968) *J. Biol. Chem.* **243**, 2454

- Cox, G. F. & Davies, D. D. (1969) *Biochem. J.* **113**, 813
- Dixon, G. H. & Kornberg, H. L. (1959) *Biochem. J.* **72**, 3P
- Giorgio, N. A., Yip, A. T., Fleming, J. & Plaut, G. W. E. (1970) *J. Biol. Chem.* **245**, 5469
- Henderson, N. S. (1968) *Ann. N.Y. Acad. Sci.* **151**, 429
- Howard, R. L. & Becker, R. R. (1970) *J. Biol. Chem.* **245**, 3186
- Kornberg, H. L., Phizackerley, P. J. R. & Sadler, J. R. (1960) *Biochem. J.* **77**, 438
- Lowenstein, J. M. & Smith, S. R. (1962) *Biochim. Biophys. Acta* **56**, 385
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
- Magar, M. E. & Robbins, J. E. (1969) *Biochim. Biophys. Acta* **191**, 173
- Moyle, J. & Dixon, M. (1956) *Biochem. J.* **63**, 548
- Parker, M. G. & Weitzman, P. D. J. (1970) *FEBS Lett.* **7**, 324
- Plaut, G. W. E. (1970) *Curr. Top. Cell. Regul.* **2**, 1
- Reeves, H. C., Brehmeyer, B. A. & Ajl, S. J. (1968a) *Bacteriol. Proc.* 117
- Reeves, H. C., Brehmeyer, B. A. & Ajl, S. J. (1968b) *Science* **162**, 359
- Rowe, J. J. & Reeves, H. C. (1971) *J. Bacteriol.* **108**, 824
- Sanwal, B. D. & Stachow, C. S. (1965) *Biochim. Biophys. Acta* **96**, 28
- Self, C. H. & Weitzman, P. D. J. (1970) *Nature (London)* **225**, 644
- Self, C. H., Parker, M. G. & Weitzman, P. D. J. (1973) *Biochem. J.* in the press
- Siebert, G., Dubuc, J., Warner, R. C. & Plaut, G. W. E. (1957) *J. Biol. Chem.* **226**, 965
- Vargues, H. & Brisou, J. (1966) *C. R. Soc. Biol.* **160**, 2195