Purification of Yeast Isocitrate Dehydrogenase

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The NAD-linked isocitrate dehydrogenase from baker's yeast was purified to homogeneity (as judged by gel filtration and polyacrylamide-gel electrophoresis) with an overall yield of 50% by using dilute solutions of the allosteric effector (AMP) to elute the enzyme specifically from CM-cellulose. This method preserves the allosteric properties of the crude enzyme. Although the pure enzyme shows only a single band on electrophoresis in the presence of sodium dodecyl sulphate, two types of subunit are observed in 8M-urea. The isoelectric point of the enzyme rises during purification, and this may reflect the partial loss of an additional low-molecular-weight component. Values are included for the amino acid composition and extinction coefficients of the pure enzyme.

Yeast isocitrate dehydrogenase is chiefly remarkable for its anomalous kinetic properties. The enzyme is activated by AMP (Hathaway & Atkinson, 1963), which increases the affinity of the enzyme for NAD^+ and isocitrate. In partially purified preparations this activation occurs without change in the Hill coefficient of 4 for isocitrate, although the co-operativity of NAD^+ binding is decreased by AMP (Atkinson *et al.*, 1965). Atkinson and co-workers have recently purified the enzyme to homogeneity in the presence of citrate, which acts as a stabilizer. Their product shows a decreased Hill coefficient of 3 for isocitrate (falling to 2 in the presence of AMP) and exhibits a hyperbolic response to NAD^+ and Mg^{2+} (Barnes *et al.*, 1971). Under other conditions these workers found that citrate-treated enzyme showed Hill coefficients as high as 8 (Kuehn *et al.*, 1970), although there are only four isocitrate binding sites per enzyme molecule (Kuehn *et al.*, 1971).

My results have been broadly in agreement with those of Atkinson's group and confirm that the activation by AMP is difficult to reconcile with the allosteric theory of Monod *et al.* (1965). However, the numerical values of the kinetic constants measured in the studies differ from those obtained by Atkinson's group, sometimes by a factor of ten or more. These discrepancies arise because the kinetic properties of the enzyme vary extensively with minor alterations in the composition of the assay system, and with the previous treatment of the enzyme. The present paper describes a purification procedure which does not involve the use of citrate buffers, and which preserves the co-operative binding of both NAD^+ and isocitrate. I have also obtained unequivocal evidence for the existence of non-identical subunits in the purified enzyme, and for AMP binding in the absence of isocitrate. These results are in superficial disagreement with the work of Barnes *et al.* (1971) and Kuehn

et al. (1971), although it can be shown that they are in fact entirely compatible.

Materials and Methods

Materials

Ampholine was supplied by LKB Produkter AB, Bromma 1, Sweden. Coenzymes were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Trisodium DL-isocitrate and Hepes [2-(N-2-hydroxyethylpiperazin-N'-yl)ethanesulphonic acid] were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Triethanolammonium chloride was prepared by titration of triethanolamine in ethanol with HCl (McKenzie, 1969). Whatman microgranular ion-exchange celluloses were supplied by H. Reeve Angel and Co. Ltd., London E.C.4, U.K.

Enzyme assays

Assays were run at 25°C in tris-HCl buffer, pH 7.6 (40 mM-tris), containing 4 mM- MgCl_2 , 2.5 mM-trisodium DL-isocitrate and 0.25 mM- NAD^+ . One unit of enzyme activity converts 1 μmol of substrate/min under these conditions. The affinity of the enzyme for its substrates falls steeply with increasing ionic strength, and it is important to control salt concentration carefully.

Assays were normally started by addition of enzyme, except at early stages in the purification, when they were started with isocitrate in order first to estimate the blank rate probably caused by alcohol dehydrogenase.

Yeast

Blocks of packed baker's yeast (*Saccharomyces cerevisiae*) (450 g) were obtained from local agents of

the Distillers Company Ltd. These were either disrupted at once, or stored at -20°C until needed. At 4°C the cells lose enzyme with a half-life of about 3 days, so it is desirable to use very fresh yeast.

Cell breakage

A motor-driven pestle and mortar (e.g. Gallenkamp End Runner Grinding Mill, model MU 300) was used to fragment the cells. Batches (300g) were ground with 1000ml of no. 11 or 12 ballotini at 25°C for 30min. About 75% of the cells are ruptured by this procedure, as judged by phase-contrast microscopy; the brilliant cytoplasm of viable cells contrasts sharply with the shadowy outlines of empty cell envelopes. Various other procedures (air drying, toluene autolysis, sonication or blending frozen cells with ballotini) were markedly less successful than grinding for samples of this size. It is important to add no liquid while the grinding is in progress, since cell rupture apparently depends on shearing forces generated as the glass beads roll against each other in the viscous mixture.

Details of enzyme preparation

Quantities are for 900g wet weight of cells.

(1) The cells were disrupted in three batches. After grinding, each batch of cells and ballotini was extracted with $2 \times 500\text{ml}$ of 10mM-cysteine. The liquid was decanted from the ballotini, which were dried and reused in subsequent preparations. (Longer grinding is needed with old beads.)

(2) Cell debris was largely removed by centrifugation for 30min at 20000g. The opalescent supernatant contains non-sedimenting material, which obstructs chromatography columns. This was removed by making the extract 40mM in CaCl_2 , and immediately adding 1.0M- K_2HPO_4 to a final concentration of 100mM. The gelatinous precipitate of calcium phosphate entrained the residual particulate material. After standing for 1h at 5°C with occasional stirring the precipitate was removed by brief centrifugation to give a clear yellow supernatant.

(3) Solid $(\text{NH}_4)_2\text{SO}_4$ (350g/litre) was added to the extract at 5°C while the pH was allowed to fall from 7 to 6.2. After 30min the precipitate was recovered by centrifuging at 20000g for 15min, and redissolved in the minimum volume of 10mM-cysteine (about 350ml) and stored at -20°C overnight.

(4) Next day the sample was diluted with its own volume of water to decrease its viscosity and transferred by gel filtration through a column (10cm $\times$ 1m) of Sephadex G-25 to buffer A, containing 20mM-triethanolamine chloride and 5mM-cysteine, titrated to pH7.6 with NaOH. The enzyme was then applied to a column of DEAE-cellulose (10cm diam. $\times$ 12.5cm high) previously equilibrated with buffer A at 25°C .

After loading, the sample was washed in with 500ml of buffer A. The enzyme was eluted with a gradient over 3 litres from 20 to 120mM-triethanolamine chloride containing 5mM-cysteine, titrated to pH7.6 with NaOH. Elution took about 1h.

The product was then precipitated by addition of 500g of solid $(\text{NH}_4)_2\text{SO}_4$ /litre and recovered by centrifugation (30min at 20000g). The precipitate was redissolved in the minimum volume (approx. 25ml) of buffer B (30mM-potassium phosphate, pH6.5, containing 1ml of 2-mercaptoethanol/litre) and stored at -20°C overnight.

(5) Next day a small insoluble residue was removed by brief centrifugation. The enzyme solution was transferred by gel filtration into buffer B as described above before being loaded on to a column of CM-cellulose previously equilibrated with the same buffer; 10g dry weight of CM-cellulose were used per gram of protein applied to the column, resulting usually in a column bed 3cm diam. $\times$ 9cm high.

Non-retarded proteins were washed from the column with about 4 column volumes of buffer B. When the effluent was free of protein the enzyme was specifically eluted with buffer B containing 1mM-AMP. (The enzyme peak had a very sharp leading edge which emerged with the AMP front. Almost all the enzyme was eluted in 1 column volume.)

(6) The enzyme was concentrated by ultrafiltration by using a PM-10 membrane (Amicon Ltd., High Wycombe, Bucks., U.K.) before removal of AMP by gel filtration on a column (2cm diam. $\times$ 30cm) of Sephadex G-25 (medium grade). Glycerol (2.5%, v/v) was added before the enzyme was stored at -20°C .

Conditions for optimum stability of the enzyme

(1) The pH should be maintained as far as possible between 6 and 7.

(2) The enzyme should be stored at concentrations not less than 1 unit/ml and preferably at 10 units/ml or above, and excessive dilution avoided during chromatography.

(3) A suitable anti-oxidant (5mM-cysteine or 15mM-2-mercaptoethanol) should be added to all solutions.

(4) The enzyme is totally inactivated by freezing, but this loss can be prevented by adding glycerol (2.5%, v/v). This precaution is not needed for an $(\text{NH}_4)_2\text{SO}_4$ 'sludge' or if total protein exceeds 2.5% (w/v).

Isoelectric focusing and polyacrylamide-gel electrophoresis

Full details of isoelectric focusing techniques and conventional gel electrophoresis are given in the following paper (Illingworth, 1972). Urea gels were prepared by adding an equal weight of urea to 6%

acrylamide gels immediately before polymerization. Samples of carboxymethylated enzyme were dissolved in 8M-urea before application to the gels, which were run in 0.1 M-tris-HCl, pH 8.1, with 0.1 M-tris-glycine in the cathode compartment.

Electrophoresis in the presence of sodium dodecyl sulphate was performed as described by Weber & Osborn (1969), by using carboxymethylated enzyme and omitting the mercaptoethanol treatment.

Enzyme samples were carboxymethylated in the minimum volume of 50mM-NaHCO₃ containing an equal weight of urea. After 15 min preincubation under nitrogen with 2mM-dithiothreitol, neutralized iodoacetic acid was added to 10mM final concentration, and the incubation continued for a further 2 h before the sample was dialysed against water.

Results and Discussion

The results of the purification procedure are summarized in Table 1. The present method gives a higher yield in fewer steps than the technique described by Barnes *et al.* (1971). Both methods make use of DEAE-cellulose chromatography. In the present procedure the enzyme emerges from the column in 50–60mM-triethanolammonium chloride, which has the same ionic strength as the 10mM-citrate buffer used by Barnes *et al.* (1971). It is apparent that citrate is no more effective in eluting the enzyme than other salts, and that the results of Barnes *et al.* (1971) cannot be described as a 'preferential elution with modifier'. Nevertheless citrate has a valuable stabilizing effect on the enzyme. High recoveries in the present method were only obtained by working quickly and avoiding

excessive dilution of the sample, whereas Barnes *et al.* (1971) were free from these restrictions.

The DEAE-cellulose column dimensions described in the Materials and Methods section are the minimum necessary for complete adsorption of the enzyme, and it may well be necessary to use more of the exchanger if the crude sample has a high protein and nucleic acid content. With larger DEAE-cellulose columns the specific activity of the eluate can be raised to about 4.5; however, this does not produce any improvement in the final specific activity of the pure enzyme. It is important to ensure that the sample applied to the DEAE-cellulose column has the same conductivity as buffer A. Traces of (NH₄)₂SO₄ frequently remain after passage through Sephadex, and in such cases the sample must be diluted with water so as to yield the correct conductivity. Assays of the Sephadex G-25 eluate always give an anomalously low value for the enzyme activity, and this is responsible for the apparent activation seen in Table 1.

The second (NH₄)₂SO₄ precipitation serves only to concentrate the enzyme, and usually causes a slight fall in specific activity.

Conditions for the AMP elution step are not critical, though the procedure suggested produces optimum results. This step is successful over the pH range of at least 6.0–6.6 and will tolerate considerable variations in the phosphate buffer concentration. It does not work with crude enzyme samples, as explained below.

At first sight the elution from CM-cellulose by AMP appears to contradict the binding studies of Kuehn *et al.* (1971), which show that the enzyme will not bind AMP in the absence of isocitrate. However,

Table 1. *Purification of yeast isocitrate dehydrogenase*

Full details are given in the Materials and Methods section. The protein concentration in the final product was determined by amino acid analysis. At earlier stages protein was estimated by using the u.v.-absorbance method of Kalckar (1947); however, in the crude samples the high *E*₂₆₀ prevented an accurate measurement.

Purification step	Volume (ml)	Enzyme activity		Protein		Specific activity (units/mg)	Yield (%)
		(units/ml)	Total (units)	(mg/ml)	Total (mg)		
1. Crude extract, clarified with calcium phosphate	2200	1.2	2640	—	—	—	100
2. (NH ₄) ₂ SO ₄ precipitate	365	7.25	2646	—	—	—	100
3. Sephadex G-25 eluate (diluted)	1500	1.18	1770	8.4	12600	0.14	67
4. DEAE-cellulose eluate	950	2.1	1995	1.31	1250	1.6	75
5. (NH ₄) ₂ SO ₄ precipitate, after desalting with Sephadex G-25	65	24.3	1580	16.2	1055	1.5	62
6. CM-cellulose eluate, after concentration and removal of AMP on Sephadex G-25	16	84.0	1345	1.83	29.3	46.0	51

the concentration of AMP used to elute the enzyme was ten times greater than the highest concentration tested in the binding studies. The Hill coefficient for AMP is 2.0, so that at low concentrations AMP binding will vary with the square of the nucleotide concentration, in which case the two sets of results may be entirely compatible.

Isocitrate alone (1 mM) will not elute the enzyme from CM-cellulose, nor will 1 mM-NAD⁺; however, 1 mM-isocitrate plus 1 mM-NAD⁺ is effective. These results are unaffected by the addition of 1 mM-MgCl₂ to the eluting buffer. Substrate elution with isocitrate has been previously used for the purification of pig liver NADP-linked isocitrate dehydrogenase (Illingworth & Tipton, 1970). In the earlier case 200 μ M-isocitrate was effective, presumably reflecting the higher binding affinity of the NADP-linked enzymes.

The purest samples prepared to date have a specific activity of 46 units/mg at 25°C. At 30°C this value rises to 63 units/mg. A 1 mg/ml enzyme solution has E_{280} 0.69 and E_{260} 0.35.

The isoelectric point of the enzyme rises during purification. The crude enzyme is isoelectric near pH 5.5, whereas after DEAE-cellulose chromatography the pI rises to 6.5 and pure enzyme has an isoelectric point near pH 7.0. These changes revealed by isoelectric focusing are reflected in the chromatographic properties of the enzyme. Crude enzyme is not retarded on CM-cellulose, even when a large excess of exchanger is used and after careful checks of pH and buffer conductivity. To elicit the change in isoelectric point the enzyme must be exposed to DEAE-cellulose: it is not sufficient to transfer the enzyme to buffer A and carry out an (NH₄)₂SO₄ fractionation at the alkaline pH value.

A number of sub-forms are obtained in isoelectric-focusing experiments. These 'isoenzymes' are artifacts produced by exposure to Ampholine, as shown in the following paper (Illingworth, 1972).

The molecular weight of the enzyme is unchanged by purification. The molecular weights of both crude enzyme [after preliminary fractionation with (NH₄)₂SO₄] and pure isocitrate dehydrogenase were estimated by gel filtration on Sephadex G-200 in 0.1 M-potassium phosphate, pH 6.8, containing 1 ml of mercaptoethanol/litre by the method of Andrews (1965). In both cases I obtained a value of 375000 $\pm$ 10%, with cytochrome *c*, pepsin, bovine serum albumin, liver alcohol dehydrogenase, yeast alcohol dehydrogenase, and apoferritin as markers. In a number of early experiments with crude enzyme I attempted to use Sepharose 6B for gel-filtration experiments in the buffer described above. To my surprise the enzyme was greatly retarded on this material and emerged from the column with an apparent molecular weight of about 25000 to 30000. This result was consistently observed even after the column was re-packed with a fresh sample of

Sepharose. Nevertheless, when the Sepharose eluate was re-run on Sephadex G-100 it was totally excluded from the gel and emerged in the void column, indicating that exposure to Sepharose 6B does not produce any permanent fall in molecular weight.

No differences were apparent between old samples of enzyme and those that had been freshly prepared. All the 'crude' samples were processed to the end of purification step 3, and loaded on to the gel without removal of (NH₄)₂SO₄.

Presumably the enzyme binds to Sepharose. Although I have not pursued this point, alternating chromatography on Sepharose and Sephadex gels has considerable potential as a very mild purification technique, since almost all other high-molecular-weight components run well ahead of yeast isocitrate dehydrogenase on Sepharose. This could be one case where Sepharose can be used for affinity chromatography without the necessity to introduce functional groups!

The pure enzyme shows only a single coincident protein and activity band on polyacrylamide-gel electrophoresis. Only one band is visible after sodium dodecyl sulphate gel electrophoresis, with an apparent molecular weight of 41000, calculated by using bovine serum albumin, catalase, chymotrypsinogen, ovalbumin and ferritin as markers. Nevertheless two well-separated bands of equal intensity are visible on gels containing 8 M-urea. Each of these bands is associated with two faint satellite bands, possibly caused by deamidation of the native enzyme, or formed by reaction with isocyanate in the urea. The same pattern is visible in samples that have not been carboxymethylated. It seems reasonable to conclude that the enzyme is composed of approximately equal numbers of two types of subunit, which have nearly the same molecular weight.

Table 2. *Amino acid composition of yeast isocitrate dehydrogenase*

These values (amino acid residues/subunit) are rounded to the nearest half-integer, and correspond to a molecular weight of about 40000. The analysis was performed with a Beckman model 120C automatic amino acid analyser, according to the procedures of Spackman *et al.* (1958).

Aspartic acid	38	Methionine	5
Threonine	30	Isoleucine	29
Serine	26.5	Leucine	31.5
Glutamic acid	30.5	Tyrosine	9
Proline	17.5	Phenylalanine	12
Glycine	31.5	Lysine	24
Alanine	30	Histidine	9
Cysteine	1.5	Arginine	16
Valine	27	Tryptophan	3

The amino acid analysis of the pure enzyme is shown in Table 2. The values for valine, isoleucine, serine and threonine were extrapolated from the analyses of samples hydrolysed for 24, 48 and 100h in order to correct for slow release and hydrolysis losses. The value for tryptophan was calculated by the method of Bencze & Schmid (1957). Titration with dithionitrobenzoic acid in 8M-urea (Ellman, 1959) indicates the presence of two reducing thiol groups per subunit of mol.wt. 40000. Two samples with known E_{280} values were hydrolysed for 24h without prior carboxymethylation or dialysis. The molar extinction coefficient was then calculated from the sample of aspartic acid + glycine + alanine + leucine and the ratios listed in Table 2.

The kinetic properties of the enzyme were investigated spectrophotometrically by using both manual assays and a double-beam version of the automatic method previously described (Illingworth & Tipton,

1969). In the latter case the results were fitted directly to the equation

$$\frac{1}{V} = \frac{1}{V_{\max.}} + \frac{1}{V_{\max.}} \cdot \frac{K}{[S]^n}$$

where V = velocity, $[S]$ = substrate concentration, n is the Hill coefficient and K is a constant. Both K and n were independently varied by an iterative computer program (J. A. Illingworth, unpublished work) so as to obtain the best fit to a straight line. The points were weighted on the basis that their absolute errors were constant (i.e. the percentage errors were assumed to be smaller for large velocities). When using this method it is not necessary to know $V_{\max.}$ but it is best to have a large number of experimental points, since two parameters must be estimated from the data.

The affinity of the enzyme for isocitrate falls steeply with increasing ionic strength. This effect has also been noted by Cennamo *et al.* (1967, 1968), who

Fig. 1. Kinetic properties of the pure enzyme

All measurements were performed at 25°C in 20mM-potassium phosphate, pH 7.5, containing 4mM-MgCl₂. No discrete points are shown, because the automatic method employed produces a continuous curve. One unit on the velocity scale corresponds to production of about 8.0 μmol of NADH/min per mg in the reaction coil. (a) Variation of velocity with NAD⁺ concentration. Curve (1) was obtained with 2mM-DL-isocitrate. The Hill coefficient was 1.4 for NAD⁺. Curve (2) shows the results with 1.66mM-DL-isocitrate. Under these conditions the Hill coefficient was 1.9 for NAD⁺. (b) Variation of velocity with isocitrate and AMP concentration. Curve (3) was measured in the absence of AMP. The isocitrate concentration scale reads from 0 to 1.6mM-DL-isocitrate, and the Hill coefficient was 3.6 for isocitrate. Curve (4) shows the effect of adding 1mM-AMP. The isocitrate concentration scale now reads from 0 to 0.16mM-DL-isocitrate, and the Hill coefficient was 2.8 for isocitrate.

attributed it to competition between buffer anions and isocitrate for enzyme binding sites. In addition to the marked instantaneous effect of added salt on the reaction rate these workers described a time-dependent loss of activity when the enzyme was diluted into the assay buffer, and this inactivation was accentuated by high salt concentrations and reversed by substrates and AMP.

Barnes *et al.* (1971) claim that their Hepes-KOH buffer system gives higher substrate-binding affinities at the expense of a decreased maximum velocity. I cannot repeat these observations. In my hands the enzyme has essentially the same $V_{\max}$ in 100mM-potassium Hepes, 20mM-potassium phosphate and 40mM-tris-HCl buffers at pH 7.5-7.6 (the substrate concentrations used were 4mM-MgCl₂, 1.35mM-NAD⁺, 1mM-AMP and 5mM-DL-isocitrate). With 4mM-MgCl₂, 0.25mM-NAD⁺ and no AMP the variation of reaction velocity with isocitrate concentration was almost identical in 100mM-potassium Hepes and 20mM-potassium phosphate buffers, as might be expected from their similar ionic strengths. It is, however, important to perform the experiments at the same time with the same batches of enzyme and substrates. I have noticed up to twofold variations in the isocitrate-binding affinity between different batches of pure enzyme assayed in phosphate buffer on different occasions. This may well be related to the variations in Hill coefficient between different enzyme samples described by Barnes *et al.* (1971), though in my hands the variations in Hill coefficient have so far been small.

Even under apparently identical conditions there are substantial variations in the curves of velocity against substrate concentration published by Hathaway & Atkinson (1963), Atkinson *et al.* (1965) and Cennamo *et al.* (1967, 1968). However, these variations are confined to numerical values only: the basic pattern of regulation described by Atkinson *et al.* (1965) has never been in dispute and indeed has been confirmed once more in the present studies.

Fig. 1 shows some of the results obtained with the automatic assay method. The enzyme concentration used in these experiments was in the range 0.4-1.0 µg/ml. There was good agreement between automatic and manual assays, and the reaction velocity was strictly proportional to enzyme concentration. The enzyme exhibits highly co-operative binding of isocitrate even after activation by AMP, but the co-operativity of NAD⁺ binding is decreased by high concentrations of isocitrate. This is the first time that the salient properties of the crude enzyme have been obtained in a highly purified preparation.

It is tempting to regard the results obtained with crude enzyme as being closer to the properties of

the enzyme *in vivo*. This is not necessarily the case. Yeast isocitrate dehydrogenase appears to possess a remarkable ability to bind a number of non-physiological compounds such as Sepharose, and some constituents of Ampholine as described in the following paper (Illingworth, 1972). During the disruption of the yeast cells the enzyme is exposed to a wide range of foreign substances such as cell-wall polysaccharides and nucleic acids, which are not present in its normal intracellular location. It is at least conceivable that chromatography on DEAE-cellulose removes this interfering material and allows the enzyme to manifest its normal kinetic and electrophoretic properties.

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