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Purification and Properties of the Nicotinamide-Adenine Dinucleotide Phosphate-Dependent Isocitrate Dehydrogenase from Pig Liver Cytoplasm

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The NADP-dependent isocitrate dehydrogenase from pig liver soluble fraction was purified over 500-fold with an overall yield of 25%. The purified enzyme, which is homogeneous by all the usual criteria, has a molecular weight of about 75000 and is composed of two identical subunits. This has been demonstrated by ultracentrifugation, fluorescence titration and peptide 'fingerprinting'. The maximal turnover number, extinction coefficients at 280nm and 260nm and amino acid analysis are described.

Three types of isocitrate dehydrogenase have been described in mammalian tissues. Mitochondria contain an NAD-linked enzyme of high molecular weight and also a much smaller enzyme that is specific for NADP. A second NADP-linked enzyme is localized in the cell cytoplasm, though this protein has received little attention compared with the mitochondrial types.

The NAD-specific enzyme has been purified from heart mitochondria (Chen & Plaut, 1963) and from liver (Plaut & Aogaichi, 1968). Enzyme from both sources is activated by ADP, which suggests a major role in the regulation of tricarboxylic acid-cycle activity.

No such clear metabolic function can be assigned to either of the NADP-linked enzymes. The mitochondrial isoenzyme is very active in heart, from which it has been purified to homogeneity (Colman, 1968). Several workers have studied this enzyme (Siebert, Dubuc, Warner & Plaut, 1957; Rose, 1960; Colman, 1967, 1968, 1969*a*,*b*,*c*) and a detailed picture of the catalytic mechanism is beginning to emerge. It has been reported that this enzyme is inhibited by folate (Magar & Homi, 1968), although the functional significance of this observation is obscure.

In liver the cytoplasmic isoenzyme appears to provide the principal path for isocitrate oxidation (Pette, 1966). The object of the present investigation was to purify this enzyme from pig liver and to compare its properties with those of the NADP-specific isoenzyme from heart.

MATERIALS AND METHODS

$NADP^+$ and NADPH were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Dithiothreitol was supplied by Calbiochem Ltd., Basingstoke, Hants., U.K., and iodo[2-^{14}C]acetic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Trisodium isocitrate was purchased from British Drug Houses Ltd., Poole, Dorset, U.K., who also supplied ultra-pure Aristar-grade urea. All other chemicals were analytical-reagent grade as supplied by British Drug Houses. Iodoacetic acid and iodoacetamide were recrystallized from *n*-heptane before use to remove traces of iodine and other decomposition products. Naphthalene Black 10B (Amido Schwartz) was obtained from G. T. Gurr Ltd., London S.W.6, U.K. Whatman Microgranular ion-exchange celluloses were supplied by H. Reeve Angel Ltd., London E.C.4, U.K.

Assay. Routine assays were performed at 25°C in 50mM-tris chloride buffer, pH8.1, containing 1mM-DL-isocitrate, 1mM-$MgCl_2$ and 200μM-$NADP^+$. The reaction was normally started by addition of enzyme and followed by monitoring the extinction of NADPH at 340nm. (At the pH optimum of 8.1 the velocity does not vary greatly with pH, and the rate is essentially linear for the first 0.5 E_{340} unit under these conditions.) All activities are expressed as μmol of NADPH produced/min at 25°C; at 30°C the rates are approximately 1.77 times as rapid. Protein was assayed by u.v. absorption by the method of Kalckar (1947).

Purification. The purification may be completed in about 2½ days, and is most easily performed on roughly 1kg of fresh pig liver.

A 1kg portion of fresh pig liver is transported from the slaughterhouse in ice and processed within 1h of the death of the animal. The liver is minced twice by an electric meat mincer and suspended in 4 litres of ice-cold distilled water containing 1mM-EDTA (sodium salt), pH7, and 1ml of 2-mercaptoethanol/l, and stirred for 10min. We do not recommend the use of a high-speed blender for macerating the tissue. Then 1kg of ice-cold chloroform is added and the extract is stirred for a further 10min, until all the globules of chloroform have disappeared and the preparation has a pink creamy appearance. The suspension is centrifuged for 15min at 20000*g* and the substantial precipitate is discarded. To the clear yellow supernatant

is added 220g of $(NH_4)_2SO_4$/l at 5–10°C; during this procedure the pH is allowed to fall from 6.8 to 6.4. After standing for 15min the solution is again centrifuged at 20000*g* for 10min, and the precipitate is discarded. A further 120g of $(NH_4)_2SO_4$/l of supernatant is added at 5–10°C, the pH falling to 6.1, and after standing for 15min the precipitate is recovered by centrifuging for 10min at 20000*g*.

The packed pellets are resuspended in the minimal volume of triethanolamine buffer, and gel-filtered into the same buffer to remove $(NH_4)_2SO_4$, by using a column of Sephadex G-25 with a bed volume of 1800ml. The triethanolamine buffer, pH7.8 (at 21°C), contains 50mM-triethanolamine hydrochloride, 0.5mM-EDTA and 1ml of 2-mercaptoethanol/l, the whole being titrated to the required pH with NaOH. [We do not recommend the use of overnight dialysis to remove $(NH_4)_2SO_4$ as considerable inactivation occurs under these conditions.] All subsequent steps may be safely performed at room temperature as the stability of the enzyme improves with purification. After dilution to a protein concentration of 30mg/ml the Sephadex G-25 eluate is applied to a column of Whatman Microgranular DEAE-cellulose previously equilibrated with the same triethanolamine buffer. At least 3g dry wt. of exchanger/g of protein is allowed, resulting in this case in a column with a bed volume of about 700ml. The enzyme may now be eluted with a gradient over 7–10 column volumes (i.e. about 5–7 litres in this case) from 50mM- to 100mM-triethanolamine chloride, both solutions containing 0.5mM-EDTA and 1ml of 2-mercaptoethanol/l and titrated to pH7.8 with NaOH. The combined active fractions are precipitated by addition of $(NH_4)_2SO_4$ to 70% saturation and stored at 5°C. We recommend that all steps up to this second $(NH_4)_2SO_4$ precipitation are completed on the first day.

The $(NH_4)_2SO_4$ sludge is recovered by centrifugation and dissolved in 10mM-sodium phosphate buffer, pH6.5, containing 1ml of 2-mercaptoethanol/l. The solution is gel-filtered to remove $(NH_4)_2SO_4$ and applied to a column of calcium phosphate gel previously poured in the same phosphate buffer. [Calcium phosphate gel is prepared in our laboratory by the method of Keilin & Hartree (1938), as quoted by Dixon & Webb (1964). We do not find it necessary to age the gel before use.] Columns are poured allowing 5g dry wt. of calcium phosphate/g of protein load. The gel is mixed with a tenfold dry-weight excess of Celite 545 filter aid (obtained from British Drug Houses Ltd.) and suspended in sufficient buffer to give a medium slurry before degassing at the water pump. The column is packed to give a final height/diameter ratio of 1:1, under a pressure of 0.5kg/cm^2, and loaded immediately with the enzyme. Elution is achieved with a gradient over 7–10 column volumes (i.e. about 2 litres in this case) from 10mM- to 100mM-sodium phosphate buffer, pH6.5, containing 1ml of 2-mercaptoethanol/l under a pressure of 0.25kg/cm^2. The combined active fractions are precipitated by addition of $(NH_4)_2SO_4$ to 70% saturation and stored at 5°C.

The precipitate is recovered by centrifugation and resuspended in 10mM-sodium succinate buffer and gel-filtered into the same buffer to remove the $(NH_4)_2SO_4$. The sodium succinate buffer has an original pH of 6.0 and contains 1mM-$MgCl_2$ and 1ml of 2-mercaptoethanol/l. A column of Whatman Microgranular CM-cellulose is equilibrated in this buffer, allowing 100g dry wt. of CM-cellulose/g of protein to be loaded. Shortly before use the buffer is made 20% (v/v) with respect to glycerol causing the apparent pH to rise to 6.1 but without interfering with the column equilibration. (This is easier than attempting to equilibrate in glycerol directly.) After the enzyme has been applied to the CM-cellulose the column is washed with the succinate buffer until protein ceases to appear in the effluent, and then the enzyme is eluted as a single, extremely sharp, peak with the same succinate buffer containing 0.2mM-sodium isocitrate.

The pure enzyme may be temporarily stabilized by addition of concentrated sodium phosphate buffer to 50mM final concentration, pH6.8. We normally store the enzyme in 20% glycerol–50mM-sodium phosphate–2.5mM-cysteine buffer, pH6.8, after gel filtration to remove isocitrate and $MgCl_2$.

As an alternative the enzyme may be crystallized from 50mM-sodium phosphate–2.5mM-cysteine buffer, pH7.0, by addition of $(NH_4)_2SO_4$ to about 40% saturation so that a slight opalescence is visible. After storage for a few days at constant temperature the solution exhibits the characteristic 'swirl' of crystallizing protein and about a week later small needle-shaped crystals are visible under the microscope.

Amino acid analysis and peptide 'fingerprinting'. Protein destined for chemical investigation was initially dialysed exhaustively against water. Some denatured protein precipitates under these conditions, but readily redissolves in 8M-urea containing dithiothreitol in twofold excess over protein thiol groups, buffered to pH8 with 100mM-$NaHCO_3$. After incubation for 15min under N_2 at 40°C, iodoacetic acid was added in at least twofold excess over total thiol groups and the incubation was continued under N_2 for 1h at 40°C. Urea and excess of reagents were removed by dialysis against water. The precipitate of carboxymethylated protein that appears under these conditions is soluble both in 1% formic acid and above pH8; however, the precipitate of carboxyamidomethylated protein prepared under similar conditions is totally intractable and has not been further investigated.

Samples (1mg) of the carboxymethylated protein were hydrolysed in 6M-HCl *in vacuo* at 105°C for 22–67h and the freeze-dried hydrolysates were investigated with a Beckman amino acid auto-analyser.

Tryptic digests were prepared by 4h incubation in 100mM-triethylammonium hydrogen carbonate at 40°C with (1% of total protein, freed from chymotryptic activity with 1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one). The digests were freeze-dried, and then redissolved in 1% formic acid before application to Whatman 3MM paper. Chromatograms were developed for 16–18h in acetic acid–butan-1-ol–pyridine–water (3:15:10:12, by vol.) and the paper strips containing the separated peptides were stitched to a sheet of Whatman 3MM paper for electrophoresis. This was performed for 45min at pH3.5 in acetic acid–pyridine–water (1:10:189, by vol.) with a potential gradient of 67V/cm. Completed 'fingerprints' are about 40cm square. Chromatograms were stained with acid ninhydrin followed by spraying with diazotized sulphanilic acid to reveal histidine peptides.

Molecular weight determinations. Gel filtration on Sephadex G-100 was performed in 100mM-potassium

phosphate buffer, pH6.8, containing 1ml of 2-mercaptoethanol/l. The column was separately calibrated with a variety of markers of known molecular weight by the method of Andrews (1964).

Ultracentrifuge runs were carried out in a Beckman model E analytical ultracentrifuge with a synthetic-boundary cell. In the case of the native enzyme the solvent was 100mM-potassium phosphate buffer, pH6.8, containing 1mM-dithiothreitol, and for the *S*-carboxymethyl subunits 20mM-diethanolamine chloride buffer, pH8.9, containing 100mM-NaCl. Schlieren optics were used throughout, and sedimentation coefficients were measured at protein concentrations of 1–8mg/ml.

Polyacrylamide-gel electrophoresis. Gels were prepared by mixing the following reagents: acrylamide, 7g; *NN'*-methylenebisacrylamide, 0.18g; *NNN'N'*-tetramethylethylenediamine, 0.04ml; ammonium persulphate, 0.07g; 0.4M-tris chloride buffer, pH8.9, to 100ml. The electrode compartments contain 5mM-tris–40mM-glycine buffer, pH8.6. In the case of urea gels 1g of Aristar urea was dissolved/ml of the gel before setting. Samples were applied by layering on to the gel surface in 20% glycerol and subjected to electrophoresis for 2h at room temperature with a voltage gradient of 33V/cm. Protein staining employed Amido Schwartz in 7% acetic acid and enzyme activity was located by staining in the following mixture: DL-isocitrate, 1mM; $MgCl_2$, 1mM; $NADP^+$, 0.1mM; phenazine methosulphate, 0.03mg/ml; Nitro Blue Tetrazolium, 0.3mg/ml; tris chloride buffer, pH8.1, 50mM. Gels were incubated in this solution for 4h at 30°C in the dark.

RESULTS

The purification steps are summarized in Table 1.

Pure pig liver isocitrate dehydrogenase migrates as a single peak in the ultracentrifuge. Only one protein band is visible after polyacrylamide-gel electrophoresis even after deliberate overloading, and this corresponds in position to enzyme activity located on duplicate gels. Only one band is visible after electrophoresis in gels containing 8M-urea. When chromatographed on Sephadex G-100 the enzyme emerges as a single peak of constant specific activity from leading edge to trailing edge. The crystalline enzyme has the same specific activity as the mother liquor within experimental errors due to the protein estimation.

In these circumstances it seems reasonable to assume that the preparation is essentially pure and homogeneous. The extinction coefficients of the native enzyme have been measured directly by freeze-drying exhaustively a sample in 100mM-triethylamine hydrogen carbonate of known optical properties, and subsequently weighing the residue. These quantities may also be estimated from amino acid composition data and the u.v. spectra of the enzyme at pH7.0 and in 100mM-NaOH by the method of Bencze & Schmid (1957). The results are as follows. A 1mg/ml solution of enzyme measured with a 1cm light-path has E_{280} 1.26 (by weighing) and 1.27 (by amino acid data). The E_{280}/E_{260} ratio is very nearly 2 at pH values near neutrality. On this basis the final specific activity of the pure enzyme in the assay system described at 25°C is 46.5μmol of NADPH formed/min per mg of enzyme.

The molecular weight of the native enzyme measured on Sephadex G-100 by the method of Andrews (1964) was 76000±10%, with horse liver alcohol dehydrogenase (mol.wt. 88000), bovine serum albumin (mol.wt. 67000), pepsin (mol.wt. 35000) and cytochrome *c* (mol.wt. 12500) as markers. In the ultracentrifuge molecular weights were estimated from sedimentation velocity experiments according to the equation:

$$\text{Mol.wt.} = \frac{s}{D} \cdot \frac{RT}{(1-\bar{v}.\rho)}$$

where s and D are the sedimentation and diffusion

Table 1. *Purification steps for NADP-dependent soluble isocitrate dehydrogenase*

The data in this table are for a single preparation from 1.4kg wet wt. of pig liver. This method gives very reproducible results, and other preparations are identical to within 10% at all stages. The final specific activity is invariant. Protein concentrations were measured by the method of Kalckar (1947) except for the final stage when the extinction coefficient at 280nm was taken as 1.26 E_{280} units/mg of protein per ml. The values for the crude homogenate refer to material centrifuged at 20000*g* for 15min and are included for comparative purposes only.

Step	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg of protein)	Total activity (units)	Total protein (g)	Yield (%)
Crude homogenate	7000	2.85	35.5	0.08	20000	250	(100)
Chloroform supernatant	5800	3.6	19.2	0.188	20900	111	105
37.5–55%-satd.-$(NH_4)_2SO_4$ fraction, dialysed	1500	11.25	21.4	0.525	16900	32.1	84.5
DEAE-cellulose eluate	238	3.82	1.0	3.8	9100	2.4	45.5
Calcium phosphate eluate	450	15.05	0.9	16.75	6760	0.5	33.8
Isocitrate eluate (gel-filtered)	52	98	2.15	46.5	5100	0.21	25.5

coefficients respectively, and R (gas constant) is 8.313erg/mol per degree. The partial specific volume $\bar{v}$ was calculated to be 0.733 from the amino acid analysis data by the method of Schachman (1957). To determine the variation of sedimentation coefficient, s, with protein concentration samples containing 1–8mg of enzyme/ml were centrifuged at 40000rev./min in 100mM-potassium phosphate buffer, pH 6.8, containing 20mM-2-mercaptoethanol. This variation proved to be slight, values ranged from 5.6S at 1mg/ml to 5.9S at 8mg/ml, all at 20.5°C. The diffusion coefficient, D, was estimated from measurements of the rate of spreading of the schlieren peak during the course of a low-speed run (9500rev./min) at a protein concentration of 5mg/ml according to the equation:

$$(\text{Peak area})^2/(\text{Peak height})^2 = 4\pi.t.(1+s\omega^2t).D$$

(where t is time in seconds, ω is the angular velocity). The diffusion coefficient given by this method was 7.6 ± 0.18 Fick Units, corresponding to a molecular weight of 75000 ± 5000 for the native enzyme.

The subunit molecular weight was estimated from a single run at 56000rev./min using carboxymethylated enzyme at a protein concentration of 3mg/ml in 20mM-diethanolamine buffer, pH 8.9, containing 100mM-NaCl. The sedimentation coefficient was 3.46S and the diffusion coefficient was estimated to be about 9.0 ± 0.5 Fick Units from the rate of spreading of the schlieren peak in the first 10min of the run. This rapid and approximate method is based on the results of Fujita (1962) and Kawahara (1969), who showed that:

$$(\text{Peak area})^2/(\text{Peak height})^2 = 4\pi.t.(1+s\omega^2t).(1-z).D$$

where $(1-z)$ is a correction for the error in measuring D due to the concentration-dependence of the sedimentation coefficient.

$$\text{If} \qquad s = s_0(1-k.c)$$

$$\text{then} \qquad z = 2.r_0.\omega^2.s_0.k.c_0.t.(\text{height/area})$$

(s_0 is the sedimentation coefficient at infinite dilution, c_0 is the protein concentration at the start of the run, r_0 is the radius at which the artificial boundary is formed and k is a constant). Kawahara (1969) made z very small by performing his experiments at low speeds; in the present case where D is large and s is small it is easier to achieve the same result by decreasing the value of t. In either case z can be ignored in calculating the results. The subunit molecular weight of 36000 determined by this method is sufficiently accurate to identify the enzyme as a dimer.

On the basis of a molecular weight of 75000 the amino acid analysis is shown in Table 2.

A tryptic peptide 'fingerprint' is shown in Fig. 1. Four strongly radioactive spots can be seen in digests prepared from [2-^{14}C]carboxymethyl-enzyme and five of the peptides stain for histidine with the Pauly reagent (diazotized sulphanilic acid). Most of the 45 peptides expected from the amino acid analysis appear on the 'fingerprint'.

Table 2. *Amino acid analysis of NADP-dependent soluble isocitrate dehydrogenase*

The data are for a single subunit of molecular weight 37500. No attempt has been made to estimate the amide content of the protein. The tryptophan values were calculated from u.v.-spectral data according to the method of Bencze & Schmid (1957).

Lysine	30	Glycine	28
Histidine	7	Alanine	25
Arginine	14	Valine	19
Cysteine	5	Methionine	11
Aspartic acid	36	Isoleucine	23
Threonine	20	Leucine	21
Serine	19	Tyrosine	14
Glutamic acid	39	Phenylalanine	12
Proline	11	Tryptophan	5

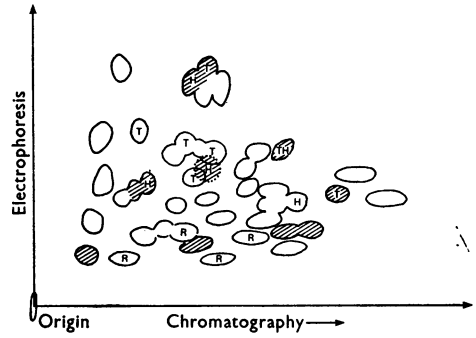

Fig. 1. Peptide 'fingerprint' prepared from a tryptic digest of *S*-carboxymethyl-isocitrate dehydrogenase, as described in the Materials and Methods section. Large peptides that stain heavily with starch–KI reagent after chlorination are shown shaded. Radioactive peptides present in digests of [2-^{14}C]carboxymethylated enzyme are marked R, those staining for histidine are marked H and the presence of tyrosine is indicated by T. The tyrosine assignments are necessarily tentative as the stain is not very intense.

The behaviour of the native enzyme has been examined in the Aminco–Bowman fluorimeter. The protein concentration used was 0.10mg/ml in a 1ml cylindrical silica cuvette. All measurements were made at 24°C in 100mM-potassium phosphate buffer, pH 7.0. All wavelength values are uncorrected for variations in lamp output and photomultiplier sensitivity with wavelength. The enzyme alone shows an excitation peak at 290nm and a fluorescence maximum at 340nm. On addition of NADPH the fluorescence at 340nm is diminished

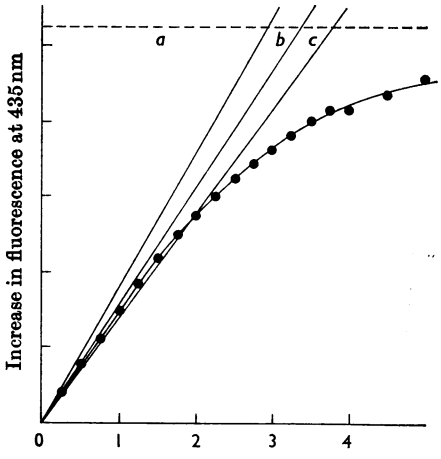

Fig. 2. Fluorescence titration of native isocitrate dehydrogenase. Portions (5 μl) of 50 μM-NADPH were added to 0.1 mg of enzyme in 1.0 ml of 100 mM-potassium phosphate buffer, pH 7.0. The fluorescence increase at 435 nm was followed in an Aminco–Bowan fluorimeter exciting at 290 nm. The results have been corrected for the dilution of the enzyme by the additions and analysed by the graphical method of Dixon (1965). The broken line (----) indicates the estimated maximal increase in fluorescence. The concentration of NADPH-binding sites is given by the distance *a*, and *b* and *c* both give the dissociation constant of the enzyme–NADPH complex. The active-site concentration indicated by these results (2.9 μM) is approximately twice the enzyme concentration of 1.33 μM, based on a molecular weight of 75000.

and a new peak appears at 435 nm. This peak does not appear on addition of $NADP^+$ to the enzyme, nor does NADPH in the absence of enzyme fluoresce at this wavelength when excited at 290 nm.

The enzyme may be titrated with NADPH. As Fig. 2 shows, the fluorescence increase at 435 nm when plotted against NADPH concentration yields a non-rectangular hyperbola, which may be analysed by the graphical method of Dixon (1965). Two molecules of NADPH are bound/molecule of enzyme, and the dissociation constant of the enzyme–NADPH complex is 0.4 ± 0.1 μM.

Carboxymethylation of the native enzyme with 30 mM-iodoacetate or -iodoacetamide results in loss of enzymic activity. With iodoacetamide this is accompanied by precipitation of the enzyme. No significant protection by substrate could be observed in our preliminary experiments, which were carried out in either tris chloride (100 mM), sodium phosphate (100 mM) or sodium succinate (100 mM) over the pH range 5.5–7.5. These studies have not been pursued, since they seem unlikely to yield useful results at the present time.

DISCUSSION

The conditions given in the purification procedure have been optimized over a considerable number of preparations and should not be varied if good results are to be obtained. In particular the calcium phosphate gel used 'batchwise' is inferior both as regards yield and purification, and the conditions for the substrate elution are critical. This enzyme is rather unstable in buffers of low ionic strength at any pH appreciably removed from its isoelectric point of about 6.5, though in 3 M-ammonium sulphate it is stable from pH 2 to pH 10 for long periods. We suspect that the inactivation is due to dissociation of the subunits. In practice this instability means that the column of CM-cellulose must be used at pH 6, when the affinity of the enzyme for the exchanger is rather low, and a very large column is needed. Were it not for this purely physical problem of large column dimensions the calcium phosphate step could be omitted entirely and the DEAE-cellulose eluate purified by substrate elution alone. In small-scale experiments the enzyme has been purified over 50-fold with essentially quantitative recovery by substrate elution, but it would be impracticable to use this step alone for large-scale work.

Considerable differences have already emerged between the purified liver enzyme and its mitochondrial partner from heart. The liver enzyme has a molecular weight 1.3 times that of the heart protein (75000 compared with 58000) (Colman, 1968) and is also considerably more active. Colman (1968) reports that the specific activity of the heart enzyme at 25°C is 29 μmol of NADPH produced/min per mg of protein under conditions that should produce essentially maximal rates. Under (different) $V_{max.}$ conditions the liver enzyme has a specific activity of 46.5 μmol/min per mg of protein at 25°C at 0.2 mM-$NADP^+$, 1 mM-DL-isocitrate and 1 mM-$MgCl_2$ at pH 8.1 in 50 mM-tris chloride buffer.

The results of the fluorescence titration indicate a very low dissociation constant for the NADPH–enzyme complex. A similar result has previously been obtained for the heart enzyme (Langan, 1960).

The liver enzyme appears to be inactivated by iodoacetate and iodoacetamide in a rather non-specific fashion, with a pH optimum above 8.0 and without protection by substrates. This is in marked contrast with the specific labelling of an active-centre methionyl residue in pig heart isocitrate dehydrogenase by iodoacetic acid at pH 5.6 found by Colman (1968), and suggests that in the present case thiol residues removed from the active site are involved.

It is clear from the results of the fluorescence titration and ultracentrifugation experiments that this enzyme is a dimer composed of two identical or

almost identical subunits. The peptide 'fingerprints' are also compatible with this model, since the total number of peptides, the number staining for histidine and the number containing cysteine are roughly half the expectation for unique polypeptides totalling 75000 in molecular weight. This conclusion has also been inferred from genetic studies (Henderson, 1965).

Colman (1968, 1969*a*) finds approx. one isocitrate or α-oxoglutarate molecule bound/molecule of the heart enzyme (mol.wt. 58000); however, this result conflicts with the ultracentrifuge studies of Magar & Robbins (1969), who found the enzyme to be a dimer.

The fluorescence titration studies also demonstrate the ability of the enzyme to bind coenzyme in the absence of other substrates or of Mg^{2+} ions. Similarly, elution from CM-cellulose indicates that isocitrate can be bound in the absence of coenzyme.

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