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Purification and properties of L-3-glycerophosphate dehydrogenase from pig brain mitochondria

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L-3-Glycerophosphate dehydrogenase (EC 1.1.99.5) was purified from pig brain mitochondria by extraction with deoxycholate, ion-exchange chromatography and $(NH_4)_2SO_4$ fractionation in cholate, and preparative isoelectric focusing in Triton X-100. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis shows that the purified enzyme consists of a single subunit of mol.wt. 75000. The enzyme contains non-covalently bound FAD and low concentrations of iron and acid-labile sulphide. No substrate-reducible e.p.r. signals were detected. The conditions of purification, particularly the isoelectric-focusing step, lead to considerable loss of FAD and possibly iron–sulphur centres. It is therefore not possible to decide with certainty whether the enzyme is a flavoprotein or a ferroflavoprotein. The enzyme catalyses the oxidation of L-3-glycerophosphate by a variety of electron acceptors, including ubiquinone analogues. A number of compounds known to inhibit ubiquinone oxidoreduction by other enzymes of the respiratory chain failed to inhibit L-3-glycerophosphate dehydrogenase, except at very high concentrations.

Mitochondrial L-3-glycerophosphate dehydrogenase (EC 1.1.99.5) is a membrane-bound enzyme which, like NADH dehydrogenase and succinate dehydrogenase, catalyses the transfer of reducing equivalents from substrate to the respiratory chain. Unlike these other dehydrogenases, however, L-3-glycerophosphate dehydrogenase in insect flight-muscle mitochondria appears to oxidize its substrate on the cytoplasmic side of the membrane (Donnellan *et al.*, 1970; Klingenberg & Bucholz, 1970), although some evidence for permeability of mitochondria to L-3-glycerophosphate has been found (Slack & Bursell, 1977). Very little is known of the structure of this enzyme. Solubilization from pig brain mitochondria by the action of phospholipase A (Ringler & Singer, 1958) or Triton X-100 (Dawson & Thorne, 1969*a*) has resulted in partially purified preparations capable of reducing ubiquinone-10, ubiquinone analogues and other electron acceptors. The enzymes contained non-covalently bound FAD and non-haem iron, but since the preparations were not homogeneous it was not possible to decide with any certainty whether either FAD or iron were prosthetic groups of the enzyme. The enzyme from both skeletal muscle and brain has been purified to homogeneity (Cole *et al.*, 1978) and, somewhat surprisingly compared with other respiratory complexes, it consists of a single polypeptide of mol.wt. 76000 when analysed by SDS/polyacrylamide-gel electrophoresis. Despite the protein homogeneity, the FAD content was variable (3–10 nmol/mg of protein) and less than expected for 1 FAD molecule per 76000 mol.wt. The non-haem iron content of 10 ng-atoms/mg of protein was similarly rather low, and it was therefore still not possible to decide whether the protein contained iron–sulphur centres in addition to FAD. Finally, the ability of this purified preparation to reduce ubiquinone or ubiquinone analogues was not reported.

The aim of the work reported here was to purify the enzyme to homogeneity in a form that would combine with the cytochrome b–c_1 segment of the respiratory chain to reconstitute antimycin-sensitive L-3-glycerophosphate–cytochrome c oxidoreductase. Successful reconstitution of this activity would constitute good evidence that the enzyme had been purified in an unmodified state. After previous attempts to purify the enzyme, we chose pig brain as source material. The enzyme proved extremely difficult to purify, and nearly homogeneous preparations could only be obtained by using preparative isoelectric focusing in the presence of Triton X-100, as also used by Cole *et al.* (1978). The small

Abbreviation used: SDS, sodium dodecyl sulphate.
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amounts of highly purified material obtained and the presence of Triton X-100, which inhibited reconstituted L-3-glycerophosphate–cytochrome *c* oxidoreductase, made these preparations unsuitable for detailed studies of the reconstitution with the cytochromes. Most of this aspect of the work was therefore carried out on a cruder enzyme which had been isolated by using deoxycholate and cholate for solubilization. The present paper describes the purification of the enzyme by this new procedure and details its properties. The following paper (Cottingham & Ragan, 1980) describes the interaction of the enzyme with ubiquinone-10 and bovine heart mitochondrial ubiquinol–cytochrome *c* oxidoreductase (Complex III).

Materials and methods

Chemicals

Ubiquinone-0 and ubiquinone-1 were gifts from Hoffman–La Roche, Basle, Switzerland. Dr. B. Trumpower, Department of Biochemistry, Dartmouth Medical School, Hanover, NH, U.S.A., provided 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone. Carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) was provided by Dr. H. Beinert, Institute for Enzyme Research, Madison, WI 53706, U.S.A. 2-Hydroxy- and 2-chloro-benzohydroxamic acids were gifts from Dr. B. Storey, Department of Biophysics and Physical Biochemistry, University of Pennsylvannia, Philadelphia, PA 19174, U.S.A. Soya-bean phosphatidylcholine and phosphatidylethanolamine were purified from crude soya-bean lipids (L-α-lecithin from Sigma) as described by Ragan & Racker (1973).

Assays

Assays of L-3-glycerophosphate dehydrogenase were based on those described by Dawson & Thorne (1969*b*). Dichlorophenol-indophenol reduction was measured at 600nm ($\varepsilon = 21000$ litre·mol^{-1}·cm^{-1}) in 1ml final volume at 22°C containing 50μmol of potassium phosphate, pH 7.6, 50μmol of DL-3-glycerophosphate, 47.5nmol of dichlorophenol-indophenol and 5–50μg of enzyme protein. The light path was 1cm. Reduction of ubiquinone analogues was measured under the same conditions, except that 100nmol of ubiquinone analogue replaced the dichlorophenol-indiphenol and the light path was 0.5cm. Absorption coefficients were obtained from the oxidized-minus-reduced difference spectra (Dawson & Thorne, 1969*b*). The values used at the measuring wavelengths were as follows: ubiquinone-0, $\varepsilon = 14400$ litre·mol^{-1}·cm^{-1} at 264nm; ubiquinone-1, $\varepsilon = 12600$ litre·mol^{-1}·cm^{-1} at 275nm; 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone, $\varepsilon = 10900$ litre·mol^{-1}·cm^{-1} at 275nm (assumed to be the same as ubiquinone-2). For assays in which phospholipid was included, soya-bean phosphatidylcholine or bovine heart cardiolipin was dispersed in water by sonication to give a final concentration of approx. 20mM (Ragan, 1978). Solutions were standardized by determining the phosphate content (Bartlett, 1969). Acid-extractable FAD and FMN were measured by the method of Faeder & Siegel (1973). Commercial FAD and FMN were purified by chromatography on Sephadex G-15. They appeared homogeneous by t.l.c. on silica gel with 5% (w/v) $Na_2HPO_4,12H_2O$ as solvent. Solutions of FAD and FMN were standardized by their A_{450} by using $\varepsilon = 11300$ litre·mol^{-1}·cm^{-1} for FAD and $\varepsilon = 12200$ litre·mol^{-1}·cm^{-1} for FMN (Cerletti & Siliprandi, 1953). Iron (Smith *et al.*, 1952), acid-labile sulphide (King & Morris, 1967), protein (Lowry *et al.*, 1951; with fraction V bovine serum albumin from Sigma as a standard) and ubiquinone-10 (Kröger & Klingenberg, 1966) were assayed as described in the references. Since the enzyme was suspended in phosphate buffer throughout purification, samples for measurement of phospholipid content were dialysed against water to remove phosphate before the phospholipids were extracted as described by Rouser & Fleischer (1967). The phospholipid content of the extracts was measured as phosphate after digestion of the sample with $HClO_4$ (Bartlett, 1969).

Phospholipid analysis

T.l.c. of phospholipid extracts was carried out on silica-gel plates, with chloroform/methanol/water (65:25:4, by vol.) as solvent (Rouser & Fleischer, 1967). Phosphatidylcholine and phosphatidylethanolamine from soya beans and bovine heart cardiolipin (Sigma) were run as markers. Spots were detected by exposure to iodine vapour. Phosphatidylethanolamine was also detected by reaction with ninhydrin.

Measurement of the solubility of ubiquinone-1 in water

Ubiquinone-1 (0.4μmol) was dispersed in 2ml of 50mM-potassium phosphate, pH 7.6, by vortex mixing for 5min. The mixture was then incubated with shaking for 4h at 20°C. After centrifugation in the 10×10ml rotor of an MSE 65 centrifuge for 15min at 30000rev./min and 20°C, the supernatant was assayed for ubiquinone-1 by the ΔA_{275} after reduction by $NaBH_4$, by using $\varepsilon = 12600$ litre·mol^{-1}·cm^{-1}.

SDS/polyacrylamide-gel electrophoresis

This was carried out as described by Weber & Osborn (1969), except that the gels (12cm×4mm internal diam.) contained 12.5% (w/v) acrylamide

and 0.34% (w/v) bisacrylamide. The molecular weight of the L-3-glycerophosphate dehydrogenase polypeptide was determined by comparison with bovine heart mitochondrial NADH dehydrogenase (EC 1.6.99.3), whose largest polypeptide has an apparent mol.wt. of 75000 (Heron *et al.*, 1979).

Results

Assay of the enzyme

We routinely assayed the enzyme by its ability to catalyse reduction of ubiquinone-1 by DL-3-glycerophosphate. Other workers have used dichlorophenol-indophenol as electron acceptor, but we hoped that the reduction of a ubiquinone analogue might be more closely related to the activity of the enzyme in the mitochondrial membrane. We also used ubiquinone-0 and a ubiquinone-2 analogue, 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone (Wan *et al.*, 1975), to test the effects of side-chain length and hydrophobicity on the catalytic activity of the enzyme.

Purification of L-3-glycerophosphate dehydrogenase

Unless otherwise stated, all operations were carried out at 0–4°C.

(*a*) *Preparation of pig brain mitochondria.* The method followed that of Ringler (1961). Cerebral hemispheres were collected shortly after the animals were killed, and stored on ice. Portions of tissue (80g) were blended with 720ml of 0.25M-sucrose/10mM-K_2HPO_4 for 1min at low speed in a Waring Commercial Blendor of 0.5-gallon (2.3 litre) capacity (Waring Products Division, New Hartford, CT, U.S.A.). The pH was adjusted to 7.6 with 1M-K_2HPO_4 and the homogenate was filtered through a wire mesh to remove large pieces of tissue. After centrifugation at 2500rev./min for 7min in the 6×1 litre rotor of the MSE Mistral LR6 centrifuge, mitochondria were sedimented by centrifugation in a Sharples Laboratory Supercentrifuge (Pennwalt Ltd., Camberley, Surrey, U.K.) at 50000rev./min and a flow rate of approx. 30litre/h. The pellet consisted of a light, loosely packed upper layer and a darker lower layer. The former was removed by washing the pellet with unbuffered 0.25M-sucrose and the dark lower layer was frozen at −20°C. Approx. 7kg of cerebral hemispheres was processed in this way.

(*b*) *Acetone drying of the mitochondria.* Following the procedure of Dawson & Thorne (1969*a*), the pig brain mitochondria were acetone-dried before solubilization of the enzyme was attempted. Specific activities towards either ubiquinone-1 or dichlorophenol-indophenol were not altered by this treatment.

The mitochondrial pellet (300–400ml in 0.25M-sucrose) was thawed and divided into two batches. Each batch was sprayed from a large (50ml) disposable syringe (without a needle) into separate 5litre batches of rapidly stirred acetone at −20°C. After further stirring for 15min, the precipitates were collected by centrifugation at 2500rev./min for 15min in the 6×1litre rotor of the MSE Mistral LR6 centrifuge. The precipitates were pooled and resuspended in 5litres of acetone at −20°C by brief homogenization in the Waring Blendor. After stirring for 15min, the precipitate was collected as above. The precipitate was washed as above with a further 5litres of acetone and then dried in a stream of air for 2h. The acetone-dried mitochondria were stored at −20°C in a vacuum desiccator over solid NaOH and paraffin wax. In this condition, activity was stable for at least 6 weeks. Typically 70g of acetone-dried powder was obtained.

(*c*) *Solubilization of L-3-glycerophosphate dehydrogenase.* Acetone-dried mitochondria (600mg) were homogenized with 10ml of 30mM-potassium phosphate, pH7.6. The suspension was stirred for 20min and then centrifuged at 18000rev./min for 15min in the 8×50ml rotor of the MSE 18 centrifuge. The precipitate was then re-extracted with a further 10ml of phosphate buffer. The final precipitate was homogenized with 10ml of 0.5M-sucrose/30mM-potassium phosphate, pH7.6. The protein concentration and enzyme activity were determined and the suspension was divided into 1ml portions. Volumes of 10% (w/v) potassium deoxycholate were then added to give final concentrations of deoxycholate between zero and 0.5mg/mg of protein. After 20min, the samples were centrifuged at 50000rev./min for 30min in the 10×10ml rotor of the MSE 65 centrifuge. The supernatants were then assayed for protein and enzyme activity. As shown in Fig. 1, almost complete solubilization was obtained at 0.4mg of deoxycholate/mg of protein. The specific activity was maximal at 0.2–0.25mg of sodium deoxycholate/mg of protein, at which concentration 70% of the activity was solubilized. A pilot experiment of this kind was performed on each batch of acetone-dried powder to determine the deoxycholate concentration needed to solubilize 70% of the activity. On a preparative scale, acetone-dried mitochondria (25g) were homogenized with 500ml of phosphate buffer and washed as described above. The final precipitate was homogenized with 600ml of 0.5M-sucrose/30mM-potassium phosphate, pH7.6, containing the required concentration of deoxycholate (typically 0.2mg/mg of protein). After stirring for 20min, the supernatant was collected by centrifugation at 40000rev./min for 90min in the 8×50ml rotor of the MSE 65 centrifuge.

The extent of purification on the preparative scale was never as great as in the pilot-scale experiment, and the specific activity in the supernatant was

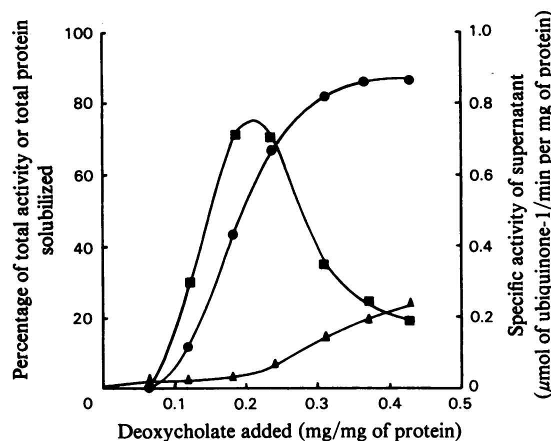

Fig. 1. *Extraction of L-3-glycerophosphate dehydrogenase with deoxycholate*

Solubilization of L-3-glycerophosphate dehydrogenase from acetone-dried powder of pig brain mitochondria was carried out as described in the Materials and methods section. The supernatant fractions were assayed for ubiquinone-1 reductase activity (●) and protein (▲), and these values were expressed as percentages of the totals present before treatment with deoxycholate. ■, Specific activity. The original specific activity was 0.05 μmol of ubiquinone-1/min per mg of protein.

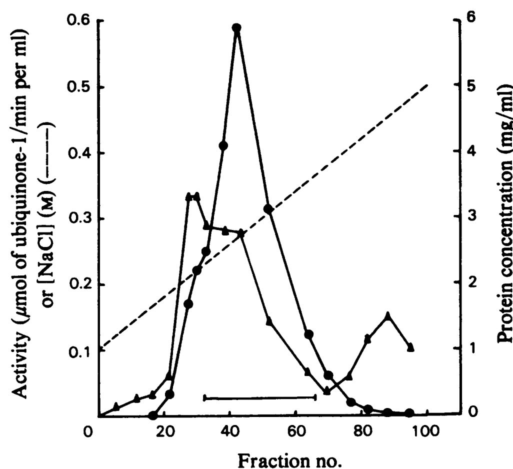

Fig. 2. *DEAE-cellulose chromatography of the deoxycholate extract*

Chromatography was performed exactly as described in the Materials and methods section. Fractions (10 ml) were assayed for ubiquinone-1 reductase activity (●) and protein (▲). The first peak of protein was turbid and was discarded. The fractions indicated by the bar were pooled for concentration and further purification. The discontinuous line shows the NaCl concentration.

between 3- and 12-fold higher than in the acetone-dried powder. Pre-extraction of the phosphate-washed acetone-dried powder with 0.1 mg of deoxycholate/mg of protein did not improve the final specific activity. Extraction of the enzyme from more concentrated suspensions of material resulted in greatly decreased specific activity.

(*d*) *Chromatography on DEAE-cellulose.* The deoxycholate extract was slightly turbid and therefore could not be concentrated by ultrafiltration.

The pooled supernatants were stirred gently with 200 ml (settled volume) of DEAE-cellulose (Whatman DE-52) equilibrated with 30 mM-potassium phosphate, pH 7.6, and 0.5% (w/v) potassium cholate. After 20 min, the slurry was allowed to settle and packed into a 30 cm × 6 cm (internal diam.) column. The column was washed with 100 ml of phosphate/cholate buffer and then with phosphate/cholate buffer containing 0.1 M-NaCl until the eluted material was no longer turbid. The column was then developed with a linear gradient of NaCl in phosphate/cholate buffer (0.1–0.5 M in 1 litre total volume). Early turbid fractions (10 ml each) were discarded and clear fractions containing enzyme activity were pooled (Fig. 2). The solution was concentrated to 25 ml by ultrafiltration through an Amicon XM 300 membrane (Amicon, Woking, Surrey, U.K.) under nitrogen at 25 lb/in^2 (170 kPa).

(*e*) *Ammonium sulphate fractionation.* The solution was adjusted to 25% saturation with $(NH_4)_2SO_4$ by addition of a saturated solution in water adjusted to pH 7.6 with NH_3. After stirring for 15 min, the precipitate was removed by centrifugation at 18000 rev./min for 15 min in the 8 × 50 ml rotor of the MSE 18 centrifuge. The enzyme was precipitated by increasing the $(NH_4)_2SO_4$ concentration in the supernatant to 40% saturation and collected as above. The precipitate was dissolved in 1–2 ml of phosphate/cholate buffer to give a clear yellow solution. The enzyme at this stage could be stored in small volumes frozen in liquid N_2. The enzyme at this stage (so-called 'cholate enzyme') defied further purification by a wide variety of techniques, including chromatography on hydroxyapatite and Sepharose CL-6B, and affinity chromatography with 6-phosphogluconate as the ligand. The latter procedure has been successful in purifying the NAD-linked L-3-glycerophosphate dehydrogenase (EC 1.1.1.8) (McGinnis & de Vellis, 1974). For reasons that are made clear in the following paper (Cottingham & Ragan, 1980), the use of non-ionic detergents such as Triton X-100 was not desirable. However, the only effective

procedure for further purification was isoelectric focusing, for which the presence of a non-ionic detergent was essential. Flat-bed isoelectric focusing in Sephadex G-75 was used in preference to the column procedure of Cole *et al.* (1978).

(*f*) *Isoelectric focusing.* Isoelectric focusing was performed in the LKB 2117 Multiphor apparatus (LKB Instruments, S. Croydon, Surrey, U.K.). The bed consisted of 5g of Sephadex G-75 equilibrated with 100ml of 0.1% (w/v) Triton X-100, 1% (w/v) pH4–6 Ampholines, 1% (w/v) pH5–7 Ampholines (Bio-Rad Laboratories, Bromley, Kent, U.K.) and 0.2% (w/v) 2-mercaptoethanol, and dried to the correct consistency as described by LKB Application Note 198. Prefocusing was conducted for 5h at a constant power of 10W. The enzyme solution from the previous stage was made 0.1% (w/v) in Triton X-100 and dialysed for 12h against 1 litre of a solution containing 0.1% (w/v) Triton X-100, 0.2% (w/v) 2-mercaptoethanol and 0.1% (w/v) glycine adjusted to pH7.0 with KOH. The dialysed solution was applied to the prefocused Sephadex bed 1cm from the cathode end and focused for 16h. The bed was fractionated into 30 strips and the enzyme eluted from each with 2.5ml of 30mM-potassium phosphate, pH7.6, containing 0.1% (w/v) Triton X-100. Each fraction was dialysed overnight against the same buffer (5 litres total volume) to remove 2-mercaptoethanol and Ampholines. The four most active fractions were pooled and the enzyme was stored frozen in liquid N_2 in small portions.

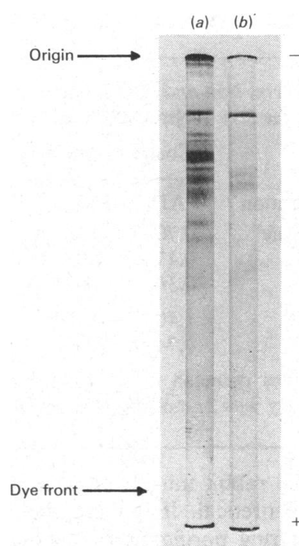

Fig. 3. *SDS/polyacrylamide gels of the 'cholate-enzyme' and 'Triton-enzyme'*
(*a*) 'Cholate-enzyme' (150µg of protein) and (*b*) 'Triton-enzyme' (32µg of protein) were electrophoresed as described in the Materials and methods section.

The enzyme migrated as a single peak of activity, with an isoelectric point at approx. pH5. Specific activity of this so-called 'Triton-enzyme' was nearly constant across the peak. Analysis by SDS/polyacrylamide-gel electrophoresis showed a major polypeptide of mol.wt. 75000, and three minor components accounting for no more than 5–10% of the total staining intensity. The 75000-mol.wt. polypeptide was also clearly visible in the gel of the enzyme before isoelectric focusing (Fig. 3). However, several other polypeptides were present in large amounts.

Catalytic properties of the enzyme

Table 1 shows the activities obtained at various stages of a single purification, and the range of yields and specific activities obtained with different purifications. Greatest variability was found in the acetone-dried powders, but this was largely eliminated after the solubilization step.

Table 2 summarizes the activities towards different electron acceptors at various stages of the

Table 1. *Purification of L-3-glycerophosphate dehydrogenase*
The results of a single preparation of enzyme are shown. The numbers in parentheses in columns 4 and 7 show the ranges obtained with eight separate purifications down to the $(NH_4)_2SO_4$ fractionation and the values from two isoelectric-focusing experiments. A unit is defined as 1µmol of ubiquinone reduced/min.

		Activity		Protein concn.			
Fraction	Volume (ml)	(units/ml)	(% of initial activity)	(mg/ml)	(% of initial protein)	Specific activity (units/mg of protein)	Purification (fold)
Acetone-dried powder	500	0.76	100	15.1	100	0.05 (0.015–0.11)	1
Phosphate-extracted acetone-dried powder	600	0.63	99 (81–100)	9.8	78	0.06 (0.02–0.19)	1.3
Deoxycholate extract	510	0.58	78 (50–85)	1.9	13	0.30 (0.11–0.39)	6
DEAE-cellulose	25	6.03	40 (20–46)	5.0	5	0.40 (0.24–0.73)	8
$(NH_4)_2SO_4$ fraction	2.2	31.4	18 (7–22)	40.2	1.2	0.78 (0.51–1.41)	15
Isoelectric focusing	8.9	1.16	2.7 (1.5–2.7)	0.4	0.05	2.90 (2.2–2.9)	54

Table 2. *Purification of activity with different electron acceptors*

K_m and $V_{max.}$ for the acceptor indicated were determined at 22°C and in the presence of 50mM-DL-3-glycerophosphate. Abbreviations n.d., not determined; DB, decylbenzoquinone analogue of ubiquinone-2; DCIP, dichlorophenol-indophenol. K_m values with DB as acceptor were too low for measurement. A unit is defined as 1 μmol of acceptor reduced/min.

	Ubiquinone-0		Ubiquinone-1		DB		DCIP	
Fraction	K_m (μM)	$V_{max.}$ (units/mg of protein)	K_m (μM)	$V_{max.}$ (units/mg of protein)	K_m (μM)	$V_{max.}$ (units/mg of protein)	K_m (μM)	$V_{max.}$ (units/mg of protein)
Mitochondria*	9.8	0.039	21	0.027	n.d.	0.029	22	0.024
Acetone-dried powder	14	0.017	20	0.048	n.d.	0.073	n.d.	n.d.
Deoxycholate extract	48	0.118	18	0.19	n.d.	0.185	n.d.	n.d.
Isoelectric focusing	n.d.	n.d.	13	2.63	n.d.	3.09	76	0.81

* The data for mitochondria were obtained with a different preparation from that used for subsequent stages of purification.

purification procedure. The increases in specific activity from mitochondria to pure enzyme differed according to the electron acceptor used. Activities obtained with the decylbenzoquinone analogue were similar to those obtained with ubiquinone-1. However, the increases in specific activity during purification were lower with either ubiquinone-0 or dichlorophenol-indophenol as acceptors. From work on the reconstitution of the enzyme with ubiquinol–cytochrome *c* oxidoreductase (Cottingham & Ragan, 1980), it appears that the activity with either ubiquinone-1 or the decylbenzoquinone analogue is a better measure of the turnover of the enzyme in the respiratory chain. The K_m for ubiquinone-1 was not greatly altered during the course of purification. It is shown below that the apparent K_m is a function of the amount of lipid associated with the enzyme, and therefore the small decrease in K_m with increasing purification could be explained by a decreasing lipid/protein ratio. The K_m for the decylbenzoquinone analogue was too low to measure, whereas the values for ubiquinone-0 and dichlorophenol-indophenol increased substantially during the course of purification.

Flavin, non-haem iron and acid-labile sulphide contents

Table 3 summarizes the results of assays of non-covalently bound FMN and FAD, iron and acid-labile sulphide in a number of preparations of the 'cholate-enzyme' or 'Triton-enzyme'. Most of the flavin was FAD, in agreement with Ringler & Singer (1958), Dawson & Thorne (1969*a*) and Cole *et al.* (1978). However, the increase in FAD content resulting from the final isoelectric-focusing step was very small and inconsistent with the protein purification estimated from SDS/polyacrylamide gels. Furthermore, a single polypeptide of 75000 mol.wt. would be expected to have an FAD content of 13.3 nmol/mg of protein. Cole *et al.* (1978) reported a rather variable FAD content of 3–10 nmol/mg of

Table 3. *Flavin, iron and acid-labile sulphide assays*

For details see the text. Abbreviation: n.d., not determined.

	Content (nmol/mg of protein)			
Enzyme preparation	FAD	FMN	Fe	S
'Cholate-enzyme'	0.70	0.10	0.8	1.3
	0.47	0.05	1.5	0.87
	0.36	0.07	1.0	1.0
'Triton-enzyme'	1.40	0.10	n.d.	n.d.
	0.74	0.05	<2*	n.d.

* No iron was detected in the 'Triton-enzyme'. The limit of sensitivity was 2 nmol/mg of protein.

protein for the rabbit muscle enzyme, again values lower than theoretical. It is clear that the enzyme loses flavin during purification, and this problem is more severe for the pig brain enzyme than for the rabbit muscle enzyme. The most obvious step at which flavin is lost is during isoelectric focusing, although losses at earlier stages of the purification cannot be ruled out. The enzyme is unstable below pH 5.5 (Fig. 4), and therefore the conditions of isoelectric focusing lead to considerable loss of total activity (Table 1), which may be related to the loss of FAD. The greater retention of FAD by the rabbit muscle enzyme was reflected by a higher final specific activity (Cole *et al.*, 1978) than was obtained with the pig brain enzyme. Addition of FAD to either the 'cholate-enzyme' or 'Triton-enzyme' did not lead to increases in specific activity.

Acid conditions also promote destruction of iron–sulphur centres. Thus the very low contents of iron in the 'Triton-enzyme' might be attributed to this. However, the 'cholate-enzyme' also contained low concentrations of both non-haem iron and acid-labile sulphide. The molar ratio of either iron or sulphide to FAD varied between 1.1 and 3, with no correlation with activity. Considering that these must represent upper limits in view of the impure nature of the enzyme, it seems unlikely that the

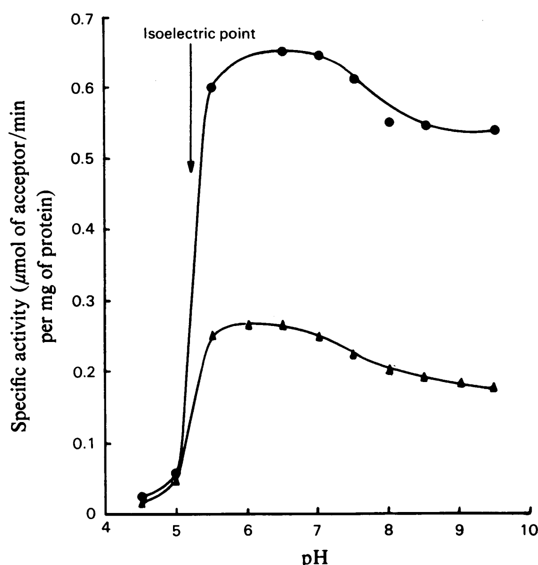

Fig. 4. *Stability of* L*-3-glycerophosphate dehydrogenase as a function of pH*

Samples of the 'cholate-enzyme' were incubated at a concentration of 1 mg of protein/ml in 150mM buffers of the indicated pH for 20h at 4°C. Activity towards both ubiquinone-1 (●) and dichlorophenol-indophenol (▲) was then measured at pH 7.6. The original specific activities at pH 7.6 were 0.68 µmol of ubiquinone-1/min per mg of protein and 0.26 µmol of dichlorophenol-indophenol/min per mg of protein. The buffers used were as follows: pH 4.5–6, sodium acetate; pH 6.5–7.5, potassium phosphate; pH 8–9.5, Tris/HCl.

enzyme contains sufficient iron or sulphide to form a single 2Fe:2S or 4Fe:4S centre. A similar conclusion may be drawn from the results of Cole *et al.* (1978), who reported a single determination of 10 ng-atoms of Fe/mg of rabbit muscle enzyme. A solution of the 'cholate-enzyme' containing 15 µM-Fe failed to show any L-3-glycerophosphate-reducible e.p.r. signals at temperatures down to 14 K.

Lipid constituents of the enzyme

A preparation of the 'cholate-enzyme' contained 70 nmol of phospholipid P/mg of protein. T.l.c. of lipid extracts revealed five components, three of which were identified as phosphatidylcholine, phosphatidylethanolamine and cardiolipin by comparison with standard markers. Since the enzyme was impure at this stage, further identifications were not pursued. The 'Triton-enzyme' contained only 20 nmol of phospholipid P/mg of protein, i.e. 1.5 mol of phospholipid P/75000 g of protein. T.l.c. revealed the presence of a single phospholipid component, which migrated with bovine heart cardiolipin, although its identity was not further investigated. Since cardiolipin contains two phosphate residues per molecule, it is possible that the 'Triton-enzyme' contains 0.75 mol of cardiolipin/mol of protein. This very low phospholipid content shows that ubiquinone reductase activity does not require the presence of a large amount of phospholipid to act as a solvent for the quinone substrate. This contrasts with the behaviour of NADH dehydrogenase, which requires phospholipid both for stabilization of the protein structure and to dissolve the ubiquinone (Ragan, 1978). It is possible that in the 'Triton-enzyme' the function of phospholipid is being carried out by Triton X-100 instead. Although this does not occur with NADH dehydrogenase, cytochrome oxidase, for example, is quite active in the presence of non-ionic detergents (see, e.g., Yonetani, 1961). Therefore it cannot be ruled out that a hydrophobic environment is necessary for the functioning of L-3-glycerophosphate dehydrogenase. Whether the remaining molecule of phospholipid is essential or merely rather firmly bound to the enzyme is a question that cannot be answered at this stage.

A preparation of the 'cholate-enzyme' also contained 0.51 nmol of ubiquinone-10/mg of protein. The presence of ubiquinone-10 was not unexpected, since the lipoprotein complexes of bovine heart mitochondria, including cytochrome oxidase (Griffiths & Wharton, 1961), contain ubiquinone-10 presumably carried along with the phospholipid. Thus the presence of ubiquinone-10 does not imply any direct association with the enzyme.

Does the enzyme reduce electron acceptors at more than one site?

By analogy with both NADH dehydrogenase and succinate dehydrogenase, the enzyme might have been expected to catalyse reduction of added electron acceptors at more than one site. For example, NADH dehydrogenase catalyses reduction of more-hydrophobic ubiquinone analogues at a site in the phospholipid phase, whereas more-hydrophilic analogues were reduced at least partly at an aqueous site (Ragan, 1978). The two sites may be distinguished by selective inhibition of reduction at the lipid site by rotenone. It is therefore this site at which ubiquinone-10 is reduced in the mitochondrial membrane. The possibility of two reduction sites in L-3-glycerophosphate dehydrogenase was suggested by the lower degree of purification of activity with the hydrophilic substrate, dichlorophenol-indophenol, compared with more hydrophobic substrates such as ubiquinone-1. A search for a reagent that would selectively inhibit reduction of either of these acceptors was unsuccessful. The enzyme was not greatly inhibited by high concentrations of thenoyltrifluoroacetone and carboxin (inhibitors of succinate–ubiquinone oxido-

reductase; Tappel, 1960; Mowery *et al.*, 1977), 2-hydroxybenzohydroxamic acid and 2-chlorobenzohydroxamic acid (inhibitors of L-3-glycerophosphate oxidation in trypanosomes; Opperdoes *et al.*, 1976) or chloroquine (an inhibitor of ubiquinone reduction by NADH; Garland *et al.*, 1972). What inhibition was observed at mM concentrations of these inhibitors was the same irrespective of the electron acceptor. In a preliminary communication (Cottingham & Ragan, 1978) we reported selective inhibition of ubiquinone reductase activity by these compounds. This was a mistake on our part. As the concentration of these compounds in the assay was increased, the total absorbance at 275 nm increased beyond the ability of the spectrophotometer to maintain its sensitivity, thereby giving the appearance of decreased rates of ubiquinone-1 reduction. A decrease in the light path to 0.5 cm prevented this problem, and a dual-wavelength spectrophotometer was used to check the results in some instances.

A second approach to the question of two reduction sites was to test the effect of added phospholipid on activity. With NADH dehydrogenase, phospholipid causes a stimulation of rotenone-sensitive ubiquinone-1 reductase activity and an inhibition of the rotenone-insensitive activity. These effects are due to alterations in the partition of the analogue by increasing the amount of the lipid phase relative to the aqueous phase (Ragan, 1978). Thus the effects of added lipid can indicate whether the reduction site is lipophilic or hydrophilic. As shown in Table 4, both soya-bean phosphatidylcholine and bovine heart cardiolipin in low concentrations caused a stimulation of ubiquinone-1 reductase activity, whereas higher concentrations were inhibitory. The reduction of the much more hydrophobic decylbenzoquinone analogue was unaffected by phospholipid in this concentration range. The partition of the latter analogue is almost completely into the lipid phase under any circumstances, and effects on activity were therefore not expected. The response with ubiquinone-1 as acceptor is more complicated. The degree of inhibition by higher concentrations of phosphatidylcholine was dependent on the ubiquinone-1 concentration. As shown in Fig. 5, the reciprocal rate was also linearly related to phospholipid concentration. This behaviour is very similar to that found for the rotenone-insensitive reduction of ubiquinone-

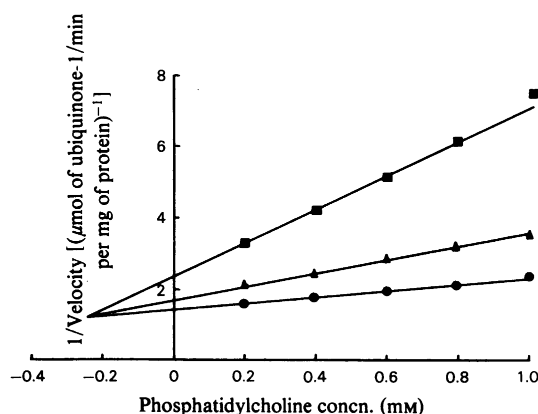

Fig. 5. *Dependence of ubiquinone-1 reductase on phosphatidylcholine concentration*
L-3-Glycerophosphate dehydrogenase (5 μg of protein) was assayed as described in the Materials and methods section, except that the ubiquinone-1 concentrations were 41 μM (●), 20.5 μM (▲) and 10.25 μM (■). Phosphatidylcholine was also present in the indicated concentrations.

Table 4. *Effect of phosphatidylcholine and cardiolipin on ubiquinone-1 reductase activity*
Assays were carried out as described in the Materials and methods section with the indicated concentrations of soya-bean phosphatidylcholine or bovine heart cardiolipin. 'Cholate-enzyme' (5 μg of protein) was used in each assay. Two different preparations were used.

Phospholipid	Concn. (μM)	L-3-Glycerophosphate–ubiquinone-1 oxidoreductase (μmol/min per mg of protein)
Phosphatidylcholine	0	0.43
	5	0.55
	10	0.61
	100	0.63
	600	0.52
	1000	0.41
Cardiolipin	0	0.62
	2.5	0.91
	7.6	0.95
	10.2	0.96

1 by NADH dehydrogenase (Ragan, 1978). Fig. 5 can be used to estimate the partition coefficient, P, for ubiquinone-1 (Ragan, 1978), since the phosphatidylcholine concentration, L, at which the lines of Fig. 5 meet, is related to P by the expression

$$L = -55.5/P$$

The value of P so obtained (2.2×10^5 at 20°C) compared favourably with the value calculated from the aqueous solubility, S, of ubiquinone-1 (1.18×10^{-5} mol/mol of water at 20°C) and the expression $P \times S = 2$ (Hill, 1974), i.e. $P = 1.7 \times 10^5$ at 20°C. Thus the inhibition of activity by phospholipid can be explained adequately if ubiquinone-1 is reduced at an aqueous site. The stimulation of activity at low phospholipid concentrations might be explained by displacement or removal of cholate, which is quite inhibitory at concentrations in the assay as low as 0.02% (w/v).

The conclusions from the phospholipid-dependence of activity are therefore somewhat contradictory. The high activity and extremely low K_m for the decylbenzoquinone analogue would argue strongly that reduction of this acceptor takes place in the lipid phase. The inhibitory effects of phospholipid on ubiquinone-1 reductase activity suggest that this analogue is reduced in the aqueous phase. Either there is one site on the enzyme, which may be approached by either hydrophobic or hydrophilic acceptors, or alternatively there are two different sites with equal catalytic activity. Since we could find no supporting evidence for two different sites, we favour the former possibility. Indeed, the existence of two sites would be surprising if the enzyme is a simple flavoprotein.

Visible absorption spectrum

Fig. 6 shows the reduced-minus-oxidized difference spectrum of the 'cholate-enzyme' after reduction by L-3-glycerophosphate. The shape is very similar to that reported by Ringler (1961). Addition of dithionite caused further bleaching in the 450nm region, but, since the enzyme was impure, such absorbancy changes cannot be necessarily ascribed to L-3-glycerophosphate dehydrogenase. Small amounts of dithionite-reducible cytochromes were also present. Assuming that the absorbancy changes at 450nm were due to FAD reduction, the substrate-reducible flavin was 0.23nmol/mg of protein or 50% of the total. The extent of reduction by dithionite corresponded to 100% of the flavin content. Unfortunately, Cole *et al.* (1978) did not report the spectrum of the substrate-reduced enzyme, only the oxidized and dithionite-reduced forms. In addition, the FAD content of the sample used was not given.

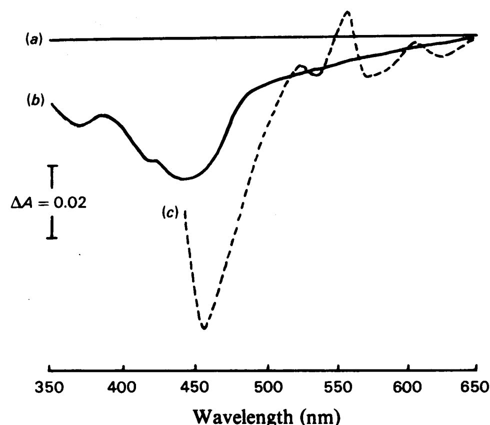

Fig. 6. *Visible absorption difference spectra of L-3-glycerophosphate dehydrogenase*
'Cholate-enzyme' (17·7mg of protein/ml, containing 0.47nmol of FAD/mg of protein) was reduced with 50mM-DL-3-glycerophosphate and then further reduced by addition of solid dithionite. (*a*), Oxidized minus oxidized; (*b*), DL-3-glycerophosphate-reduced minus oxidized; (*c*), dithionite-reduced minus oxidized.

Discussion

The subunit composition and molecular weight of the enzyme are in good agreement with the findings of Cole *et al.* (1978). L-3-Glycerophosphate dehydrogenase is therefore much the most simple respiratory-chain dehydrogenase system described. NADH–ubiquinone oxidoreductase consists of at least 26 polypeptides (Heron *et al.*, 1979), and succinate–ubiquinone oxidoreductase consists of three (Yu *et al.*, 1977) or four polypeptides (Capaldi *et al.*, 1977). The acyl-CoA dehydrogenase system seems to consist of three separable enzymes, acyl-CoA dehydrogenase itself, the electron-transferring flavoprotein and its dehydrogenase, a ferroflavoprotein catalysing oxidation of reduced electron-transferring flavoprotein by ubiquinone analogues (Ruzicka & Beinert, 1975).

The three examples above all involve iron–sulphur centres on the pathway from substrate to ubiquinone, and there is good evidence that iron–sulphur centres are the immediate electron donors to ubiquinone (see, e.g., Ohnishi, 1979). The absence of sufficient iron or acid-labile sulphide in purified L-3-glycerophosphate dehydrogenase to form a binuclear or tetranuclear structure is rather surprising and needs further investigation. In view of the loss of FAD incurred on purification, simultaneous loss of iron–sulphur centres would not be unexpected, and with the preparation of the enzyme described in the present paper, or that of Cole *et al.* (1978), a clear answer cannot be given. However,

the enzyme before the acidic isoelectric-focusing step also has a very low iron/flavin ratio, and the yield of enzyme activity at this stage would be inconsistent with wholesale destruction of iron–sulphur centres (a considerable proportion of the total enzyme units are discarded in fractions of low purity rather than lost through inactivation). The enzyme of Cole *et al.* (1978) has an absorption spectrum resembling that of a ferroflavoprotein. However, the loss of absorption on treatment with mersalyl was extremely small, which might indicate that the bulk of the chromophore was not an iron–sulphur centre. The iron content of the Cole *et al.* (1978) preparation and the enzyme described in the present paper might be sufficient to form a centre containing a single iron atom, as found, for example, in rubredoxin. If so, it would be the only example ever found in mitochondrial electron-transfer systems (see, e.g., Ohnishi, 1979).

An alternative explanation that must be considered is that the isolated enzyme has lost polypeptides with associated iron–sulphur centres, which are required for reduction of ubiquinone-10 in the respiratory chain. The reduction of ubiquinone analogues could therefore occur through a non-physiological pathway similar to the rotenone-insensitive reduction of such compounds by NADH dehydrogenase (see e.g., Ragan, 1978). In the following paper (Cottingham & Ragan, 1980), it will be shown that both the 'cholate-enzyme' and the purified 'Triton-enzyme' are capable of reconstitution with ubiquinone-10 and mitochondrial ubiquinol–cytochrome *c* oxidoreductase to give antimycin-sensitive L-3-glycerophosphate–cytochrome *c* oxidoreductase activity. The maximum turnover of this system is close to that expected from the ubiquinone-1 reductase activity of the dehydrogenase. Thus the ability of the isolated enzyme to transfer electrons to the respiratory chain is unimpaired compared with its ability to donate electrons to other acceptors. There is therefore no evidence that other polypeptides are involved in electron transfer between the single-subunit dehydrogenase and the rest of the respiratory chain.

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