Plant mitochondrial 2-oxoglutarate dehydrogenase complex: purification and characterization in potato

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The 2-oxoglutarate dehydrogenase complex (OGDC) in potato (*Solanum tuberosum* cv. Romano) tuber mitochondria is largely associated with the membrane fraction of osmotically ruptured organelles, whereas most of the other tricarboxylic acid cycle enzymes are found in the soluble matrix fraction. The purification of OGDC from either membrane or soluble matrix fractions resulted in the increasing dependence of its activity on the addition of dihydrolipoamide dehydrogenase (E3). A 30-fold purification of OGDC to apparent homogeneity and with a specific activity of 4.6 $\mu\text{mol}/\text{min}$ per mg of protein in the presence of exogenously added E3 was obtained. SDS/PAGE revealed that the purified complex consisted of three major polypeptides with apparent molecular masses of 48, 50 and 105 kDa. Before the gel-filtration purification step, E3 polypeptides of 57 and 58 kDa were identified by immunoreaction as minor proteins associated with OGDC. The N-terminal sequence of the 57 kDa

protein was identical with that previously purified as the E3 component of the pyruvate dehydrogenase complex from potato. The 105 kDa protein was identified as the 2-oxoglutarate dehydrogenase subunit of OGDC by N-terminal sequencing. The N-terminal sequences of the 50 and 48 kDa proteins shared 90–95 % identity over 20 residues and were identified by sequence similarity as dihydrolipoamide succinyltransferases (OGDC-E2). The incubation of OGDC with [U-¹⁴C]2-oxoglutarate resulted in the reversible succinylation of both the 48 and the 50 kDa protein bands. Proteins previously reported as subunits of complex I of the respiratory chain from *Vicia faba* and *Solanum tuberosum* are proposed to be OGDC-E2 and the possible basis of this association is discussed.

Key words: dihydrolipoamide succinyltransferase, lipoamide dehydrogenase, succinyl-CoA.

INTRODUCTION

The mitochondrial 2-oxoglutarate dehydrogenase complex (OGDC) occupies a central point in cellular metabolism within the tricarboxylic acid cycle. The complex has been characterized in mammals, fungi and bacteria as a large multienzyme assembly that catalyses the oxidative decarboxylation of 2-oxoglutarate (2-OG) to form succinyl-CoA and NADH by the sequential operation of three separate enzymes: 2-oxoglutarate dehydrogenase (OGDC-E1, EC 1.2.4.2), dihydrolipoamide succinyltransferase (OGDC-E2, EC 2.3.1.61) and dihydrolipoamide dehydrogenase (E3, EC 1.8.1.4). OGDC exists as a polymeric structure with a molecular mass of approx. 2000 kDa that comprises an E2 core of 24 subunits, to which are attached E1 and E3 homodimers [1,2]. E3 binds directly to the E1 subunits of mammalian OGDC [3,4]. In contrast, the binding of E3 to the related pyruvate dehydrogenase complex (PDC) occurs via a separate E3-binding protein (E3BP) attached to the dihydrolipoamide acetyltransferase (PDC-E2) core [5].

In contrast, very little information is available on the structure, function or subunit composition of the plant OGDC. 2-OG-dependent respiration in plant mitochondria is known to require thiamine pyrophosphate (TPP), NAD⁺ and ADP [6,7]; competition between PDC and OGDC for intra-mitochondrial NAD⁺ and CoA has been reported [8,9]. Partial purification of OGDC has been reported from cauliflower mitochondria with specific activities of 1–3 $\mu\text{mol}/\text{min}$ per mg of protein [10,11]. The cauliflower OGDC is activated by AMP [12], owing to the

interaction of this nucleotide with the E1 component, which lowers the K_m for 2-OG and increases the V_{max} of the overall OGDC reaction [13,14]. No effect of AMP on the activity of OGDC from non-plant sources has been reported. OGDC exhibits approximately the same affinities for NAD⁺ and NADH as other plant mitochondrial NAD⁺-linked dehydrogenases [15]. Preparations of OGDC from cauliflower showed an association of an NADH oxidase, contamination with mitochondrial PDC (mPDC) activity and loss of E3 activity during purification [10,11]. No information on the proteins present in these partial purifications has been presented.

Only one E3 gene containing mitochondrial targeting sequences has been isolated from pea; the predicted 56 kDa polypeptide that it encodes has been proposed to participate in the catalytic function of PDC, OGDC and the glycine decarboxylase complex in plant mitochondria [16–18]. However, several recent reports have suggested the presence of multiple E3 proteins in plant mitochondria. Conner et al. [19] described two E3 populations in potato mitochondria with apparent molecular masses of 56 and 58 kDa. We have identified two E3 subunits of the same sizes associated with the purified potato mPDC [20]. Multiple E3 polypeptides are also associated with the partly purified maize mPDC [21]. The differential association of these E3 polypeptides with different multienzyme dehydrogenases in mitochondria has not until now been investigated in plants.

Here we investigate the activity and localization of OGDC in potato mitochondria, present the purification of OGDC to near homogeneity from potato tuber mitochondria and identify the

Abbreviations used: E3, dihydrolipoamide dehydrogenase (EC 1.8.1.4); E3BP, E3-binding protein; mPDC, mitochondrial PDC; 2-OG, 2-oxoglutarate; OGDC, 2-oxoglutarate dehydrogenase complex; OGDC-E1, 2-oxoglutarate dehydrogenase subunit of OGDC (EC 1.2.4.2); OGDC-E2, dihydrolipoamide succinyltransferase subunit of OGDC (EC 2.3.1.61); ORF, open reading frame; PDC, pyruvate dehydrogenase complex; PDC-E2, dihydrolipoamide acetyltransferase subunit of PDC (EC 2.3.1.12); PEG-6000, 6000 Da poly(ethylene glycol); TPP, thiamine pyrophosphate.

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major polypeptide components by N-terminal sequencing and ^{14}C -succinylation studies. *Arabidopsis* nucleotide sequences encoding these subunits are identified by N-terminal sequence similarity. The two E3 polypeptides found in potato mitochondria are shown to be associated with both OGDC and mPDC from potato mitochondria and evidence is provided that a polypeptide identified as a component of complex I in plants is likely to be OGDC-E2.

MATERIALS AND METHODS

Isolation and fractionation of potato mitochondria

Potato tubers (*Solanum tuberosum* cv. Romano) were purchased locally; 5–10 kg was used for mitochondrial preparations by the method of Millar et al. [20]. Fractionation experiments were performed with 20–25 mg of mitochondrial protein, which was diluted to 2 mg/ml in 25 mM KH_2PO_4 , pH 6.7. This suspension was then freeze-thawed twice in liquid N_2 and centrifuged at 25000 *g* for 30 min at 4 °C; the pellet was retained (25000 *g* pellet). The supernatant was then centrifuged at 100000 *g* for 1 h; the pellet was retained (100000 *g* pellet) and the supernatant was centrifuged again at 200000 *g* for 3 h. These pelleted fractions, together with the final 200000 *g* supernatant, were then assayed for enzyme activities of membrane and soluble mitochondrial components.

Enzyme activities

O_2 consumption was measured in an O_2 electrode (Hansatech, King's Lynn, Norfolk, U.K.) in 1 ml of reaction medium containing 0.3 M mannitol, 10 mM Tes/KOH, pH 7.5, 5 mM KH_2PO_4 , 10 mM NaCl, 2 mM MgSO_4 and 0.1 % BSA. Pyruvate (5 mM), malate (0.5 mM), 2-OG (5 mM), NAD^+ (0.5 mM), TPP (0.05 mM) and ADP (0.1–1 mM) were added as indicated. OGDC activity was measured as NADH formation at 340 nm in reaction medium containing 75 mM Tes/NaOH, pH 7.5, 0.05 % (w/v) Triton X-100, 0.5 mM MgCl_2 , 2 mM NAD^+ , 0.12 mM lithium CoA, 0.2 mM TPP, 2.5 mM cysteine/HCl, 1 mM AMP and 1 mM sodium 2-OG with or without the addition of 3 units of pig lipoamide dehydrogenase (EC 1.8.1.4) as indicated. PDC was assayed as described in [20], NAD^+ -malic enzyme as described in [22], cytochrome *c* oxidase as described in [23], citrate synthase as described in [24], aconitase and fumarase as described in [25], NAD^+ -dependent isocitrate dehydrogenase as described in [26] and NADH:ubiquinone oxidoreductase as NADH-dependent FeCN reduction as described in [27]. Malate dehydrogenase was assayed as oxaloacetate-dependent NADH oxidation in 10 mM oxaloacetate/0.2 mM NADH/10 mM MgCl_2 /50 mM Tes/KOH (pH 7.2). Lipoamide dehydrogenase was measured at 340 nm as L,D -lipoamide (1 mM)-dependent NADH (0.2 mM) oxidation or as L,D -dihydrolipoamide (1 mM)-dependent NAD^+ (2 mM) reduction in 100 mM Tes/KOH, pH 8.5. All enzyme activities were measured at 25 °C. Protein concentrations were determined by the method of Bradford [28], with BSA as standard.

Purification of OGDC

Isolated mitochondria were diluted to 3–5 mg/ml protein in a solution of 25 mM KH_2PO_4 /KOH (pH 6.7)/1 mM NAD^+ /0.1 mM TPP/5 mM dithiothreitol, freeze-thawed once in liquid N_2 , stirred on ice for 15 min and then centrifuged at 40000 *g* for 30 min.

For the preparation of OGDC from the soluble matrix fraction, the 40000 *g* supernatant was centrifuged at 200000 *g* for 180 min.

The amber pellet was resuspended in OGDC buffer [25 mM Tes/KOH (pH 7.0)/1 mM NAD^+ /0.1 mM TPP/5 mM dithiothreitol] to a final concentration of 4–5 mg/ml protein.

For the preparation of OGDC from the membrane fraction, the 40000 *g* pellet was resuspended in OGDC buffer to a final concentration of 5 mg/ml and sonicated on ice for 10 s in five bursts (2 s each) with a Soniprep 150 (Sanyo, Loughborough, Leics., U.K.). Most membrane material was removed by centrifugation at 40000 *g* for 30 min. The supernatant was then removed and centrifuged at 200000 *g* for 180 min. Unless indicated otherwise, all these steps were performed at 4 °C.

Samples of the 200000 *g* pellets from either the soluble fraction or the membrane fraction were then slowly warmed to 25 °C, 10 mM MgCl_2 was added and the precipitated material was collected by centrifugation at 20000 *g* for 15 min at 4 °C. This pellet, which contained the OGDC activity, was resolubilized in OGDC buffer supplemented with 2 mM EDTA. The supernatant was again warmed to 25 °C and 0.1 vol. of 50 % (w/v) 6000 Da poly(ethylene glycol) (PEG-6000) was added, after which it was centrifuged at 20000 *g* for 15 min at 4 °C. The supernatant was again warmed to 25 °C, 10 mM MgCl_2 was added and the precipitated material was collected by centrifugation at 20000 *g* for 15 min. The precipitated OGDC activity was resolubilized in OGDC buffer supplemented with 2 mM EDTA; insoluble material was removed by further centrifugation at 20000 *g* for 15 min at 4 °C.

Protein gel electrophoresis, N-terminal sequencing and FPLC

The protein subunit composition of the purified potato OGDC was further characterized by electrophoresis under denaturing, reducing conditions on 0.1 % SDS/12 % (w/v) polyacrylamide gels by the method of Laemmli [29]. The apparent molecular masses of the component polypeptides are based on the average of three independent experiments with standard protein markers from Novex (San Diego, CA, U.S.A.). For immunoreaction experiments, proteins were electroblotted from SDS/PAGE gels to nitrocellulose membranes and blocked in 5 % (w/v) casein. Chemiluminescence was used for the detection of horseradish peroxidase-conjugated secondary antibodies. For N-terminal sequence analysis, proteins were separated by SDS/PAGE, electroblotted to PVDF membranes and sequenced by automated Edman degradation on an Applied Biosystems protein sequencer (Model 494A). Superose 6 gel-filtration and MonoQ anion-exchange columns were used on an FPLC system from Pharmacia LKB (Uppsala, Sweden).

Succinylation of OGDC

Succinylation was performed by a modification of the procedure reported by Jilka et al. [30]. Purified potato OGDC (30 μg of protein) was incubated at 30 °C for 200 s in the presence of 0.2 mM $[\text{U-}^{14}\text{C}]$ 2-OG (10.4 GBq/mmol) in a volume of 30 μl containing 1 mM MgCl_2 , 1 mM NAD^+ , 0.2 mM TPP, 1 mM AMP and 60 mM Tes/NaOH, pH 7.5. Reactions were terminated by the addition of *N*-ethylmaleimide to a final concentration of 10 mM. After incubation for 30 min at 25 °C, samples were denatured in non-reducing SDS sample buffer at 50 °C for 2 h; the OGDC subunits were then separated by SDS/PAGE. Gels were stained with Coomassie Blue, destained and vacuum-dried on Whatman no. 3MM paper. Dried gels were exposed directly to Kodak Biomax X-ray film and developed after 7–15 days in accordance with standard protocols.

Analytical ultracentrifugation

Samples of OGDC (1.0 mg/ml) in 25 mM KH_2PO_4 , pH 7.0, were centrifuged at 20 000 g (16 000 rev./min) for 90–120 min at 20 °C in a Beckman Optima XL-A Analytical Ultracentrifuge. Absorbance scans of the samples at 280 nm, recorded at 10 min intervals, were analysed by using Origin 2.8 software employing the second-moment method for the determination of concentration boundary points. With the use of data from four to six absorbance scans, the sedimentation coefficient (s) of a sample was calculated from the slope of plots of $\ln(r_{\text{boundary}})$ against $\omega^2 t$, where r_{boundary} is the radius of the concentration boundary from the centre of rotation, ω is the angular velocity and t is the elapsed time of centrifugation.

RESULTS

NAD⁺-linked respiration in isolated potato mitochondria and separation of tricarboxylic acid cycle enzymes by differential centrifugation

The rate of 2-OG-dependent O_2 consumption by isolated potato mitochondria was double the rate of pyruvate-dependent O_2 consumption in the presence of saturating concentrations of ADP (Table 1). The apparent activity of OGDC and mPDC in the organelles can be calculated from a knowledge of the rate of O_2 consumption and the enzymes of the tricarboxylic acid cycle that are operating in the oxidation of the substrate provided (Table 1). Such calculations show that OGDC was operating at nearly 4-fold the rate of mPDC during oxidation of their respective substrates in intact mitochondria. However, measurements of OGDC and mPDC activities in matrix extracts or solubilized whole potato mitochondria show a greater rate of NAD⁺ reduction by mPDC than by OGDC. Activities of mPDC and OGDC in extracts were measured in the presence of 3 units of added pig E3. The addition of exogenous E3 stimulated

Table 1 Oxidation of pyruvate and 2-OG by intact potato mitochondria and the activities of PDC and OGDC in potato mitochondrial extracts

In-organellar (IO) NAD⁺ reduction was calculated from O_2 consumption data on the basis of the following assumptions: all the NADH was derived from OGDC activity during the oxidation of 5 mM 2-OG in the presence of 20 mM malonate; and 50% of the NADH derived from mPDC and 50% from MDH during the oxidation of 5 mM pyruvate plus 0.5 mM malate at pH 7.5. These assumptions were based on the following evidence. Succinate-dependent O_2 consumption by potato mitochondria was inhibited by 96% by the addition of 20 mM malonate, showing that the latter effectively prevents further NADH reduction steps in the tricarboxylic acid cycle after the OGDC reaction. A 1:1 molar stoichiometry was observed between O_2 consumption and citrate accumulation in the medium during O_2 consumption dependent on 5 mM pyruvate plus 0.5 mM malate at pH 7.5, showing that citrate is the end product of the tricarboxylic acid cycle under these conditions. Solubilized mitochondria (SM) were formed by the addition of 0.1% (w/v) Triton X-100 to mitochondrial samples. Matrix extracts (ME) were prepared by freeze-thawing mitochondria in 25 mM Tes (pH 7.5) and removing membranes by centrifugation at 25 000 g for 30 min. O_2 consumption and OGDC and mPDC activities (in the presence of 3 units of pig E3) were measured as described in the Materials and methods section. Malate and malonate were not present during assays of OGDC and mPDC in extracts. Values given are means $\pm$ S.E.M.

Substrate	Intact mitochondria		Mitochondrial extracts [NAD ⁺ reduction (nmol of NADH/min per mg of protein)]	
	O_2 consumption (n-atom O/min per mg of protein)	IO NAD ⁺ reduction (nmol of NADH/min per mg of protein)	SM	ME
Pyruvate (+ malate)	260 $\pm$ 12	130 $\pm$ 6	160 $\pm$ 20	145 $\pm$ 14
2-OG (+ malonate)	500 $\pm$ 40	500 $\pm$ 40	140 $\pm$ 36	40 $\pm$ 3

Table 2 Distribution of tricarboxylic acid cycle enzymes after fractionation of potato tuber mitochondria by successive differential centrifugations

Different fractions of mitochondrial protein were obtained and enzymes and protein were assayed as outlined in the Materials and methods section. Percentages are based on the sum of activities of each enzyme across all fractions and represent yields of 91–104% from the activities measured in whole mitochondrial homogenates. Results of a typical experiment are presented and results from enzymes with very similar distributions were pooled and percentage ranges given for the activities in each fraction. Abbreviations: COX, cytochrome *c* oxidase; SDH, succinate dehydrogenase; A, aconitase; CS, citrate synthase; MDH, malate dehydrogenase; ICDH, NAD⁺-dependent isocitrate dehydrogenase; ME, NAD⁺-dependent malic enzyme.

Enzyme	Activity or protein (% of total)			
	25 000 g pellet	100 000 g pellet	200 000 g pellet	200 000 g supernatant
COX and SDH	94–95	4–5	1–2	0
OGDC	77	22	1	0
PDC	11	43	38	8
Fumarase	1	2	48	50
A, CS, MDH, ICDH and ME	0–3	0–2	1–7	88–99
Protein	47	5	21	27

OGDC activity by 25–50% but did not significantly affect mPDC activity (results not shown). The activities of both complexes were maximal at pH 7.5, as judged by assays at 0.25 pH unit intervals over the pH range 6.5–8.0 (results not shown). Much of the calculated OGDC activity in the organelles could thus not be quantitatively accounted for during measurements in mitochondrial extracts even in the presence of exogenous E3 and at optimum pH (Table 1). A similar problem was encountered by Bowman et al. [6], who reported that OGDC activity in mung bean mitochondrial extracts was below the level of detection, whereas a significant rate of 2-OG oxidation was measured in intact mitochondria.

These results suggest that, unlike mPDC, OGDC is not primarily found in the soluble matrix fraction of potato mitochondria and it seems to be more active in intact mitochondria than after mitochondrial disruption (Table 1). To investigate this phenomenon further, we measured the fractionation of OGDC activity compared with other tricarboxylic acid cycle enzyme activities in potato tuber mitochondria after a series of differential centrifugation steps (Table 2). After freeze-thawing of whole potato mitochondrial extracts under hypo-osmotic conditions and centrifugation at 25 000 g for 30 min, 77% of OGDC activity was associated with the membrane pellet. This contrasted with other enzyme activities in the 25 000 g pellet that accounted for only 11% of total mPDC activity and less than 3% of six other tricarboxylic acid cycle enzymes measured (Table 2). Approximately 95% of the membrane marker enzyme activities succinate dehydrogenase and cytochrome *c* oxidase were associated with the 25 000 g membrane pellet. The remaining OGDC activity pelleted at 100 000 g , the PDC activity pelleted at 100 000–200 000 g and small, soluble tricarboxylic acid cycle enzymes were found in the supernatant of the 200 000 g centrifugation. The activity of OGDC, but not that of mPDC, increased 40–60% after the addition of pig E3 to assays of fractions in which these complexes were found (results not shown).

Purification of potato OGDC

In an effort to investigate further the composition and the function of OGDC from plants, this fractionation information (Table 2) was used to develop a scheme for the purification of

Table 3 Purification of OGDC from potato tuber mitochondria

Details of each purification step are outlined in the Materials and methods section. Results of a typical experiment are presented. OGDC activity measurements were all made in the presence of exogenous E3.

Fraction	Volume (ml)	Activity ($\mu\text{mol}/\text{min}$)	Protein (mg)	Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)	Recovery (%)
Freeze-thawed mitochondria	48	24.5	200	0.154	—
Matrix fraction	46	4.9	120	0.041	20
200 000 <i>g</i> pellet	4.5	4.8	38.5	0.125	20
MgCl ₂ pellet	0.7	4.3	4.44	0.96	17
EDTA/PEG/Mg ²⁺ pellet	0.6	3.6	0.79	4.55	15
NaCl/gel filtration	0.3	3.0	0.65	4.60	12
Membrane fraction	5.0	18.5	80	0.23	76
Sonication/50 000 <i>g</i> supernatant	13.0	6.2	10.5	0.59	25
200 000 <i>g</i> pellet	0.9	5.0	3.06	1.63	20
PEG/Mg ²⁺ pellet	0.3	3.7	0.95	3.90	15

OGDC from potato tuber mitochondria (Table 3). Initially, the soluble and membrane fractions from freeze-thawed purified mitochondria were separated by centrifugation at 25 000 *g* for 30 min.

The OGDC and mPDC activities in the supernatant were separated from the bulk of matrix protein by centrifugation at 200 000 *g* for 3 h. After resuspension of the pellet in OGDC buffer, the addition of 10 mM MgCl₂ caused the precipitation of a fraction that was removed by centrifugation at 20 000 *g* for 15 min. This precipitate contained over 90 % of the OGDC activity but less than 3 % of the mPDC activity present; it could be solubilized by the addition of 2 mM EDTA in OGDC buffer. This solubilized sample also contained the remainder of the cytochrome *c* oxidase activity; assays showed a low level of NADH-dependent O₂ consumption that was inhibited by 95 % on the addition of antimycin A or KCN (results not shown). The OGDC activity in this fraction remained soluble on the addition of 5 % (w/v) PEG-6000; after centrifugation to remove PEG-insoluble material, the OGDC activity could again be precipitated by the addition of 10 mM MgCl₂. This preparation had a specific activity of 4.55 $\mu\text{mol}/\text{min}$ per mg of protein (Table 3); no NADH oxidase or cytochrome *c* oxidase activity was detected (results not shown).

Washing the 25 000 *g* membrane pellet with NaCl at concentrations between 0 and 500 mM, adding EGTA at concentrations between 0 and 20 mM and altering the pH over the range 4.5–9.0 failed to removed more than 5–10 % of the remaining OGDC from the membrane fraction (results not shown). The addition of 5 mM CHAPS caused maximal release of OGDC (approx. 50 %), however, additions of more than 10 mM CHAPS caused the sedimentation of OGDC activity with the membranous material at 25 000 *g* (results not shown). After the addition of CHAPS, OGDC activity and the remaining mPDC activity could not be adequately separated (results not shown). After sonication and centrifugation for 30 min at 25 000 *g*, 30–35 % of the OGDC activity remained in the supernatant (Table 3). This fraction exhibited a 3-fold increase in OGDC specific activity over the initial membrane pellet. Subsequent MgCl₂/PEG fractionation and re-solubilization in the presence of EDTA produced a sample with a specific activity similar to that obtained from the soluble fraction (Table 3). Aliquots of OGDC (1.5–5 mg/ml protein) isolated from membrane or soluble fractions could be

stored for several months at -80°C with only 5–15 % loss in activity.

The OGDC activity of the purified complex was greatly dependent on the addition of exogenous E3. Pig E3 was routinely used for this purpose and its addition increased OGDC activity 5–10-fold, depending on the OGDC preparation. To determine whether potato E3 allowed a greater catalytic rate for the overall OGDC reaction, this enzyme was partly purified from the 200 000 *g* supernatant of the OGDC purification by a separation protocol with a MonoQ column by the method of Turner et al. [16]. At pH 6.8, the potato E3 was eluted from the MonoQ column at a KH₂PO₄ concentration of 220 mM (results not shown). Half-maximal activation of OGDC with the partly purified potato E3 was achieved with approximately one-third of the amount of pig E3 that was required for half-maximal activation. The E3 amount in these calculations was based on relative rates of lipoamide-dependent NADH oxidation or dihydrolipoamide-dependent NAD⁺ reduction by the two E3 enzymes (results not shown). The apparent V_{max} of OGDC was only 6–9 % higher in the presence of the partly purified potato E3 than in the presence of the commercially available pig E3 (results not shown). Consequently, pig E3 was used in all assays of OGDC presented. Poulsen and Wedding [11] performed their experiments on cauliflower OGDC in the presence of pig E3. They also confirmed that, although the cauliflower E3 had a higher affinity for the plant OGDC, there was no significant difference between the apparent V_{max} values for OGDC measured in the presence of the two different E3 enzymes.

The rate of NAD⁺ reduction by the OGDC preparation in the presence of other 2-oxo acids such as pyruvate, isovalerate and isohexanoate (1 mM) was less than 2 % of the 2-OG-dependent rate. K_m values (means $\pm$ S.E.M.) of the purified OGDC activity were 30 ± 4 μM for 2-OG, 6 ± 3 μM for TPP and 14 ± 4 μM for CoA, calculated by using double-reciprocal plots of data from initial-rate experiments ($n = 3$). AMP (1 mM) increased the activity of OGDC by 40–65 % at saturating 2-OG concentrations. These kinetic values and the effect of AMP addition are similar to published results for the cauliflower OGDC [13].

Incubation of the purified OGDC with 1 M NaCl/1 % (w/v) n-dodecyl- β -D-maltoside/25 mM Mops (pH 7.5) for 1 h at 4°C and separation on a Superose 6 gel-filtration column yielded a large complex that was eluted several ml after the void volume on this column (Figure 1). Immediately after separation, the eluted OGDC was completely dependent on added pig E3 for NAD⁺ reduction (results not shown) and had an activity of 4.6 $\mu\text{mol}/\text{min}$ per mg of protein (Table 3). Activity of the gel-filtered OGDC was lost progressively in storage, even at -80°C .

Comparison of the gel-filtration profile of OGDC with superimposed separations of other large mitochondrial complexes of known approximate molecular masses provides an estimate of the size of OGDC. Complex I of the respiratory chain from mitochondria has an approximate molecular mass of 1000 kDa [31] and can be identified in whole solubilized mitochondrial membrane separations by its NADH-dependent FeCN reduction activity and its characteristic protein component profile after separation by SDS/PAGE [27,31]. The mitochondrial PDC has an approximate molecular mass of 6000–8000 kDa [32–34] and we have purified this large complex from potato mitochondria [20]. Aliquots of purified mPDC or aliquots of total mitochondrial membrane solubilized in 1 % (w/v) n-dodecyl- β -D-maltoside were separated by Superose 6 gel filtration. Superimposition of the elution profile of PDC activity, the FeCN-reducing activity of complex I in whole membrane extracts and OGDC activity revealed that OGDC was eluted after mPDC but before complex I (Figure 1). Sedimentation velocity analysis of

Figure 1 Superimposition of gel-filtration sizing of OGDC, mPDC and NADH:ubiquinone oxidoreductase activities isolated from potato mitochondria

Activities of mPDC (■), OGDC (●) and NADH:ubiquinone oxidoreductase (complex I) (▲) were measured in eluted fractions as described in the Materials and methods section and are expressed as percentages of their peak activity. Enzymes were eluted at 0.3 ml/min from a Superose 6 column (5000–10 kDa separation range) in 25 mM Mops (pH 7.5)/100 mM NaCl/0.1% n-dodecyl- β -D-maltoside. Solutes voiding from this column were eluted at the end of fraction 0. Each fraction represents 1 ml of column eluate.

Figure 2 Separation by SDS/PAGE of purified OGDC and mPDC from potato mitochondria, and immunodetection of E3 subunits

Potato OGDC from the final PEG/MgCl₂ step (A), potato OGDC after treatment with NaCl and gel filtration (B) and purified potato mPDC (C) were separated by SDS/PAGE and duplicate gel lanes were either stained with Coomassie Blue (lanes 1) or transferred to nitrocellulose and incubated with polyclonal antibodies raised against pea E3 (lanes 2).

the gel-filtration-purified OGDC, in the absence of detergent, showed a single peak with a sedimentation coefficient at 20 °C of 25 ± 2 S. This confirms, along with the gel-filtration experiments, that the OGDC has a molecular mass of several thousand kilodaltons.

Protein gel electrophoresis of potato OGDC and immunoreaction with E3

SDS/PAGE of the MgCl₂/PEG-purified OGDC from potato revealed that the complex consisted of three major polypeptides with apparent molecular masses of 105, 50 and 48 kDa and a range of other polypeptides including a prominent 57 kDa protein (Figure 2A, lane 1). Polyclonal antibodies raised against the pea E3 protein [35] reacted with two protein bands at 57–58 kDa

(Figure 2A, lane 2). After the treatment with 1 M NaCl and gel filtration, the OGDC sample was completely dependent for activity on added E3; it appeared as a homogeneous preparation of the three major polypeptides (Figure 2B, lane 1) and very little reaction with E3 antibodies was observed (Figure 2B, lane 2). The mPDC purified from potato mitochondria [20] also contained E3 polypeptides at 57–58 kDa (Figure 2C, lane 1) and incubation with the pea E3 antibodies revealed a similar ratio of these two polypeptides (Figure 2C, lane 2) to that observed in the OGDC preparation (Figure 2A, lane 2). The exposure times of the immunoblots varied and were optimized for showing the intensities of the two bands in each lane rather than for comparisons between lanes.

N-terminal sequencing and identification of OGDC components

To identify the OGDC polypeptides, protein bands from SDS/PAGE-separated OGDC (Figure 2A) were electroblotted to PVDF membranes and excised; the N-terminal sequence of each was determined (Table 4).

The sequence of the 105 kDa protein shared 70% identity with the sequence near the N-terminus of the protein sequence predicted from an *Arabidopsis* open reading frame (ORF) (EMBL accession no. AL021684). The precursor protein predicted by this ORF has a calculated molecular mass of 110 kDa and shares 46–48% identity with 2-oxoglutarate dehydrogenase protein sequences from *Schizosaccharomyces pombe* (EMBL O74378), *Saccharomyces cerevisiae* (SwissProt P20967) and *Homo sapiens* (SwissProt Q022128).

The sequence from the 57 kDa protein was identical with the N-terminal sequence from a potato mitochondrial matrix protein (SwissProt P80503) reported by Jansch et al. [31] and also the N-terminus of a component of the purified potato PDC [20]. These were both identified as E3 on the basis of a greater than 90% identity with amino acid sequences derived from soya bean and pea cDNA sequences (EBML Q41219, SwissProt P31023).

The 50 kDa protein band yielded a single N-terminal sequence, whereas the 48 kDa protein band reproducibly yielded a very similar sequence, with Ala replacing the Ser residue at position 2 and an equal yield of Leu and Thr at position 7. All three sequences share 70% identity with a sequence near the N-terminus of a protein predicted from an *Arabidopsis* cDNA (Genbank AJ223803), an identical genomic ORF (Genbank AB010071) and an expressed sequence tag sequence (Genbank T46184). The predicted full-length proteins from *Arabidopsis* share 45–60% sequence identity with dihydrolipoamide succinyltransferase sequences from *Fugu rubripes* (SwissProt Q90512), *Rattus norvegicus* (SwissProt Q01295) and *S. cerevisiae* (SwissProt P19262). An N-terminal sequence from a 50 kDa *Arabidopsis* mitochondrial-membrane-associated protein matches the *Arabidopsis* predicted ORF and cDNA sequences (L. C. Packman and P. Dupree, personal communication) and shows the mature protein starting at an analogous position to the start of the N-terminal sequence from potato (Table 4). In addition, an N-terminal sequence of a 46 kDa protein (SwissProt PQ0778) from a purified preparation of mitochondrial complex I from *Vicia faba* shares a high degree of sequence identity with the plant OGDC-E2 sequences.

Succinylation of OGDC

To determine whether the 48 and 50 kDa E2 proteins of OGDC identified by N-terminal sequencing were functional succinyltransferases, the purified complex was incubated with [U-¹⁴C]-2-OG and TPP in the absence of CoA to succinylate the lipoyl

Table 4 N-terminal sequences of the 105, 57, 50 and 48 kDa potato mitochondrial OGDC subunits and comparison with similar plant sequences

Residues shown in bold indicate identity with the potato N-terminal sequences. Abbreviations: ORF, open reading frame from genome sequencing; NTS, N-terminal sequence; EST, expressed sequence tag (cDNA fragment). The low-level signals in the first round of degradation of the 50 and 48 kDa protein proteins (marked X) might be Cys residues on the basis of signal similarity to Cys degradations in proteins of known sequence, but these have not been confirmed by derivatization.

Details	Accession number	Amino acid sequence	Sequence identity
105 kDa NTS (this study)		AQAAPVPRPVXLSKKTDSFX	
<i>Arabidopsis thaliana</i> ORF	AL021684	ES AAPVPRPVPLSKLTDSFL	15/20
57 kDa NTS (this study)		ASGSDENDVVVIGGGPGGYV	
<i>Pisum sativum</i> cDNA	P31023	ASGSDENDVVIIGGGPGGYV	19/20
50 kDa NTS (this study)		XNSGDLVDVAVPYMGESIS	
48 kDa NTS (this study)		XANSGLVDVAVPYMGESIS	19/20
48 kDa NTS (this study)		XANSGLTVDAVVPYMGESIS	18/20
<i>A. thaliana</i> ORF/cDNA	AJ223803/AB010071	SSDSGDVVEAVVPHMGESIT	14/20
<i>A. thaliana</i> NTS	*	SSDSGDVVEAVVPHMGESIT	12/17
<i>A. thaliana</i> EST	T46184	SAETGDTVEAVVPHMGESIT	14/20
<i>V. faba</i> NTS	PQ0778	XXDNGDTVVAVVXL	6/11

* L. C. Packman and P. Dupree (personal communication).

Figure 3 Reversible succinylation of proteins in purified plant mitochondrial OGDC

OGDC was incubated as outlined in the Materials and methods section. [^{14}C]2-OG (0.2 mM) was added; after 200 s, 50 mM 2-OG and 2 mM CoA were added. Samples were taken at specified time points and the reaction was stopped by the addition of 10 mM *N*-ethylmaleimide and by dissociation in SDS/PAGE sample buffer. After the separation of OGDC proteins (30 μg per lane) by SDS/PAGE followed by staining with Coomassie Blue (lane 1), ^{14}C incorporation into OGDC polypeptides was revealed by autoradiography. Time points presented are 0 s (lane 2) and 200 s (lane 3) after the addition of 0.2 mM [^{14}C]2-OG and 200 s (lane 4) after the subsequent addition of 50 mM 2-OG and 2 mM CoA.

chains of proteins accepting succinyl groups from OGDC-E1. Autoradiographic detection of ^{14}C incorporation revealed labelling of both the 48 and 50 kDa protein bands (Figure 3). The subsequent addition of CoA and dilution of the ^{14}C -labelled substrate with unlabelled 2-OG resulted in a rapid loss of incorporation of ^{14}C in both protein bands.

DISCUSSION

In bacteria, 2-oxoacid dehydrogenase complexes are found in the soluble fraction of the cytoplasm [36,37]. From mammals, purification of these complexes was originally undertaken from the soluble fraction obtained after rehydration of freeze-dried purified mitochondrial samples [38]. Yields from bovine heart were later found to be markedly improved by the inclusion of a

non-ionic detergent, such as Triton X-100 [39]; subsequent work showed that both OGDC and PDC are associated with the inner mitochondrial membrane in mammals [40]. The differential membrane association of the 2-oxoacid dehydrogenases in potato mitochondria reported here has facilitated the purification of OGDC without contamination by mPDC, which was a common problem in earlier partial purifications of OGDC from plants [10,11].

The reason for this differential association is unclear; extension of this study beyond potato and with the use of various incubation conditions will be required to establish whether this is a common feature of the plant complexes. However, the previous reports of contamination with NADH oxidase of OGDC preparations [10,11] but not PDC preparations [20,21,41] suggest a tendency for the differential membrane association of OGDC in plants. Whether this membrane interaction is due to a general hydrophobicity of OGDC or due to a more specific interaction with a membrane component is not currently known in plants. The high degree of identity between a protein associated with complex I from *V. faba* [42] and the potato OGDC-E2 subunit raises the possibility that this subunit of complex I is in fact OGDC-E2. Further, a report on the purification of complex I from potato has also noted that a 49–50 kDa protein doublet was associated with the complex [27]. These molecular masses are nearly identical with those identified as the potato OGDC-E2 components in Figure 2. These observations are consistent with an association observed in mammalian mitochondria between a proportion of the total OGDC and complex I [43]. This association has been shown to be relatively weak, being easily dissociated by the addition of glycerol or detergent but not NaCl, and has been interpreted to be part of the metabolon organization of the tricarboxylic acid cycle [44]. A metabolon association of complex I and OGDC might offer explanations for the 2-OG dependent high O_2 consumption sustained by OGDC in potato mitochondria (Table 1). The activation of OGDC by AMP already suggests that OGDC conformations with varying V_{max} values exist [12–14]. One such conformation that has not been stabilized *in vitro* might be favoured through supramolecular interactions of OGDC with other mitochondrial matrix or membrane components that lead to the high rates of OGDC activity in intact mitochondria.

Table 5 Comparison of the N-terminal sequence regions of PDC-E2, PDC-E3BP and OGDC-E1 enzymes obtained by direct N-terminal sequencing or deduced from published nucleotide sequences

Hyphens represent spacing to allow alignment of the sequences. The PALSP/GTM motif and similar regions in OGDC-E1 sequences are shown in bold, as are the Pro and Gly residues of interest as outlined by Rice et al. [4].

Protein	Source	Accession number	N-terminal sequence
OGDC-E1	<i>A. thaliana</i>	AL021684	ESAAPVPRPVPLSKLTDS SFLDGTS --SVYL
	<i>H. sapiens</i>	Q02218	SAPV-----AAE PFLSGTS --SNYV
	<i>B. taurus</i>	G254064	TAPV-----AAE PFLSGTS --GNYV
	<i>S. cerevisiae</i>	P20967	IVGRGLA-----TTGTDNFLS-TSNATYID
PDC-E2	<i>A. thaliana</i>	AB010071	TGPISQTV----LAMP PALSPTM SHGNVV
	<i>H. sapiens</i>	P36957	SLP PP -----QKVPL PSLSPTM QAGTIA
	<i>S. cerevisiae</i>	P12695	ASY PE TI-----IGM PALSPTM TQGMIA
PDC-E3BP	<i>H. sapiens</i>	Q99783	GD PIK -----ILM PSLSPTM EEGNIV
	<i>S. cerevisiae</i>	P16451	AVKT-----FSM PAMSPTM EKGIV

Recently, the biochemical pathway responsible for the lipoylation of the E2 components in mPDC, OGDC and the branched-chain 2-oxoacid dehydrogenase complex in both *Escherichia coli* and mammals has been shown to involve the transfer of lipoic acid from the acyl carrier protein via an acyl lipoyl transferase to acetyl and succinyl transferases [45]. This acyl carrier protein has been shown to be an integral protein in complex I of the respiratory transport chain from mammals and yeast [46,47] and recently also from plants [48]. This metabolic link might form an additional basis for the association of OGDC components or indeed a proportion of the entire OGDC complex with complex I in mitochondria. It is not yet clear whether lipoyl attachment occurs only before or during assembly of the 2-oxoacid dehydrogenases, or whether 'running repairs' might occur to complexes after assembly and operation.

Because PDC and OGDC share the E3 content of plant mitochondria, their relative affinities for this subunit are potentially of importance in the operation of these two complexes in plant mitochondria. The immunological evidence (Figure 2) shows that the two E3 proteins from potato are associated with purified PDC and OGDC in a similar ratio, suggesting that these potential E3 isoenzymes are not complex-specific. However, there is a large difference in the amount of E3 associated with each complex. In our previous purification of PDC from potato mitochondria, a large proportion of E3 remained bound to the complex throughout the purification process, including during precipitation and gel filtration of the complex. Prominent proteins associated with mPDC were found to be E3 proteins; the addition of pig E3 did not increase mPDC activity significantly [20]. During the purification of OGDC, the E3 subunit is progressively lost from the complex: the final preparation is devoid of any 57–58 kDa proteins (Figure 2) and is completely dependent on the addition of E3 for OGDC activity. Similar weak associations of E3 have been noted for the plant glycine decarboxylase complex [16] and the mammalian branched-chain 2-oxoacid dehydrogenase complex [49].

One reason for this weak association might be related to the method by which E3 is attached to the OGDC complex. The predicted *Arabidopsis* OGDC-E2 sequence (Genbank AB010071), identified by similarity to the potato N-terminal sequences, lacks the E1/E3-binding motif (PX₁₇GXXGXXGRX-XXXX) that is found widely in acetyltransferases and succinyltransferases. Rice et al. [4] showed that the mammalian OGDC-E2 also lacks this motif and proposed that OGDC-E1 is

responsible for binding the E3 component to OGDC. They showed sequence similarity between the extreme N-terminus of OGDC-E1 and the corresponding regions of PDC-E2 and E3BP proposing that a 'lipoyl-domain-like' motif without a subsequent lysine for lipoyl attachment was present at the N-terminus of the mammalian OGDC-E1. The characteristic PALSP/GTM motif found in yeast and mammalian PDC-E2 and E3BP sequences was replaced with Pro-Phe-Leu-Ser-Gly-Thr-Ser in the mammalian OGDC-E1 sequence; a proline residue at position 3 or 4 and a glycine at position 20–21 were conserved in the mature protein sequence (Table 5). Recently, McCartney et al. [3] have presented direct evidence for the binding of E3 to the bovine OGDC via the extreme N-terminus of the OGDC-E1 subunit. Similar sequence features to those described by Rice et al. [4] are also present in the predicted *Arabidopsis* OGDC-E1 sequence shown in comparison with mammalian and yeast sequences in Table 5. Four Pro residues are present near the extreme N-terminus of the predicted mature *Arabidopsis* OGDC-E1 sequence and a SFLDGTS motif aligns with similar sequences across all those presented. The *Arabidopsis* OGDC-E1 sequence lacks the Gly residue at position 20–21, as also does the human OGDC-E1 (Table 5). The potato N-terminal sequence for OGDC-E1 shows conservation of at least three of the four Pro residues and contains the first two residues of the SFLDGTS motif at its extreme C-terminus (Table 4). Notably, both plant OGDC-E1 sequences contain an extended region of four to seven amino acids between the N-terminal Pro residue and the PALSP/GTM-like motif that is absent from mammalian and yeast OGDC-E1, E3BP and PDC-E2 sequences (Table 5). The possibility that these differences contribute to the weak binding of E3 by plant OGDC requires further investigation.

The membrane localization of OGDC, its potential interaction with complex I, and its weak association with E3, might all have direct implications for the operation of this enzyme in plant mitochondria, where it functions both in the oxidative tricarboxylic acid cycle and as a determinant of the 2-OG concentration that feeds the anaplerotic delivery of carbon skeletons for nitrogen assimilation in plants. The characterization of OGDC from a higher plant source presented here and the identification of plant genes encoding its constituent polypeptides provide the basis required for research into these issues.

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