Purification and partial characterization of spinach leaf glycerate kinase

Leszek A. Kleczkowski and Douglas D. Randall

Department of Biochemistry, University of Missouri, Columbia, MO 65211, USA

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Glycerate kinase from spinach leaves was purified to near homogeneity using PEG/$MgCl_2$ fractionation, ion exchange, molecular sieving and affinity chromatography. The purified enzyme is a monomer of M_r 40000, shows a pI-value of 4.8 and a broad pH optimum of 6.5–8.5 and is specific for D-isomer of glycerate. The high activity of crude enzyme (~ 150 μmol . h^{-1} . mg chl^{-1}) indicates that glycerate kinase does not limit the oxidative photosynthetic carbon cycle.

Glycerate kinase *Photorespiration* *Photosynthesis* *PEG fractionation* Spinacea oleracea *L*

1. INTRODUCTION

Glycerate kinase (GK) has been proposed to be the terminal enzyme in the oxidative photosynthetic carbon cycle [1–3]. This pathway accounts for a 10–50% reduction in net photosynthesis in many plant species [2,3]. There are two possible roles for GK: to direct carbon recovered from the oxidative photosynthetic carbon cycle back to the reductive photosynthetic carbon cycle and/or to provide precursors for sucrose synthesis [2–7]. Despite its critical position, GK is the least characterized photorespiratory enzyme and has not been purified from any plant tissue. This report describes a purification procedure of spinach GK and presents some molecular properties of the purified enzyme.

2. MATERIALS AND METHODS

2.1. *Extraction and purification of GK*

Spinach leaves were purchased from a local market. All purification procedures were performed at 0–4°C. Extraction medium contained 40 mM Tricine (pH 7.8), 2 mM $MgCl_2$, 1 mM EDTA, 2% insoluble polyvinylpyrolidine (acid-washed), 1 mM benzamidine, 1 mM ϵ-aminocaproic acid, 14 mM 2-mercaptoethanol and 5 μM leupeptin. The DEAE-cellulose, Sephadexes G-100 and G-75SF and the Affi-Gel Blue (Bio-Rad) columns were equilibrated and eluted with buffer A containing 20 mM Tricine (pH 7.8), 2 mM $MgCl_2$ and 14 mM 2-mercaptoethanol. Affinity matrix-dye ligand Green A (Amicon) column was equilibrated and eluted with buffer B containing 20 mM MOPS (pH 6.75), 2 mM $MgCl_2$ and 14 mM 2-mercaptoethanol.

Both native and dissociating polyacrylamide gel electrophoreses were done as in [8]. The proteins in polyacrylamide gels were detected by silver staining (Bio-Rad) and/or Coomassie Blue. Protein and chlorophyll concentrations were determined by the procedures in [9] and [10], respectively.

2.2. *Glycerate kinase assay*

The complete (1 ml) reaction mixture contained 100 mM Tricine (pH 7.8), 10 mM $MgCl_2$, 0.1 mM NADH, 10 mM ATP, 5 mM D,L-glycerate, 5 units of glyceraldehyde-3-phosphate dehydrogenase and 5 units of phosphoglycerate kinase. Lactate dehydrogenase (5 units) and pyruvate kinase (3.5 units) were sometimes used as coupling enzymes in

Abbreviations: GK, glycerate kinase; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate

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a reaction mixture of 100 mM Tricine (pH 7.8), 10 mM $MgCl_2$, 0.1 mM NADH, 5 mM ATP, 2.5 mM phosphoenolpyruvate, 60 mM KCl and 5 mM D,L-glycerate. Both coupling enzyme systems gave identical rates for glycerate kinase. Reactions were initiated by addition of glycerate. Assays were done at 25°C by monitoring NADH oxidation at A_{340} with a recording spectrophotometer. One unit of GK activity was defined as the amount of enzyme required to oxidize 1 μmol NADH/min under maximum rate conditions.

3. RESULTS

Spinach leaves (1300 g) were homogenized in a Waring blender for 30 s in the presence of 1200 ml extraction medium, and the homogenate squeezed through 4 layers of cheesecloth and 1 layer of Miracloth and centrifuged at 15000 × *g* for 20 min. The supernatant was fractionated using PEG/$MgCl_2$. Solid PEG 8000 (275 g/l) was added to the enzyme solution and equilibrated for 90 min followed by addition of $MgCl_2 \cdot 6\ H_2O$ (5 g/l). The suspension was further equilibrated for 30 min and then centrifuged at 15000 × *g* for 10 min. The pellet was discarded and 2-mercaptoethanol (1 ml/l) and PEG 8000 (250 g/l) were added followed by a 30 min equilibration. GK was precipitated by addition of $MgCl_2 \cdot 6\ H_2O$ (5 g/l), equilibrated for 30 min and the precipitated enzyme collected by centrifugation at 15000 × *g* for 10 min. The enzyme was redissolved in buffer A and insoluble material removed by centrifugation at 10000 × *g*. The PEG/$MgCl_2$-fractionated enzyme was chromatographed on a DEAE-cellulose column (1.5 × 20 cm), using a 500 ml linear gradient of 0–0.3 M NaCl. Gk eluted at about 0.12 M NaCl. The most active fractions were pooled, concentrated using a Diaflo PM10 filter (Amicon) and applied to a calibrated Sephadex G-75SF column (2.5 × 55 cm). The most active fractions from Sephadex G-75SF were pooled, chromatographed on an Affi-Gel Blue column (1.8 × 5.5 cm) and eluted with a 50 ml linear gradient of 0–3 mM ATP. The fractions with greatest GK activity eluted at about 1 mM ATP and were pooled, concentrated and desalted with medium B using a Diaflo PM10 filter, and applied to a second affinity matrix of Green A (1 × 3 cm column). Glycerate kinase activity was eluted with 1 mM ATP. The results of the purification procedure are summarized in table 1.

The final enzyme preparation exhibited one band on native PAGE when silver-stained (fig. 1, lane A). Under dissociating SDS–PAGE, a single band was observed at M_r 39500 when stained with Coomassie Blue (fig. 1, lane C), however with silver-stain minor contaminants could be detected (fig. 1, lane D). Only one band which coincided with activity, was observed when purified enzyme was analyzed by isoelectric focusing on agarose. Glycerate kinase could be eluted from both PAGE and agarose gels and its activity closely corresponded to the single stainable band on the gels. Chromatography of undissociated GK on calibrated Sephadex G-100 and G-75SF columns gave M_r values of 37500 and 40500, respectively (fig. 2). The M_r-values reported for native and SDS-treated enzyme indicate that the protein is a monomer of about 40000. The purified protein showed a single pI of 4.8 and a broad pH optimum of 6.5–8.5 (fig. 3). Using lactate dehydrogen-

Table 1

Purification summary of spinach leaf glycerate kinase

Fraction	Volume (ml)	Total units	Units/mg protein	Yield (%)	Enrichment
Extract					
crude	1640	1888	0.16	100	1
centrifuged	1545	1914	0.18	101	1
PEG/$MgCl_2$	96	1130	3.27	60	21
DEAE-cellulose	85	873	13.99	46	88
Sephadex G-75SF	18	599	31.61	32	199
Affi-Gel Blue	35	322	60.93	17	383
Green A	6	162	203.0	8.6	1274

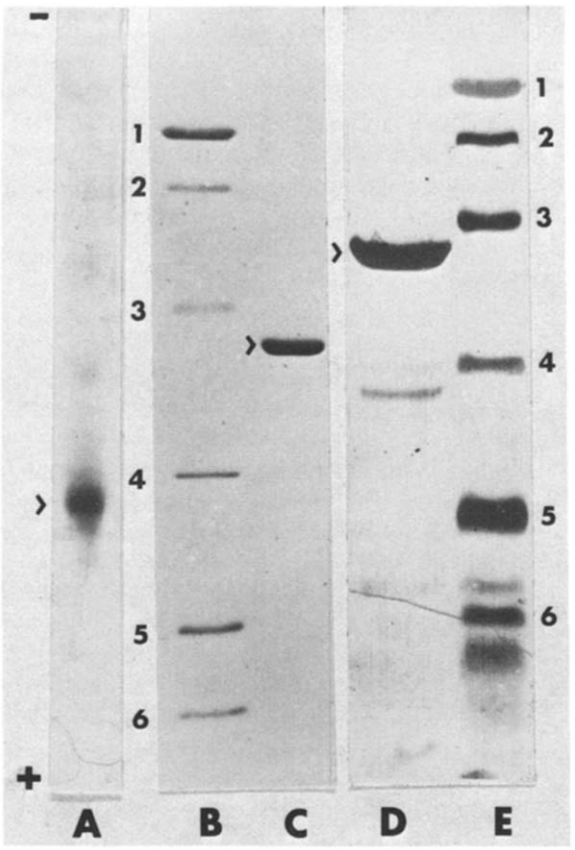

Fig. 1. Polyacrylamide gel electrophoresis of purified glycerate kinase. (A) Electrophoresis under non-dissociating conditions. A 3-μg sample of the enzyme from Green A column was loaded onto 7.5–12.5% gradient PAGE. Gel was stained with silver reagent (Bio-Rad). (B–E) Electrophoresis under dissociating conditions. The purified enzyme was mixed with an equal volume of the solution of 6 M urea, 4% 2-mercaptoethanol and 4% SDS, heated at 80°C for 30 min and applied to a 12.5% polyacrylamide gel; lane B, 20 μg of standard proteins; lane C, 6 μg of purified GK; both B and C lanes were stained with Coomassie blue; lane D, 12 μg of purified GK; lane E, 20 μg of standard proteins. Both D and E lanes were stained with silver reagent (Bio-Rad). Standard proteins kit (Bio-Rad) contained: phosphorylase *b* (1) - M_r 92 500; BSA (2) – 66 200; ovalbumin (3) - M_r 45 000; carbonic anhydrase (4) - M_r 31 000; soybean trypsin inhibitor (5) - M_r 21 500 and lysozyme (6) - M_r 14 400. The bands corresponding to glycerate kinase are indicated by arrows.

ase/pyruvate kinase as coupling enzymes, the apparent K_m for D-glycerate was 0.285 mM. Activity with L-glycerate as substrate is due to D-glycerate

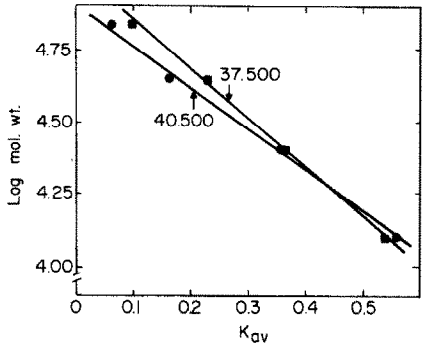

Fig. 2. Estimation of M_r of glycerate kinase by gel filtration chromatography. Sephadex G-75SF and Sephadex G-100 columns (2.5 × 55 cm and 2.5 × 47 cm, respectively) were calibrated with standard proteins from Boehringer Mannheim: cytochrome C (M_r 12 500); chymotrypsinogen (M_r 25 000); albumin (M_r 45 000) and albumin (M_r 68 000). Exclusion volumes were determined with Dextran Blue. The flow rates for Sephadex G-75SF and Sephadex G-100 were 18 and 15 ml/h, respectively. Arrows indicate that K_{av}-values for glycerate kinase and corresponding M_r-estimations. Sephadex G-75SF (●), Sephadex G-100 (■).

contamination of L-glycerate. In contrast to the highly purified enzyme obtained from a Green A column, the partially purified GK was unstable when maintained at low protein concentration (<0.2 mg/ml) at 0–4°C, losing 40% or more of its activity during a 24-h period. The purified enzyme

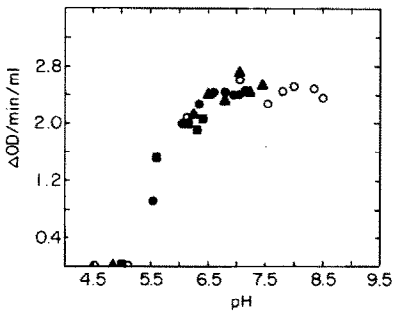

Fig. 3. The pH dependency of purified glycerate kinase. Four different buffers were used at 100 mM final concentration: Tricine (○), PIPES (●), MES (■) and MOPS (▲). Other components (PGAPK/GAP DH system) were essentially as described in section 2. Each point represents the average of two assays. The pH-values shown on the graph were obtained after completion of each assay.

was totally inactive after overnight storage in a freezer unless BSA (3 mg/ml) was added.

4. DISCUSSION

GK was purified to a specific activity of 203 units/mg protein with a 1250-fold enrichment. The native enzyme appears to be a monomer of M_r 40 000 with a broad pH optimum of 6.5–8.5 and a pI-value of 4.8. During the purification procedure the glycerate and ATP-dependent activity was associated exclusively with single activity peaks suggesting that there is only one form of GK in spinach leaves. On the other hand, instability of partially purified enzyme at low protein concentration (0–4°C) and stability of the purified GK at the same conditions does not eliminate a possible labile isozyme being lost during purification.

The specific activities of GK in crude homogenates (150 μmol . h^{-1} . mg chl^{-1}) were considerably higher than those obtained for spinach enzyme in [11] (11.6 μmol . h^{-1} . mg chl^{-1})and in [6] (40 μmol . h^{-1} . mg chl^{-1}) and 1/4 of the unusually high activity (576 μmol . h^{-1} . mg chl^{-1}) reported in [5] where a ^{14}C-assay was used. Our activity is similar to that reported for another C_3 plant, wheat (100 μmol . h^{-1} . mg chl^{-1}) [12]. Authors in [6] postulated that the level of glycerate kinase appeared to be insufficient to permit the high rate of carbon flow attributed to the glycolate pathway (oxidative photosynthetic carbon cycle) in vivo. The apparent diversity in reported rates is probably due to the different methods of preparation of crude samples and possibly to different methods of assay. The rates of GK activity in crude extracts reported here indicate that glycerate kinase is not a serious bottleneck in the oxidative photosynthetic carbon cycle as suggested in [6].

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