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SUBSTRATE STEREOSPECIFICITY OF LEAF GLYCERATE KINASE FROM C_3 AND C_4 PLANTS

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Key Word Index—*Zea mays*, Graminane, *Spinacia oleracea*; Chenopodiaceae, maize, spinach; substrate specificity, glycerate carrier, photorespiration, photosynthesis

Abstract—Glycerate kinase (EC 2.7.1.31) purified from maize (*Zea mays*) leaves showed apparent rates with both D- and L-stereoisomers of commercially available glycerate. The apparent K_ms for D- and L-glycerate were 0.12 and 1.5 mM, respectively. The V_{max} with D-glycerate was about 40% higher than with the L-stereoisomer. On the other hand, the enzyme was inactive with L-glycerate made enzymically from hydroxypyruvate (by lactate dehydrogenase), while it did react with the hydroxypyruvate-derived D-glycerate (by hydroxypyruvate reductase). Exhaustive phosphorylation of glycerate by glycerate kinase resulted in the total utilization of D-glycerate, whereas commercial L-glycerate was utilized only in 5–8%. Analogous results were obtained for purified glycerate kinase from spinach (*Spinacia oleracea*) leaves and for the enzyme from crude leaf preparations of maize and spinach. The data suggest that the apparent rates of glycerate kinase with commercial L-glycerate result from a small contamination of this compound by the D-stereoisomer. This finding is discussed with respect to previous reports on substrate specificity of leaf glycerate kinase and a membrane-bound glycerate-transporting carrier from chloroplasts.

INTRODUCTION

Leaf glycerate kinase (GK) catalyses the apparently irreversible reaction: glycerate + ATP $\xrightarrow{Mg^{2+}}$ 3-phosphoglycerate (3-PGA) + ADP [1–3]. The enzyme is localized exclusively in chloroplasts of mesophyll cells in both C_3 and C_4 plants [1, 4]. In the former group of plants, GK activity is intimately associated with the operation of the oxidative photosynthetic carbon pathway (photorespiration) [5, 6] and utilizes glycerate produced by hydroxypyruvate reductase (HPR) from hydroxypyruvate, which in turn is derived from phosphoglycolate, the first product of the photorespiratory cycle. In C_4 species, which show low, if any, rates of photorespiratory CO_2-evolution [6, 7], GK is probably linked to a 3-PGA phosphatase activity, and utilizes glycerate derived from the reductive photosynthetic carbon pathway (Calvin cycle) [3, 8, 9]. Both HPR and 3-PGA phosphatase form a D-stereoform of glycerate as a product [6, 10–12], and thus D-glycerate is the natural substrate for GK.

Studies with purified or partially purified GK from leaves of spinach and rye (C_3 plants) have established that the enzyme apparently does not show absolute stereospecificity with respect to glycerate and may react with both D- and L-forms of this compound [1, 2, 13, 14]. This is surprising since enzymes generally exhibit absolute stereospecificity when they act upon substances containing asymmetric centres [15, 16]. Recent data from our own laboratory [2] have indicated that the reactivity of GK with commercially available L-glycerate may be due to contamination of this compound by small amounts of its D-stereoisomer. The strongest evidence in favour of this presumption came from the fact that purified homogeneous spinach GK phosphorylated less than 7% of the L-glycerate supplied to the assay mixture, while the D-form was utilized to about 100% [2]. However, similar studies carried out by Chaguturu [14] with partially purified spinach GK indicated a full utilization of both stereoisomers of commercial glycerate. Thus, the possibility existed that leaves contain two isozymes of GK which differ in substrate specificity, and which might be resolved by the purification scheme reported for the homogeneous GK [2].

In the present investigation, we re-examined the substrate specificity of plant GK by studying the D- and L-glycerate-dependent rates of purified and crude leaf GKs from C_3 (spinach) and C_4 (maize) plants. Both D- and L-glycerate were prepared enzymically from hydroxypyruvate and their effects were compared to those of commercial compounds. The results establish that leaves contain no kinase capable of the utilization of L-glycerate.

RESULTS AND DISCUSSION

The substrate stereospecificity of GK was studied using three different sets of coupling enzymes to link formation of products of GK to NADH oxidation (Fig. 1).

Abbreviations: EL, enolase; GAP-DH, glyceraldehyde phosphate dehydrogenase; GK, glycerate kinase, HPR, hydroxypyruvate reductase, LDH, lactate dehydrogenase, PGA, phosphoglycerate, PGA-PK, phosphoglycerate phosphokinase; PGM, phosphoglyceromutase; PK, pyruvate kinase.

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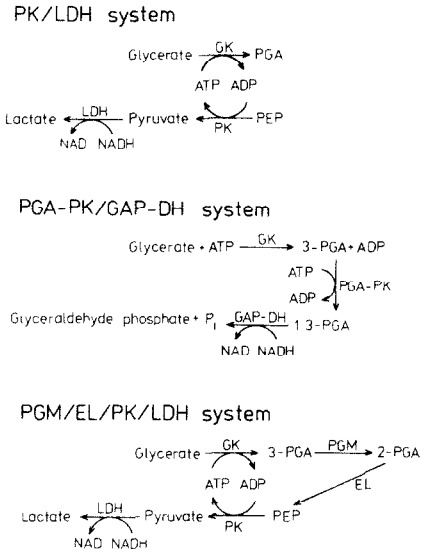

Fig 1 An outline of assays of glycerate kinase with the use of coupling enzymes

Assays containing pyruvate kinase and lactate dehydrogenase (PK–LDH system) were designed to monitor ADP formation, those with phosphoglycerate phosphokinase and glyceraldehyde phosphate dehydrogenase (PGA-PK–GAP-DH system) to measure 3-PGA production, and those containing phosphoglyceromutase, enolase, PK and LDH (PGM–EL–PK–LDH system) to follow the formation of both 3-PGA and ADP All three assay systems were found to be effective in monitoring rates of GK activity with commercial (from Sigma) D- and L-glycerate, and this was true for either purified or crude leaf GK from maize and spinach However, in experiments intended to evaluate the extent of consumption of both D- and L-glycerate (Table 1), the latter was phosphorylated by only some 58%, while the utilization of the D- form was close to 100% These results strongly indicated that the apparent reactivity with commercial L-glycerate was due to some impurity, most probably that of D-glycerate. In previous studies on purified spinach GK, under conditions optimized for a very accurate assay of glycerate consumption, L-glycerate was utilized to about 6 7% [2]

There is a fundamental discrepancy between our results and those of Chaguturu [14], who claimed that partially purified spinach GK could completely consume commercial L-glycerate (also from Sigma) In our studies, in several repetitions of the L-glycerate-dependent assays, the utilization of this compound was always less than 10% of that of D-glycerate (e g Table 1) Since we used either crude leaf or purified spinach GK, our results are incompatible with the presence of an L-glycerate-utilizing kinase in this species

When the kinetic properties of purified maize GK were studied using Lineweaver–Burke reciprocal plots (Fig 2), the K_m value for D-glycerate was more than 10-fold lower than that for the L-stereoisomer (0 12 and 1 5 mM, respectively), while the V_{max} with D-glycerate was only about 40% higher than with the L-form The points obtained with L-glycerate were subsequently adjusted for a supposed 6.7% contamination of L-glycerate by the D-form [2] The adjusted points fitted quite accurately the line obtained with D-glycerate alone (Fig 2) and thus strongly indicated that the apparent rates with L-glycerate were actually due to the D-glycerate impurity This was further supported by the fact that purified maize GK showed no reactivity with hydroxypyruvate-derived L-glycerate (made by LDH [10, 17]), while it did react with hydroxypyruvate-derived D-glycerate (made by HPR [10, 11, 17]) In both cases the reaction mixture contained 5 mM ATP, 10 mM $MgCl_2$, 0 03–0 10 mM glycer-

Table 1 Exhaustive phosphorylation of commercial D- and L-stereoisomers of glycerate by leaf glycerate kinase

	Stereoisomer of glycerate	Utilization of glycerate (%)	Coupled enzymes
Purified maize GK	D	92–97	A*, B†
	L	7	A
Crude maize GK	D	97	C‡
	L	5	C
Purified spinach GK	D	90–100	A, B, C
	L	6–7	A, B, C
Crude spinach GK	D	102	C
	L	8	C

Assays (except for purified spinach GK) contained, in a given coupling enzyme system, 0 01–0 03 unit of GK, 5 mM ATP, 10 mM $MgCl_2$ and 0 04–0 17 mM of either D- or L-glycerate The respective reaction mixtures for purified spinach GK contained 1 unit of GK, 1 mM ATP, 2 mM $MgCl_2$ and 0 164 mM of either D- or L-glycerate [2] Reactions were initiated with glycerate and monitored till completion Control assays containing all components but glycerate were always carried out to correct for the non-specific oxidation of NADH

*PK–LDH assay system

†PGM–EL–PK–LDH assay system

‡PGA-PK–GAP-DH assay system

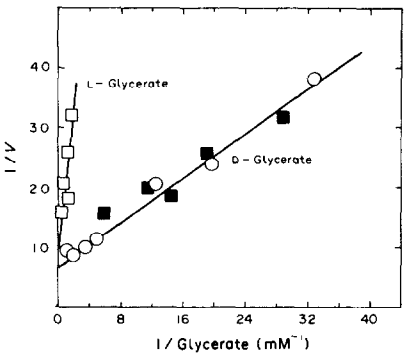

Fig. 2. Kinetics of purified maize leaf glycerate kinase with commercial D- and L-stereoisomers of glycerate. Assays were carried out using 0.02 unit of GK in the PGA-PK–GAP-DH coupling enzyme system Concentrations of ATP and $MgCl_2$ were fixed at 3 0 and 5.0 mM, respectively. Solid squares represent points obtained after adjustment for 6.7% D-glycerate impurity in stocks of L-glycerate

ate, 0.01 unit of GK and the rest of the components of either the PK-LDH or the PGA-PK–GAP-DH system. The absolute discrimination between the enzymically-made D- and L-glycerate was also demonstrated for purified and crude leaf spinach GK (using the same conditions). These data, together with the results presented in Table 1, indicate that leaves of both C_3 and C_4 species contain no kinase which can utilize the L-form of glycerate

Kinetic constants obtained in the present study for maize GK (with commercial glycerate) were fairly similar to those previously found for partially purified GKs from rye and spinach leaves [1, 14] (Table 2). Of particular interest are the values calculated for the ratios of K_m s and V_{max}s determined with D- and L-glycerate. Assuming that L-glycerate does not serve as a substrate for GK, a V_{max} with D-glycerate should theoretically be exactly the same as that with the D-glycerate-contaminated L-stereoform, while the K_ms ratio should correspond to the level of the contamination. This assumption, however, does not take into consideration a possible inhibitory effect of L-glycerate. The presence of the very large excess of L-glycerate over the D-form in stocks of the former compound (about 15:1 [2]) is most probably responsible for the lower V_{max} found for GK with commercial L-glycerate (Table 2) It should be pointed out that in studies by Chaguturu [14], a V_{max} value determined with D-glycerate was the same as that with D,L-glycerate (a 1 1 racemic mixture), which suggests that the inhibition by the L-form becomes apparent only at high L-glycerate/D-glycerate ratios. The inhibitory effect of L-glycerate implies that GK does have some (although very low) affinity for this compound and can bind a portion of the L-form structure even though the fit is not such as to allow a reaction to take place. Concerning the K_ms ratios, the values of 0 07–0.11 (Table 2) rather accurately reflect the level of D-glycerate impurity in stocks of commercial L-glycerate (5–8%, Table 1), and they would probably be even more accurate if an adjustment for L-glycerate inhibition were made (K_m for commercial L-glycerate would increase, resulting in a lower K_ms ratio).

The lack of absolute discrimination between both stereoforms of commercial glycerate was also previously observed for a chloroplast membrane-bound carrier, transporting glycerate across the inner membrane into the stroma [18–20]. In studies by Edwards and Walker [19], the addition of either D- or L-glycerate to illuminated wheat chloroplasts caused a substantial O_2-evolution, indicating that both forms could be transported into the chloroplasts, and that they could be further metabolized to triose phosphates, utilizing ATP and NADPH generated by the light reactions of photosynthesis. Kinetic constants calculated from these studies (Table 2) reflect the properties of a rate limiting step of the glycerate to triose phosphate conversion—most probably those of the glycerate carrier [18, 19] or, perhaps, those of GK or some other enzyme of glycerate metabolism through the Calvin cycle [20, 21]. Regardless of the nature of such a limiting step, the values calculated for V_{max}s and K_ms with both forms of commercial glycerate (Table 2) show the same tendency as observed for GK, i.e. relatively small difference between V_{max} values and a strong apparent preference for D-glycerate (low K_ms ratio) Because of the very complex and indirect assay system, monitoring glycerate-dependent rates of O_2-evolution from intact chloroplasts, it is probably not surprising that the constants (especially the ratio of K_ms) differ from those calculated for GK If these differences, however, were to be taken to represent the actual reactivity with L-glycerate, the activity in question would have to belong to the glycerate carrier which precedes the GK step.

In the light of the results of the present investigation, as well as in the previous report on substrate specificity of purified spinach GK [2], we believe that most, if not all,

Table 2. Apparent kinetic constants of leaf glycerate kinase and the glycerate carrier obtained with the use of commercial D- and L-glycerate

	K_m(mM)			
	D-form	L-form	K_m(D)/K_m(L)	V_{max}(D)/V_{max}(L)
Maize GK	0.12	1 5	0.08	1.4
Spinach GK [14]	0.09	1.33	0.07	1 9
Rye GK [1]	0.18	1 59	0 11	1 9
Wheat glycerate carrier [19]	0.3*	1.5*	0 20*	2.0*

In all studies hemicalcium salts of D- and L-glycerate from Sigma were utilized.

*Kinetic constants were determined indirectly based on measurements of glycerate-dependent O_2-evolution from illuminated chloroplasts in the absence of bicarbonate.

of the apparent effects of commercial L-glycerate are related to its contamination by low levels of D-glycerate. Were the L-form a true alternate substrate for GK this would lead to serious reconsiderations of our knowledge of stereospecificity of several enzymes, not only GK. The fact that commercial L-glycerate was previously found to serve as a substrate for GK in assays using the PGA-PK–GAP-DH system [1, 2, 13, 14], and that it induced some photosynthetic O_2-evolution in the absence of bicarbonate in chloroplasts [19] led to the suggestion [14, 19] that L-glycerate was not only recognized by GK but also that the L-forms of 3-PGA and 1,3-PGA could serve as substrates for PGA-PK and GAP-DH, respectively Rates of purified spinach GK with commercial L-glycerate could also be monitored using PGM-EL–PK-LDH assay system [2], thus suggesting reactivity of PGM and EL with L-3-PGA and L-2-PGA, respectively To our knowledge, at least GAP-DH and EL have been shown to utilize exclusively the D-stereoforms of their substrates [22, 23], which is incompatible with the apparent reactivities of these enzymes in the coupled assays of GK with commercial L-glycerate (e.g Table 1), and which further supports our contention that the observed rates are due to the D-glycerate impurity

EXPERIMENTAL

Plant material Maize (*Zea mays* L) seedlings were grown as described in ref [8] Spinach leaves were purchased in a local grocery store

Reagents D- and L-Glycerate as well as GAP-DH, PK, LDH, PGM (all from rabbit muscle), PGA-PK, EL (both from yeast) and HPR (spinach) were from Sigma, U S A The HPR was further purified to homogeneity as described in ref [24] Phosphoenolpyruvate, ATP and NADH were from P-L Biochemicals

Extraction of crude leaf glycerate kinase Leaf extracts of maize and spinach were prepared as described in ref [24] Samples of extracts were desalted on small Sephadex G-25 column Prior to assays, 100 mM 2-mercaptoethanol was added to the desalted maize preparations to assure optimal activity of GK (the enzyme is thiol-activated in maize and some other C_4 plants [8, 9])

Purification of leaf glycerate kinase Maize GK was purified as described in ref [3] The enzyme was at least 60% pure as found by electrophoresis in dissociating conditions [3] Spinach GK, purified as described in ref [2], was essentially homogeneous as determined by electrophoresis in dissociating conditions [2] and by western immunoblots using rabbit antibodies prepared against this protein [3, 9]

Enzymic production of D- *and* L-*glycerate* D- and L-Glycerate were produced enzymically from hydroxypyruvate as products of the reactions catalysed by HPR and LDH, respectively [10, 11, 17] Reaction mixtures contained, in 1 0 ml, 100 mM Tricine (pH 7 8), 0 3 mM NADH, 0 08–0 24 mM hydroxypyruvate and two units of either HPR or LDH Reactions were initiated with hydroxypyruvate and monitored spectrophotometrically at 340 nm (25°) till the exhaustion of hydroxypyruvate For both HPR and LDH, the amount of oxidized NADH was stoichiometrically identical to that of hydroxypyruvate present in the reaction mixture at the beginning of assays After completion of reactions, mixtures were boiled at 100 for 15 min and centrifuged to remove denatured proteins Supernatants were kept frozen at −20°

Assay of glycerate kinase Assays of GK were carried out spectrophotometrically using three different sets of coupling enzymes to link 3-PGA and/or ADP formation to NADH oxidation monitored at 340 nm [2, 3] (see Fig 1)

(a) Pyruvate kinase–lactate dehydrogenase system The assay contained, in 1.0 ml, 100 mM Tricine (pH 7 8), 1 mM phosphoenolpyruvate, 60 mM KCl, 0 2 mM NADH, five units each of PK and LDH, varying amounts of GK and, unless otherwise indicated, 5 mM ATP, 10 mM $MgCl_2$, and 5 mM glycerate

(b) Phosphoglycerate phosphokinase/glyceraldehyde phosphate dehydrogenase system The assay contained, in 1 0 ml, 100 mM Tricine (pH 7 8), 0 2 mM NADH, five units each of PGA-PK and GAP-DH, varying amounts of GK and, unless otherwise indicated, 5 mM ATP, 10 mM $MgCl_2$ and 5 mM glycerate

(c) Phosphoglyceromutase–enolase–pyruvate kinase–lactate dehydrogenase system The assay contained, in 1 0 ml, 100 mM Tricine (pH 7 8) 5 mM ATP, 10 mM $MgCl_2$, 0 1 mM glycerate, 0 25 mM NADH, 60 mM KCl, 0 02 unit of GK and in excess of six units each of PGM, EL, PK and LDH

In all three assay systems described, the reaction was initiated by addition of glycerate One unit of GK activity was defined as amount of the enzyme required to oxidize 1 μmol NADH/min under assay conditions of 5 mM ATP, 10 mM $MgCl_2$ and 5 mM D-glycerate, using the PK–LDH or PGA-PK-GAP-DH coupling enzyme systems All coupling enzymes were desalted on small Sephadex G-25 columns

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