

# Variations in $K_m(\text{CO}_2)$ of Ribulose-1,5-bisphosphate Carboxylase among Grasses

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## ABSTRACT

A survey of the  $K_m(\text{CO}_2)$  values of ribulose-1,5-bisphosphate carboxylase from 60 grass species shows that enzyme from  $C_3$  grasses consistently exhibits lower  $K_m(\text{CO}_2)$  than does that from  $C_4$  grasses. Systematically ordered variation in  $K_m(\text{CO}_2)$  of ribulose-1,5-bisphosphate carboxylases from  $C_3$  and  $C_4$  grasses is also apparent and, among  $C_4$  grasses, this shows some correlation with  $C_4$  types.

RuBP<sup>3</sup> carboxylase (EC 4.1.1. 39) is the fundamental carboxylating enzyme of photosynthesis. Comparisons among reported  $K_m(\text{CO}_2)$  values for this enzyme suggest that the  $C_3$  and  $C_4$  photosynthetic pathways may be distinguishable in terms of substrate affinity for  $\text{CO}_2$  (2, 6, 15, 22); however, published figures are not reliably interpretable because  $K_m(\text{CO}_2)$  estimations may depend upon the assay conditions (4, 26). As part of a comparative and systematic survey of RuBP carboxylase in grasses, an attempt has been made here to discover the extent of  $K_m(\text{CO}_2)$  variation, and whether it primarily reflects different photosynthetic pathways, taxonomic relationships, or ecology. Concentration has initially been on grasses (Poaceae), a family whose taxonomic relationships have been thoroughly studied and which incorporates both  $C_3$  and  $C_4$  plants, including different  $C_4$  types (8–11).

## MATERIALS AND METHODS

**Plant Material.** Plants listed in Table I were grown from seeds or collected from the field, and their identities were conscientiously checked with reference to appropriate regional floristic works.

**Enzyme Preparation and Assay.** RuBP carboxylase was extracted in 100 mM Bicine Buffer (pH 8.0), containing 25 mM  $\text{MgCl}_2$  and 1 mM DTT, and partially purified by elution through Sephadex G-25 in the same buffer. The enzyme was preactivated in 5 mM  $\text{NaHCO}_3$  and then assayed by measuring the fixation of [<sup>14</sup>C]bicarbonate (2). The reaction mixture containing 100 mM Bicine and 25 mM  $\text{MgCl}_2$  (pH 8.0) was prepared  $\text{CO}_2$ -free and flushed with  $\text{N}_2$ . Assays (total volume, 400  $\mu\text{l}$ ) were performed in 1-ml stoppered vials (Pierce Reacti-vials No. 13221) which had been flushed with  $\text{N}_2$ . Reaction was started by injection of 5  $\mu\text{l}$  preactivated enzyme and stopped after 1 min at 25 C by injection of 0.2 ml 2 N  $\text{HCOOH}$ . Bicarbonate concentration ranged from 0.4 to 16.5 mM, with RuBP fixed at 0.5 mM. The bicarbonate introduced into the assay solution with the enzyme aliquot was

taken into consideration when calculating  $\text{HCO}_3^-$  concentration and specific radioactivity. To minimize this correction for unlabeled  $\text{HCO}_3^-$ , only 5  $\mu\text{l}$  of the extract was used for assay, and  $\text{NaHCO}_3$  used for activation was limited to 5 mM. The possibility that full activation may not have been reached in extracts and that this may have produced variations in  $K_m(\text{CO}_2)$  values was checked by using 10 mM  $\text{NaHCO}_3$  in some preactivation conditions. This had no detectable effect on the  $K_m(\text{CO}_2)$  values of RuBP carboxylase from either  $C_3$  or  $C_4$  plants; hence, 5 mM was chosen as the activation level for all assays reported here. The  $K_m$  values were statistically calculated using Wilkinson's method (30). The  $\text{CO}_2$  concentration then was calculated from the pH and  $\text{HCO}_3^-$  concentration using the Henderson-Hasselbach equation and the pK value of 6.37 at 25 C (27).

## RESULTS AND DISCUSSION

The  $K_m(\text{CO}_2)$  values of RuBP carboxylases extracted from 60 grass species, representing all the main taxonomic groups and including all the known types of  $C_4$  plants, have been determined, i.e. 35  $C_3$ , 24  $C_4$  (12 NADP-ME, 7 PCK, and 5 NAD-ME), and one  $C_3$ - $C_4$  intermediate (8–11). The results are summarized in Table I, where the sample of grasses is arranged according to the best available information on taxonomic relationships above generic level, regarding both the contents of the major groupings and tribes, and (in so far as this can be achieved in a linear arrangement) the sequence of their presentation (16, 28). The  $C_3$  grasses exhibit lower  $K_m(\text{CO}_2)$  values, ranging from 13 to 26  $\mu\text{M}$   $\text{CO}_2$ , than do their  $C_4$  counterparts, where the values vary from 28 to 63  $\mu\text{M}$   $\text{CO}_2$ . Significantly, the  $C_3$ / $C_4$  distinction in terms of  $K_m(\text{CO}_2)$  holds good within the eu-panicoid assemblage where genera, which, in the context of the family as a whole, are taxonomically very closely related, have yielded low values (13 to 18  $\mu\text{M}$   $\text{CO}_2$ ) or high values (28 to 63  $\mu\text{M}$   $\text{CO}_2$ ) strictly in accord with the difference in photosynthetic pathway. Likewise, *Triraphis mollis*, a  $C_4$  danthonioid, has yielded a higher  $K_m(\text{CO}_2)$  (39  $\mu\text{M}$   $\text{CO}_2$ ) than its  $C_3$  relatives, *Cortaderia selloana* (14  $\mu\text{M}$   $\text{CO}_2$ ) and *Danthonia pallida* (19  $\mu\text{M}$   $\text{CO}_2$ ). Evidently, variation in  $K_m(\text{CO}_2)$  is primarily associated with the distinction between  $C_3$  and  $C_4$  photosynthetic pathways, and questions of taxonomic relatedness even at subfamily level are largely overridden by this consideration. If the taxonomic groups have any phylogenetic import, it must be concluded that grass RuBP carboxylases have shown remarkable flexibility regarding evolutionary modification of their kinetic properties.

There is no obvious correlation between the  $K_m(\text{CO}_2)$  of the enzyme and the natural habitats of grass species. For example, species from sand dunes, such as *Festuca littoralis*, *Zoysia macrantha* and *Spinifex hirsutus*, species from alpine regions (1500 m), e.g. *Poa hiemata*, and grasses from aquatic habitats, e.g. *Phragmites australis* and *Oryza sativa*, exhibit  $K_m(\text{CO}_2)$  values in line with their photosynthetic pathways. Although our sample covers dif-

<sup>3</sup> Abbreviations: RuBP, ribulose 1,5-bisphosphate; NADP-ME, NADP-malic enzyme; NAD-ME, NAD-malic enzyme; PCK, phosphoenolpyruvate carboxylase.

Table 1.  $K_m(\text{CO}_2)$  of RuBP Carboxylase from Grasses

$C_4$  species are indicated by bold face, and those with an asterisk have been biochemically determined to be PCK, NAD-ME, or NADP-ME types (8-10). The remainder are determined by anatomical criteria (11).

Species	$K_m(\text{CO}_2)$ $\mu\text{M}$	Species	$K_m(\text{CO}_2)$
Bamboos		<i>Triraphis mollis</i> * (PCK)	39 ± 5
<i>Arundinaria</i> sp.	26 ± 5	Arundineae	
Oryzoids		<i>Arundo donax</i>	16 ± 3
<i>Oryza sativa</i> cv Baru	17 ± 2	<i>Phragmites australis</i>	20 ± 4
<i>Oryza sativa</i> cv Calrose	17 ± 3	Doubtful affinities	
Pooideae		<i>Microlaena stipoides</i>	21 ± 4
Triticeae		<i>Tetrarrhena juncea</i>	20 ± 2
<i>Hordeum vulgare</i>	15 ± 3	Chloridoids	
<i>Secale cereale</i>	13 ± 2	<i>Chloris truncata</i> (PCK)	34 ± 2
<i>Triticum aestivum</i>	15 ± 2	<i>Sporobolus virginicus</i> (PCK)	41 ± 7
Bromeae		<i>Zoysia macrantha</i> (PCK)	34 ± 4
<i>Bromus arenarius</i>	16 ± 2	<i>Eleusine coracana</i> (NAD-ME)	41 ± 5
<i>Bromus unioloides</i>	17 ± 3	<i>Eragrostis chloromelas</i> (NAD-ME)	46 ± 3
Agrostideae		Panicoids <i>sensu lato</i>	
<i>Anthoxanthum odoratum</i>	20 ± 1	Eu-panicoids	
<i>Deyeuxia quadriseta</i>	25 ± 2	<i>Entolasia stricta</i>	18 ± 2
<i>Holcus lanatus</i>	24 ± 3	<i>Isachne globosa</i>	13 ± 4
<i>Lagurus ovatus</i>	22 ± 5	<i>Oplismenus aemulus</i>	15 ± 3
<i>Phalaris brachystachya</i>	19 ± 3	<i>Panicum bisulcatum</i>	15 ± 4
<i>Polypogon monspeliensis</i>	19 ± 2	<i>Panicum milioides</i> ( $C_3$ - $C_4$ )	13 ± 2
Aveneae		<i>Brachiaria lorentziana</i> (PCK)	28 ± 2
<i>Amphibromus neesii</i>	19 ± 3	<i>Panicum maximum</i> * (PCK)	37 ± 5
<i>Avena sativa</i>	20 ± 2	<i>Spinifex hirsutus</i> (PCK)	34 ± 9
Meliceae		<i>Panicum decompositum</i> * (NAD-ME)	59 ± 5
<i>Glyceria declinata</i>	17 ± 3	<i>Panicum miliaceum</i> * (NAD-ME)	58 ± 6
Poeae		<i>Panicum stapfianum</i> * (NAD-ME)	63 ± 8
<i>Cynosurus echinatus</i>	24 ± 12	<i>Axonopus compressus</i> (NADP-ME)	61 ± 15
<i>Festuca arundinacea</i>	20 ± 6	<i>Echinochloa crus-galli</i> * (NADP-ME)	57 ± 21
<i>Festuca littoralis</i>	19 ± 3	<i>Panicum antidotale</i> * (NADP-ME)	53 ± 3
<i>Lolium perenne</i>	19 ± 2	<i>Panicum lanipes</i> * (NADP-ME)	45 ± 1
<i>Poa helmsii</i>	21 ± 4	<i>Pennisetum typhoides</i> * (NADP-ME)	54 ± 3
<i>Poa hiemata</i>	20 ± 2	<i>Setaria geniculata</i> (NADP-ME)	51 ± 2
Arundinoids, danthonioids, etc.		Andropogonoids	
Stipeae		<i>Bothriochloa macra</i> (NADP-ME)	51 ± 5
<i>Anisopogon avenaceus</i>	18 ± 3	<i>Cymbopogon refractus</i> (NADP-ME)	52 ± 11
<i>Nassella trichotoma</i>	20 ± 3	<i>Imperata cylindrica</i> (NADP-ME)	62 ± 8
<i>Stipa mollis</i>	20 ± 5	<i>Sorghum vulgare</i> * (NADP-ME)	50 ± 4
Danthonieae		<i>Themeda australis</i> (NADP-ME)	45 ± 9
<i>Cortaderia selloana</i>	14 ± 2	<i>Zea mays</i> * (NADP-ME)	56 ± 5
<i>Danthonia pallida</i>	19 ± 2		

ferent ploidy levels in several grass groups [e.g. Agrostideae, Panicoids *sensu lato* (cf refs 17-21)], there is no indication here of correlation between ploidy and  $K_m(\text{CO}_2)$  as described by Garret (7) and by Rathnam and Chollet (23) for cultivars of *Lolium*. However, among both  $C_3$  and  $C_4$  genera, there is some taxonomic pattern. Within the Pooideae ( $C_3$ ), the tribes Triticeae, Bromeae, and Meliceae exhibit lower  $K_m(\text{CO}_2)$  values (13 to 17  $\mu\text{M}$   $\text{CO}_2$ ) than do the Agrostideae, Aveneae, and Poeae (19 to 25  $\mu\text{M}$   $\text{CO}_2$ ). The Triticeae and Bromeae share a number of peculiarities, both morphological and physiological, and the  $K_m(\text{CO}_2)$  values lend some support to a recent proposal to distinguish them from other Pooideae at supertribal level (13, 16, 25, 29). Among the  $C_4$  grasses, the chloridoids exhibit lower  $K_m(\text{CO}_2)$  values (34 to 46  $\mu\text{M}$   $\text{CO}_2$ ) than do the andropogonoids (45 to 62  $\mu\text{M}$   $\text{CO}_2$ ), whereas the  $C_4$  eu-panicoids exhibit a wider range (28 to 63  $\mu\text{M}$   $\text{CO}_2$ ) which overlaps those of the chloridoids and andropogonoids. However, this systematic variation among the  $C_4$  genera correlates to some extent with the different  $C_4$  types in that carboxylases isolated from PCK species tend to show lower  $K_m(\text{CO}_2)$  values (28 to 41  $\mu\text{M}$   $\text{CO}_2$ ) than do those from NAD-ME species (41 to 63  $\mu\text{M}$   $\text{CO}_2$ )

and NADP-ME species (45 to 62  $\mu\text{M}$   $\text{CO}_2$ ). The difference is detectable within both the groups (chloridoids and eu-panicoids) which exhibit mixtures of  $C_4$  types and, here too, phylogenetic considerations are apparently being outweighed by functional aspects. The two NAD-ME chloridoids have given lower values than the three NAD-ME eu-panicoids, and it is not possible to distinguish the carboxylases from NAD-ME and NADP-ME species with respect to the  $K_m(\text{CO}_2)$  values.

Variation in  $K_m(\text{CO}_2)$  between the  $C_3$  and  $C_4$  species and among  $C_4$  species may be functionally related to variations in the concentration of  $\text{CO}_2$  in the histological framework within which the enzyme normally operates. Cyanobacteria and unicellular green algae seem to have  $\text{CO}_2$ -concentrating mechanisms, endowing them with a higher affinity for  $\text{CO}_2$  during photosynthesis (3, 14). There, the lower substrate affinity of the enzyme is masked by the ability to concentrate  $\text{CO}_2$ , and high-affinity enzymes have apparently not evolved. In  $C_3$  higher plants, there appears to be no ability to concentrate  $\text{CO}_2$ ; hence, affinity for external  $\text{CO}_2$  during photosynthesis largely rests on the  $K_m(\text{CO}_2)$  of the enzyme.  $C_3$  plants seem to have evolved an enzyme with higher affinity for

CO<sub>2</sub>. C<sub>4</sub> plants concentrate CO<sub>2</sub> within the bundle sheath (PCK) cells where RuBP carboxylase is located (12). If they evolved from C<sub>3</sub> ancestors with high CO<sub>2</sub> affinity, as seems likely, it appears that, with development of an over-riding concentrating mechanism, the enzyme's CO<sub>2</sub> affinity decreased again. Such reversal might have had certain selective advantages. First, the enzyme from C<sub>3</sub> plants seems to be inhibited by CO<sub>2</sub> concentrations in excess of about 60 μM, unlike the enzyme for C<sub>4</sub> plants where activity continues to respond to CO<sub>2</sub> concentrations in excess of 180 μM (data not shown). Second, lower affinity for CO<sub>2</sub> may be associated with a high turnover number of enzyme [cf *Anabaena variabilis* and *Rhodospirillum rubrum*, where values in excess of four turnovers/s·active site have been reported, compared with the values of less than 2 generally reported from higher plant enzyme (1, 5, 24)]. Less enzyme or protein investment would achieve the same CO<sub>2</sub>-saturated rates of photosynthesis as in C<sub>3</sub> plants. Variations in  $K_m(\text{CO}_2)$  among C<sub>4</sub> species may be related to histologically derived differences between C<sub>4</sub> types regarding CO<sub>2</sub> accumulation: perhaps NAD-ME and NADP-ME forms are better equipped to concentrate CO<sub>2</sub> and/or prevent CO<sub>2</sub> leakage from the bundle sheath cells than are the PCK types.

There is considerable interest in breeding C<sub>3</sub> crop plants, including cereals, with reduced levels of photorespiration and O<sub>2</sub> inhibition of photosynthesis. The RuBP oxygenase function of RuBP carboxylase appears to be responsible for both these effects, so plants with reduced RuBP oxygenase activity and specific chemical inhibitors of this activity are being sought. However, the correlative changes which seem to have occurred during evolution of CO<sub>2</sub>-concentrating mechanisms and of CO<sub>2</sub> affinity of the enzyme in grasses and algae suggest that selection pressure has operated on the  $K_m(\text{CO}_2)$  of the enzyme, enhancing its efficiency at lower CO<sub>2</sub> concentrations. Little is known about any concomitant changes in RuBP oxygenase activity, but comparison of properties of the *A. variabilis* enzyme with that from higher plants suggests that it does not increase in affinity or activity to the same extent as the carboxylase function (1). Evolutionary modifications in CO<sub>2</sub> affinity of the enzyme as exemplified in Table I would probably have occurred even in the absence of elevated O<sub>2</sub> levels in the atmosphere. Therefore, selective modification of RuBP carboxylase aimed at improving productivity of C<sub>3</sub> grasses (and of C<sub>3</sub> crops in general) should perhaps be directed towards screening C<sub>3</sub> forms for carboxylase mutants with even higher affinities for CO<sub>2</sub> than they now possess. This approach seems promising, considering the flexibility regarding  $K_m(\text{CO}_2)$  demonstrated by this enzyme during its evolution.

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