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Variations in $K_m(CO_2)$ of Ribulose-1,5-bisphosphate Carboxylase among Grasses

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ABSTRACT

A survey of the $K_m(CO_2)$ values of ribulose-1,5-bisphosphate carboxylase from 60 grass species shows that enzyme from C_3 grasses consistently exhibits lower $K_m(CO_2)$ than does that from C_4 grasses. Systematically ordered variation in $K_m(CO_2)$ 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[3] carboxylase (EC 4.1.1. 39) is the fundamental carboxylating enzyme of photosynthesis. Comparisons among reported $K_m(CO_2)$ values for this enzyme suggest that the C_3 and C_4 photosynthetic pathways may be distinguishable in terms of substrate affinity for CO_2 (2, 6, 15, 22); however, published figures are not reliably interpretable because $K_m(CO_2)$ 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 $K_m(CO_2)$ 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 $MgCl_2$ and 1 mM DTT, and partially purified by elution through Sephadex G-25 in the same buffer. The enzyme was preactivated in 5 mM $NaHCO_3$ and then assayed by measuring the fixation of [^{14}C]bicarbonate (2). The reaction mixture containing 100 mM Bicine and 25 mM $MgCl_2$ (pH 8.0) was prepared CO_2-free and flushed with N_2. Assays (total volume, 400 μl) were performed in 1-ml stoppered vials (Pierce Reacti-vials No. 13221) which had been flushed with N_2. Reaction was started by injection of 5 μl preactivated enzyme and stopped after 1 min at 25 C by injection of 0.2 ml 2 N 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 HCO_3^- concentration and specific radioactivity. To minimize this correction for unlabeled HCO_3^-, only 5 μl of the extract was used for assay, and $NaHCO_3$ 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 $K_m(CO_2)$ values was checked by using 10 mM $NaHCO_3$ in some preactivation conditions. This had no detectable effect on the $K_m(CO_2)$ 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 CO_2 concentration then was calculated from the pH and HCO_3^- concentration using the Henderson-Hasselbach equation and the pK value of 6.37 at 25 C (27).

RESULTS AND DISCUSSION

The $K_m(CO_2)$ 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 $K_m(CO_2)$ values, ranging from 13 to 26 μM CO_2, than do their C_4 counterparts, where the values vary from 28 to 63 μM CO_2. Significantly, the C_3/C_4 distinction in terms of $K_m(CO_2)$ 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 μM CO_2) or high values (28 to 63 μM CO_2) strictly in accord with the difference in photosynthetic pathway. Likewise, *Triraphis mollis,* a C_4 danthonioid, has yielded a higher $K_m(CO_2)$ (39 μM CO_2) than its C_3 relatives, *Cortaderia selloana* (14 μM CO_2) and *Danthonia pallida* (19 μM CO_2). Evidently, variation in $K_m(CO_2)$ 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 $K_m(CO_2)$ 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 $K_m(CO_2)$ values in line with their photosynthetic pathways. Although our sample covers dif-

[3] Abbreviations: RuBP, ribulose 1,5-bisphosphate; NADP-ME, NADP-malic enzyme; NAD-ME, NAD-malic enzyme; PCK, phosphoenolpyruvate carboxykinase.

Table I. *$K_m(CO_2)$ 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	$K_m(CO_2)$	Species	$K_m(CO_2)$
	μM		
Bamboos		***Triraphis mollis**** (PCK)	39 ± 5
Arundinaria sp.	26 ± 5	Arundineae	
Oryzoids		*Arundo donax*	16 ± 3
Oryza sativa cv Baru	17 ± 2	*Phragmites australis*	20 ± 4
Oryza sativa cv Calrose	17 ± 3	Doubtful affinities	
Pooideae		*Microlaena stipoides*	21 ± 4
Triticeae		*Tetrarrhena juncea*	20 ± 2
Hordeum vulgare	15 ± 3	Chloridoids	
Secale cereale	13 ± 2	***Chloris truncata*** (PCK)	34 ± 2
Triticum aestivum	15 ± 2	***Sporobolus virginicus*** (PCK)	41 ± 7
Bromeae		***Zoysia macrantha*** (PCK)	34 ± 4
Bromus arenarius	16 ± 2	***Eleusine coracana*** (NAD-ME)	41 ± 5
Bromus unioloides	17 ± 3	***Eragrostis chloromelas*** (NAD-ME)	46 ± 3
Agrostideae		Panicoids *sensu lato*	
Anthoxanthum odoratum	20 ± 1	Eu-panicoids	
Deyeuxia quadriseta	25 ± 2	*Entolasia stricta*	18 ± 2
Holcus lanatus	24 ± 3	*Isachne globosa*	13 ± 4
Lagurus ovatus	22 ± 5	*Oplismenus aemulus*	15 ± 3
Phalaris brachystachya	19 ± 3	*Panicum bisulcatum*	15 ± 4
Polypogon monspeliensis	19 ± 2	*Panicum milioides* (C_3-C_4)	13 ± 2
Aveneae		***Brachiaria lorentziana*** (PCK)	28 ± 2
Amphibromus neesii	19 ± 3	***Panicum maximum**** (PCK)	37 ± 5
Avena sativa	20 ± 2	***Spinifex hirsutus*** (PCK)	34 ± 9
Meliceae		***Panicum decompositum**** (NAD-ME)	59 ± 5
Glyceria declinata	17 ± 3	***Panicum miliaceum**** (NAD-ME)	58 ± 6
Poeae		***Panicum stapfianum**** (NAD-ME)	63 ± 8
Cynosurus echinatus	24 ± 12	***Axonopus compressus*** (NADP-ME)	61 ± 15
Festuca arundinacea	20 ± 6	***Echinochloa crus-galli**** (NADP-ME)	57 ± 21
Festuca littoralis	19 ± 3	***Panicum antidotale**** (NADP-ME)	53 ± 3
Lolium perenne	19 ± 2	***Panicum lanipes**** (NADP-ME)	45 ± 1
Poa helmsii	21 ± 4	***Pennisetum typhoides**** (NADP-ME)	54 ± 3
Poa hiemata	20 ± 2	***Setaria geniculata*** (NADP-ME)	51 ± 2
Arundinoids, danthonioids, etc.		Andropogonoids	
Stipeae		***Bothriochloa macra*** (NADP-ME)	51 ± 5
Anisopogon avenaceus	18 ± 3	***Cymbopogon refractus*** (NADP-ME)	52 ± 11
Nassella trichotoma	20 ± 3	***Imperata cylindrica*** (NADP-ME)	62 ± 8
Stipa mollis	20 ± 5	***Sorghum vulgare**** (NADP-ME)	50 ± 4
Danthonieae		***Themeda australis*** (NADP-ME)	45 ± 9
Cortaderia selloana	14 ± 2	***Zea mays**** (NADP-ME)	56 ± 5
Danthonia pallida	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 $K_m(CO_2)$ 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 $K_m(CO_2)$ values (13 to 17 μM CO_2) than do the Agrostideae, Aveneae, and Poeae (19 to 25 μM CO_2). The Triticeae and Bromeae share a number of peculiarities, both morphological and physiological, and the $K_m(CO_2)$ 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 $K_m(CO_2)$ values (34 to 46 μM CO_2) than do the andropogonoids (45 to 62 μM CO_2), whereas the C_4 eu-panicoids exhibit a wider range (28 to 63 μM 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 $K_m(CO_2)$ values (28 to 41 μM CO_2) than do those from NAD-ME species (41 to 63 μM CO_2) and NADP-ME species (45 to 62 μM 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 $K_m(CO_2)$ values.

Variation in $K_m(CO_2)$ between the C_3 and C_4 species and among C_4 species may be functionally related to variations in the concentration of CO_2 in the histological framework within which the enzyme normally operates. Cyanobacteria and unicellular green algae seem to have CO_2-concentrating mechanisms, endowing them with a higher affinity for CO_2 during photosynthesis (3, 14). There, the lower substrate affinity of the enzyme is masked by the ability to concentrate CO_2, and high-affinity enzymes have apparently not evolved. In C_3 higher plants, there appears to be no ability to concentrate CO_2; hence, affinity for external CO_2 during photosynthesis largely rests on the $K_m(CO_2)$ of the enzyme. C_3 plants seem to have evolved an enzyme with higher affinity for

CO_2. C_4 plants concentrate CO_2 within the bundle sheath (PCK) cells where RuBP carboxylase is located (12). If they evolved from C_3 ancestors with high CO_2 affinity, as seems likely, it appears that, with development of an over-riding concentrating mechanism, the enzyme's CO_2 affinity decreased again. Such reversal might have had certain selective advantages. First, the enzyme from C_3 plants seems to be inhibited by CO_2 concentrations in excess of about 60 μM, unlike the enzyme for C_4 plants where activity continues to respond to CO_2 concentrations in excess of 180 μM (data not shown). Second, lower affinity for CO_2 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_2-saturated rates of photosynthesis as in C_3 plants. Variations in $K_m(CO_2)$ among C_4 species may be related to histologically derived differences between C_4 types regarding CO_2 accumulation: perhaps NAD-ME and NADP-ME forms are better equipped to concentrate CO_2 and/or prevent CO_2 leakage from the bundle sheath cells than are the PCK types.

There is considerable interest in breeding C_3 crop plants, including cereals, with reduced levels of photorespiration and O_2 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_2-concentrating mechanisms and of CO_2 affinity of the enzyme in grasses and algae suggest that selection pressure has operated on the $K_m(CO_2)$ of the enzyme, enhancing its efficiency at lower CO_2 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_2 affinity of the enzyme as exemplified in Table I would probably have occurred even in the absence of elevated O_2 levels in the atmosphere. Therefore, selective modification of RuBP carboxylase aimed at improving productivity of C_3 grasses (and of C_3 crops in general) should perhaps be directed towards screening C_3 forms for carboxylase mutants with even higher affinities for CO_2 than they now possess. This approach seems promising, considering the flexibility regarding $K_m(CO_2)$ demonstrated by this enzyme during its evolution.

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