Plant Cell Physiol. 39(2): 212–219 (1998)

Distribution of Fallover in the Carboxylase Reaction and Fallover-Inducible Sites among Ribulose 1,5-Bisphosphate Carboxylase/Oxygenases of Photosynthetic Organisms

Koichi Uemura [1, 7], Hiroshi Tokai [2], Takashi Higuchi [1, 2, 8], Hiroshi Murayama [1, 2], Hiroshi Yamamoto [1, 2, 9], Yukito Enomoto [3], Shoko Fujiwara [4, 10], Jin Hamada [5] and Akiho Yokota [1, 6, 11]

[1] *Plant Molecular Physiology Laboratory, Research Institute of Innovative Technology for the Earth (RITE), Kizu, Kyoto, 619-02 Japan*
[2] *Department of Agricultural Chemistry, University of Osaka Prefecture, Sakai, Osaka, 593 Japan*
[3] *Marine Biology Station, Faculty of Science, Kobe University, Tuna, Hyogo, 656-24 Japan*
[4] *National Instutute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Tsukuba, 305 Japan*
[5] *Department of Community Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama, 930-01 Japan*
[6] *Graduate School of Biological Sciences, Nara Institute of Science and Technology, Takayama, Ikoma, Nara, 630-01 Japan*

The biphasic reaction course, fallover, of carboxylation catalysed by ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) has been known as a characteristic of the enzyme from higher land plants. Fallover consists of hysteresis in the reaction seen during the initial several minutes and a very slow suicide inhibition by inhibitors formed from the substrate ribulose-1,5-bisphosphate (RuBP). This study examined the relationship between occurrence of fallover and non-catalytic RuBP-binding sites, and the putative hysteresis-inducible sites (Lys-21 and Lys-305 of the large subunit in spinach RuBisCO) amongst RuBisCOs of a wide variety of photosynthetic organisms. Fallover could be detected by following the course of the carboxylase reaction at 1 mM RuBP and the non-catalytic binding sites by alleviation of fallover at 5 mM RuBP. RuBisCO from *Euglena gracilis* showed the same linear reaction course at both RuBP concentrations, indicating an association between an absence of fallover and an absence of the non-catalytic binding sites. This was supported by the results of an equilibrium binding assay for this enzyme with a transition state analogue. Green macroalgae and non-green algae contained the plant-type, fallover enzyme. RuBisCOs from Conjugatae, *Closterium ehrenbergii, Gonatozygon monotaenium* and *Netrium digitus*, showed a much smaller decrease in activity at 1 mM RuBP than the spinach enzyme and the reaction courses of these enzymes at 5 mM RuBP were almost linear. RuBisCO of a primitive type Conjugatae, *Mesotaenium caldariorum*, showed the same linear course at both RuBP concentrations. Sequencing of *rbc*L of these organisms indicated that Lys-305 was changed into arginine with Lys-21 conserved.

Key words: Conjugatae — Fallover — Non-catalytic substrate-binding sites — Photosynthetic organisms — Ribulose 1,5-bisphosphate — Ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39).

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO; EC 4.1.1.39) reveals its activity after the previous activation of the enzyme by CO_2 and Mg^{2+} (Lorimer et al. 1976, Andrews and Lorimer 1987). The activated enzyme from plant sources shows an apparently biphasic reaction course with time in the presence of 1 mM or less ribulose 1,5-bisphosphate (RuBP) comprised of the initial burst which slows down gradually in several minutes and a subsequent course where RuBisCO loses its activity much more slowly compared to the initial burst (Andrews et al. 1990, Edmondson et al. 1990a, b, Robinson and Portis 1989, Yokota 1991, Yokota and Kitaoka 1989, Zhu and Jensen 1991a, b). The biphasic reaction course has been called "fallover". It has been proposed that the initial rapid decrease in activity is due to reaction hysteresis accompanying a change in protein conformation of RuBisCO (Yokota 1991). The latter is the slow inhibition phase where suicide inhibitors including xylulose 1,5-bisphosphate is formed from RuBP on the catalytic sites of the enzyme. Hysteresis is always accompanied by the subsequent slow decrease in activity by the suicide reaction. Fallover has been seen in RuBisCOs from C_3 and C_4 plants. Lys-21 and Lys-305 from the large subunit of the spinach enzyme have been identified as two of the residues involved in a change of the protein conformation in hysteresis (Yokota and Tokai 1993). The activity after the fallover increases about 50%

Abbreviations: CABP, 2-carboxyarabinitol 1,5-bisphosphate; RuBP, ribulose 1,5-bisphosphate; RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase.

[7] On leave from Research and Development Center, Unitika Ltd., 23 Kozakura, Uji, Kyoto, 611 Japan.

[8] Present address: Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Tsutsumidori-Amamiyamachi, Sendai, 981 Japan.

[9] Present address: National Institute for Basic Biology, Myodaiji, Okazaki, 444 Japan.

[10] Present address: Department of Environmental Biology, Tokyo Pharmaceutical University, Hachioji, Tokyo, 192-03 Japan.

[11] To whom correspondence should be addressed.

in the presence of more than 1 mM RuBP (Yokota 1991). It has been proposed that the increase is due to the alleviation of the decrease of the activity in fallover through the binding of RuBP to the non-catalytic RuBP-binding sites (Yokota 1991, Yokota et al. 1994, 1996). The non-catalytic RuBP-binding sites have been designated as the regulatory sites affecting the enzymatic activity. RuBisCO showing fallover and the characteristic of the regulatory sites will be described as the fallover-type enzyme in this paper.

In contrast to the enzyme from higher land plant sources, RuBisCOs from green microalgae including *Euglena gracilis* (Yokota 1991, Yokota and Kitaoka 1989), cyanobacteria (Andrews and Ballment 1984, Asami et al. 1983), and a photosynthetic bacterium (McFadden 1974) do not show fallover. Residues 21 and 305 which are lysine in the spinach enzyme, are other amino acid residues in RuBisCOs of green microalgae and photosynthetic bacteria (Yokota and Tokai 1993). RuBP of more than 1 mM does not increase the activity of the *Euglena* enzyme (Yokota 1991). This type of RuBisCO will be grouped as the linear-type enzyme here.

It is of interest to know at which evolution step and how RuBisCO evolved from the linear-type enzyme to the fallover-type. To this end, we analyzed the courses of the carboxylase reaction with time for RuBisCOs from a wide variety of photosynthetic organisms. It was found that RuBisCOs showing the biphasic reaction course were accompanied by the occurrence of the non-catalytic RuBP-binding sites. Distribution of fallover in RuBisCO among photosynthetic organisms followed the phylogenetic tree for the evolution for the gene of the large subunits of the enzyme. Comparison of the reaction courses and amino acid residues at the sites in question suggested that Conjugatae might be the transition organism in the evolution of the RuBisCO functions in the photosynthetic organisms containing Chl *a* and *b*.

Materials and Methods

Materials—The cultured cells of *Alcaligenes eutrophus* were obtained from Saibu Gas Company (Fukuoka, Japan). Chlorophyceae, *Bryopsis maxima*, was a gift from Dr. S. Miyamura, Tsukuba University, and Charophyceae *Chara fragilos* was from Dr. T. Bando, Kyoto University of Education. Axenic strains of Charophyceae, *Closterium ehrenbergii* M-16-4a(+), *Gonatozygon monotaenium* LB1253 UTEX (strain of University of Texas), *Netrium digitus* Ned-1 (strain 41-13 of Dr. S. Ohtani, Shimane University) and *Mesotaenium caldariorum* IAM C-309 (strain of Tokyo University) were cultured with CA medium (*Netrium*) and C medium (the others) as reported (Ichimura 1971, Hamada 1978). Cryptophyceae *Cryptomonas tetrapyrenoidosa* was grown in the VT medium (Provasoli and Pintner 1959) and Protoflorideophyceae *Porphyridium cruentum* and *Cyanidium caldarium* in the EMS (Watanabe et al. 1988) and Allen's media (Allen 1959), respectively. Chlorophyceae *Chlamydomonas reinhardtii* C-9 was cultured in the 3/10 HSM medium (Sueoka et al. 1967). These cultures were bubbled with sterile air under illumination at 80 μmol m^{-2} s^{-1}. Sea-water Florideophyceae *Gelidium amansii* and Chlorophyceae *Ulva pertusa* and *Enteromorpha linza* were collected at the sea shore near Iwaya of Awaji Island, Hyogo, Japan on May 15, 1992. These were stored at −80°C after briefly washing with distilled water. *Escherichia coli* JM-109 transformed with pCV-23 carrying *rbc*A-*rbc*B was grown as reported (Viale et al. 1990). All of these organisms were stored at −80°C until use.

RuBP was from Sigma Co., and other chemicals were of analytical grades. 2-Carboxyarabinitol-1,5-bisphosphate (2-CABP) was synthesized and purified as reported (Pierce et al. 1980).

Preparation of extracts—Gelidium, Ulva, Bryopsis and *Enteromorpha* were ground with a chilled mortar and pestle in 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol, 2% (w/v) polyvinylpolypyrrolidone, 1 mM phenylmethylsulfonylfluoride, and 10 μM leupeptin. *Closterium, Gonatozygon, Netrium, Mesotaenium, Chlamydomonas, Cryptomonas, Porphiridium, Cyanidium,* and *Alcaligenes* were disintegrated by sonication for 2 min in all in the above buffer devoid of polyvinylpolypyrrolidone. The homogenates were centrifuged at 10,000 × *g* for 10 min. The supernatants obtained were used for the RuBisCO assay, since fallover can be well followed with leaf extracts (Yokota et al. 1990) and the chloroplast stroma (Yokota and Tsujimoto 1992). Induction of the RuBisCO genes in *E. coli* was done with isopropyl β-D-thiogalactopyranoside as reported by Viale et al. (1990). The extract of the *E. coli* cells was prepared as reported (Viale et al. 1990).

Purification of RuBisCO—RuBisCOs of spinach and *Euglena gracilis* Z were purified as reported previously, and were reactivated with dithiothreitol before assay (Yokota 1991). RuBisCO of *Netrium* was purified as following. The supernatant obtained by sonication and centrifugation mentioned in "Preparation of extract" was precipitated with polyethylene glycol-4000 between 10 and 20% in the presence of 10 mM $MgCl_2$. Precipitates were dissolved in a small volume of 50 mM HEPES-KOH buffer (pH 7.6) containing 1 mM EDTA and 1 mM dithiothreitol, and farther purified by a gel-filtration chromatography on Superdex pg as reported previously (Uemura et al. 1996).

Assay of RuBisCO—RuBisCOs were assayed by the method reported previously (Yokota 1991), except for the enzyme of *Chromatium* that was assayed by the method of Viale et al. (1990). The protein concentration in the reaction mixtures was adjusted so that less than 0.1 mM RuBP was consumed in 10 min. The data points in the time course experiments were the average of two determinations. The reaction courses in the individual figures are representatives of several repeated experiments.

Equilibrium binding assay—Equilibrium ligand-binding assays with the carbamylated form of spinach and *Euglena* RuBisCOs were done as reported previously for the spinach enzyme (Yokota et al. 1991).

Amino acid sequence analysis—Total DNA from *Netrium, Closterium* and *Mesotaenium* were isolated as has been described by Sekimoto et al. (1994) and 1,011 bp fragments of *rbc*L were amplified by *Taq* polymerase-mediated PCRs. Two primers correspond to positions 1-24 (5′-ATG(GT)C(AT)CCACA(AG)AC-(AGTC)GA(AG)AC(TA)AA-3′) and 1011-987 (5′-C(AC)CCT-TC(AT)AGTTTACCTACAACAGT-3′) were designed based on the reported sequences of *rbc*L of *Spirogyra maxima* (Genbank: L11057), *Chlamydomonas reinhardtii* (Dron et al. 1982) and *Marchantia polymorpha* (Ohyama et al. 1986). Amplified DNA fragments were purified by electrophoresis, and were directly sequenced using the *Taq* dideoxy terminator cycle sequencing

kit (Perkin Elmer) and a DNA sequencer (Perkin Elmer; model 373A). Six primers (above two primers, and primers correspond to positions 207–190 (5′-CACAGTAGTCCATGTTCC-3′), 787–806 (5′-CCTATTATTATGCATGACTA-3′), 715–731 (5′-TACCTA-AACGCTACTGC-3′) and 158–140 (5′-GCTTCTTCTGGTGGT-ACTC-3′) were used for nucleotide sequencing.

Measurement of protein—The amount of purified RuBisCO was measured by the dye-binding method using purified spinach RuBisCO as the standard (Yokota 1991). The bulk amount of protein was measured by the same method with bovine serum albumin as the standard.

Results

The activity of RuBisCO from spinach decreases with reaction time to about 50% of the initial value when the enzyme reaction is started at 1 mM RuBP or less (Yokota 1991, Yokota and Tsujimoto 1992). The loss of the activity in fallover is repressed about 50% in the presence of more than 1 mM RuBP (Fig. 1a). This is because RuBP at the latter concentrations binds to the regulatory, non-catalytic sites to repress the loss of the activity in fallover (Yokota 1991, Yokota et al. 1991, 1994, 1996). Accordingly, we are able to examine the occurrence of fallover by analyzing the reaction course at 1 mM RuBP, and the presence of the regulatory, non-catalytic binding sites, are tested by comparing the reaction courses at 1 and 5 mM RuBP.

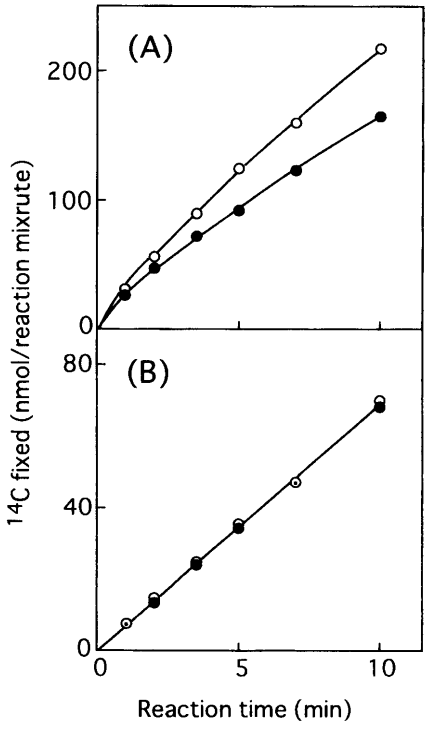

Fig. 1 Courses of the carboxylase reaction of RuBisCOs from spinach (A) and *Euglena* (B) with different concentrations of RuBP. The concentration of RuBP was 1 (closed circles) and 5 mM (open circles).

RuBisCO from *Euglena* showed quite similar linear reaction courses with both 1 and 5 mM RuBP (Fig. 1b). The same was true for the *Chlamydomonas* and *Chromatium* enzymes (data not shown). This indicated an absence of both fallover and regulatory sites in these RuBisCOs. The absence of the regulatory sites in *Euglena* RuBisCO was confirmed by measurement of equilibrium binding of the transition state analogue 2-CABP (Fig. 2). The dissociation constant of the catalytic sites of activated RuBisCO for 2-CABP is near to 10^{-13} M and RuBisCO forms a stable quaternary complex with 2-CABP in the presence of CO_2 and Mg^{2+} (Pierce et al. 1980, Schloss 1988). Increasing concentrations of 2-CABP caused additional binding of 2-CABP to spinach RuBisCO; totally 16 molecules of 2-CABP bound to 1 molecule of spinach RuBisCO in the presence of saturating concentrations (over 100 μM) of 2-CABP. This indicates that the quaternary complex of spinach RuBisCO with 2-CABP still binds one more 2-CABP per protomer in the presence of more than 100 μM 2-CABP, as reported in Yokota et al. 1991. This extra eight 2-CABP-binding sites also bind RuBP with much higher dissociation constants than the catalytic sites (Yokota et al. 1994). On the contrary, *Euglena* RuBisCO bound eight molecules of 2-CABP per 1 molecule of RuBisCO even in the presence of 150 μM 2-CABP (Fig. 2). Since *Euglena* RuBisCO forms a stable quatenary complex with 2-CABP using its catalytic sites like the spinach enzyme (Yokota and Canvin 1985), the absence of the binding of eight more 2-CABP indicates that the *Euglena* enzyme does not possess the non-catalytic RuBP-binding sites or the sites are hidden not to bind extra sugar phosphates. These observations support the kinetic results in Fig. 1.

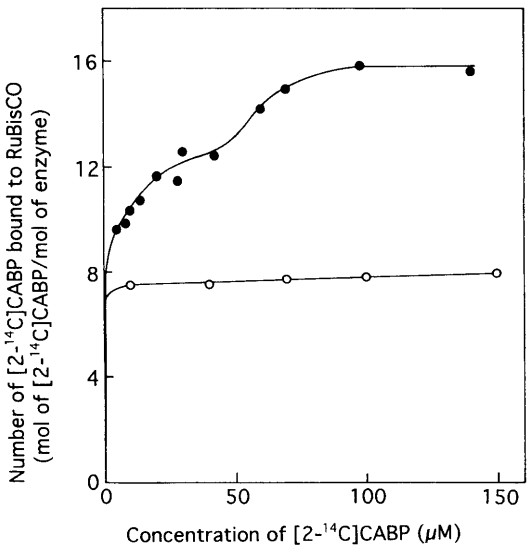

Fig. 2 Equilibrium binding of 2-CABP to the carbamylated form of RuBisCOs from spinach (closed circles) and *Euglena* (open circles).

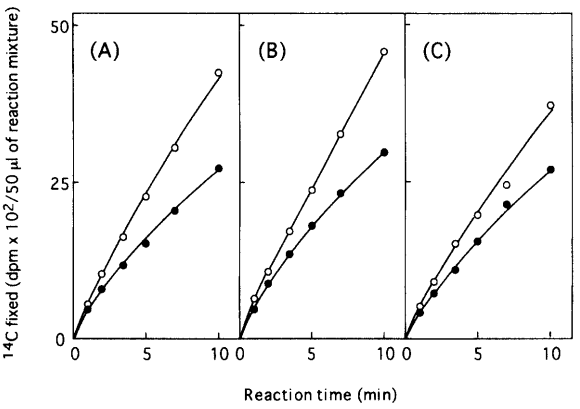

Fig. 3 Reaction courses of RuBisCOs of green macroalgae, *Enteromorpha* (A), *Ulva* (B), and *Chara* (C). Symbols were the same as in Fig. 1.

RuBisCOs from green macroalgae, *Enteromorpha* and *Ulva* showed a 40% and a 43% decreases in the activity, respectively, at 1 mM RuBP, and these decrease was alleviated by 5 mM RuBP as in the spinach enzyme (Fig. 3). RuBisCO of *Bryopsis* was also fallover-type (data not shown).

In the phylogeny of green microalgae to the land plants, Charophyceae locates between them (Picket-Heaps 1975, Graham 1993). *Chara* which belongs to Charales, the most developed order in Charophyceae, contained follover-type RuBisCO (Fig.3). A Conjugatae *Closterium* which belongs to more primitive order, Conjugatales, in Charophyceae, had RuBisCO the activity of which decreased only 15% in the carboxylase reaction at 1 mM RuBP (Fig. 4). The slight loss of the activity was removed at 5 mM RuBP and the course was nearly linear. RuBisCOs of other Conjugatae *Netrium* and *Gonatozygon* were quite resembled the *Closterium* enzyme. This was the same when purified enzyme from *Netrium* was used for the reaction (data not shown). The RuBisCO from the most primitive Conjugatae examined in this study, *Mesotaenium*, showed the completely linear-type course both at 1 mM and 5 mM RuBP like the *Euglena* enzyme (Fig. 4).

The results presented above clearly show that the reaction course of RuBisCO is closely related to the evolution stage in photosynthetic organisms having Chl *a* and *b*. Photosynthetic bacteria and green microalgae had the linear-type enzyme, and green macroalgae and land plants contained the fallover-type enzyme. RuBisCOs of Conjugatae may be the intermediate type between them. In this context, it is interesting to analyze the reaction courses of RuBisCOs of organisms having other auxiliary pigments than Chl *b* (Fig. 5). Unicellular, primitive red algae, *Cyanidium* and *Porphyridium*, and red macroalga *Gelidium* contained the fallover-type enzyme. Cryptophyceae, *Cryptomonas* also had fallover-type RuBisCO. In addition to these non-green algae, *Alcaligenes eutrophus* also contained the fallover-type enzyme. Fallover of these enzymes was, of course, accompanied by the occurence of the regulatory, non-catalytic RuBP-binding sites, as inferred from their responses to 5 mM RuBP.

Our next step was to compare the reaction course and

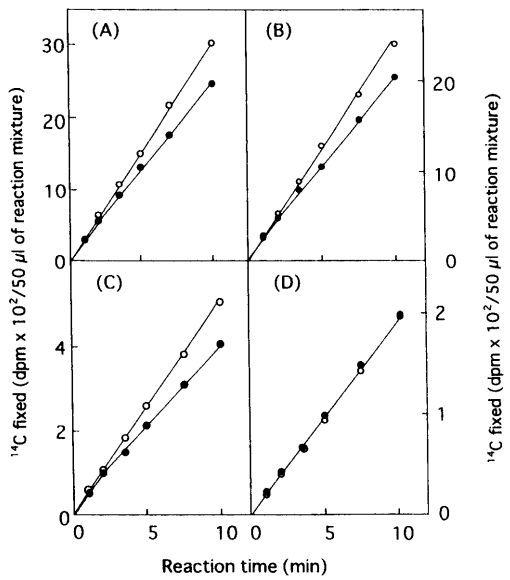

Fig. 4 Reaction courses of RuBisCOs of *Closterium* (A), *Netrium* (B), *Gonatozygon* (C) and *Mesotaenium* (D). Symbols were the same as in Fig. 1.

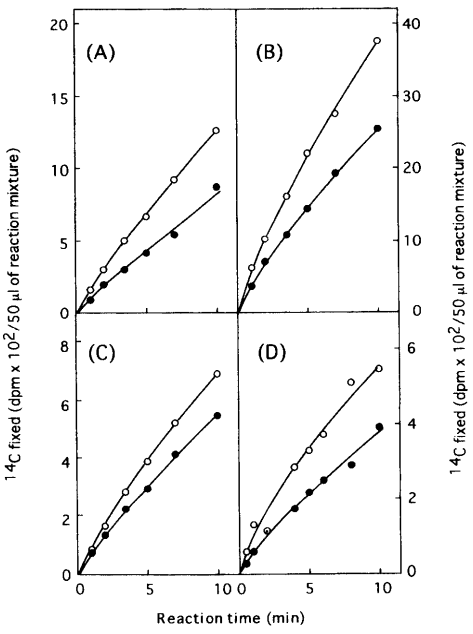

Fig. 5 Reaction courses of RuBisCOs of non-green algae and a chemolithotroph, *Cryptomonas* (A), *Porphyridium* (B), *Gelidium* (C) and *Alcaligenes* (D). Symbols were the same as in Fig. 1.

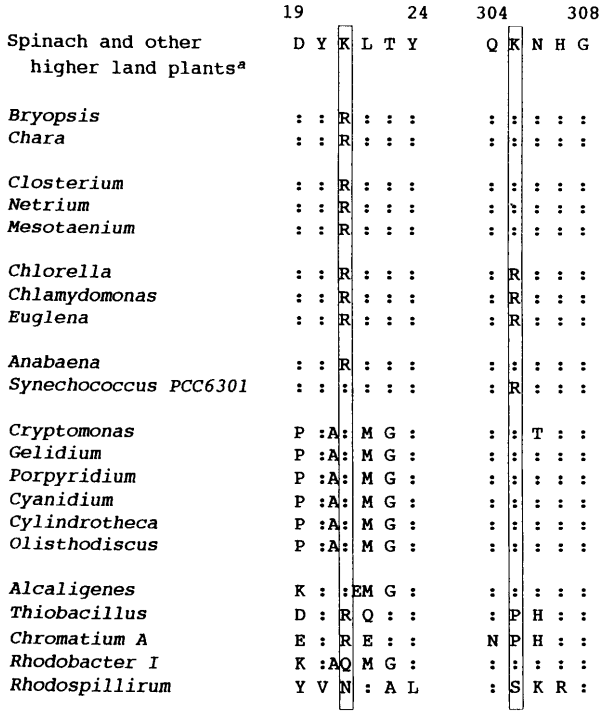

Fig. 6 Alignment of the deduced amino acid sequences of RuBisCOs around Lys-21 and Lys-305. The sequences of RuBisCOs were from following sources: Spinach and other land higher plants, *Chlamydomonas reinhardtii, Euglena gracilis, Synechococcus* PCC6301 and photosynthetic bacteria (Yokota and Tokai 1993); *Bryopsis maxima* (Kono et al. 1991); *Chlorella ellipsoidea* (Yoshinaga et al. 1988); *Anabaena* 7120 (Curtis and Haselkorn 1983); *Cryptomonas* F (Douglas et al. 1990); *Cyanidium caldarium* and *Olisthodiscus luteus* (Fujiwara et al. 1993). Sequences of RuBisCOs of *Chara connivens* and *Gelidium americanum* were obtained from GenBank, accession number L13476 and L22459. [a] Lys-21 is changed into arginine in alfalfa in the land higher plants.

the occurrence of the hysteresis-inducible sites amongst RuBisCOs of a wide variety of photosynthetic organisms. To this end, we determined nucleotide sequences of *rbc*L of the organisms included in Conjugatae and deduced the amino acid sequences. The deduced amino acid sequences near the sites hysteresis-inducible in spinach RuBisCO (Yokota and Tokai 1993) are aligned with the sequences of the enzymes of other organisms in Fig. 6. Lys-21 and Lys-305 (this numbering of the residues are for spinach RuBisCO) are conserved in RuBisCOs of higher land plants except alfalfa (Yokota and Tokai 1993). Both sites are changed into arginine in such green microalgae as *Euglena* (Gingrich and Hallick 1985), *Chlamydomonas* (Dron et al. 1982) and *Chlorella* (Yoshinaga et al. 1988). The intermediate-type enzymes of the organisms included in Conjugatae conserved Lys-305 but the site corresponding to Lys-21 in the spinach enzyme was arginine.

Discussion

There have been three ideas for the mechanism of fallover seen with plant RuBisCO. One is that the decrease in the activity is due to the suicide inhibition by tight binding of inhibitors including xylulose bisphosphate which are formed on the catalytic sites during reaction from RuBP by the enzyme itself (Andrews et al. 1990, Edmondson et al. 1990a, b, Robinson and Portis 1989, Zhu and Jensen 1991a, b). RuBP itself has been inferred to be a candidate for the inhibitor (McCurry et al. 1981, Mott and Berry 1986), since decarbamylated inactive RuBisCO forms a stable, inactive complex with RuBP (Jordan and Chollet 1983). The third idea is that the change in the protein conformation of RuBisCO from a high- to low-activity form in the course of the reaction (hysteresis) and the subsequent suicide inhibition is the cause of the partial loss of the activity in fallover (Yokota 1991, Yokota et al. 1996). Hysteresis has been known in many other enzymes (Neet and Ainslie 1980). The exact mechanism has received a strong attention, but is still under a subject of debate (Hartman and Harpel 1993, 1994).

Two lysine residues have been identified to be involved in the hysteretic protein conformational change of spinach RuBisCO (Yokota and Tokai 1993). Surveying the hysteresis sites and occurence of fallover among RuBisCOs of a wide variety of photosynthetic organisms may give us an answer for the debate. Lys-21 and Lys-305 of the large subunits are well conserved in RuBisCOs from higher plants, but are changed into arginine or proline in the enzymes of green microalgae and some photosynthetic bacteria (Fig. 6). The present study clearly showed that RuBisCOs of *Alcaligenes, Cryptomonas, Cyanidium, Porphyridium* and *Gelidium* were the fallover-type enzymes (Fig. 5). The two lysine residues are also well conserved in RuBisCOs of these organisms. RuBisCOs of γ-purple bacteria, cyanobacteria, green microalgae including the primitive Conjugatae, *Mesotaenium*, were linear-type. *Closterium* and several other organisms included in Conjugatae contained interesting RuBisCO, in which the activity was slightly decreased in the course of the reaction at 1 mM RuBP but a higher concentration of RuBP strongly eliminated fallover (Fig. 4). In other words, the enzyme of this organism was an intermediate between the fallover- and linear-type RuBisCOs. These results strongly suggest that fallover was aquired during the evolution of RuBisCO in green organisms. In RuBisCOs of Conjugatae, Lys-305 was conserved but Lys-21 was changed into arginine (Fig. 6). Green macroalgae *Chara, Bryopsis, Ulva* and *Enteromorpha* contained the fallover-type enzyme (Fig. 3). RuBisCOs of the former two organisms conserve Lys-305 but the Lys-21 site is arginine as like the enzyme of Conjugatae (see Fig. 6 for references). It is reasonable to deduce that these lysine residues are important for occur-

rence of fallover or in the hysteretic conformational change of RuBisCO. However, there must be additional sites involved in fallover as reported recently (Lee et al. 1993). These additional sites may be more critical residues in induction of fallover in alfalfa and cyanobacterial RuBisCOs, where only one of the two lysine residues is conserved although the reaction courses of these RuBisCOs follow the above criteria discussed above.

RuBisCOs that showed fallover in the reaction course responded to a higher concentration of RuBP. The response is due to the binding of RuBP to the regulatory, non-catalytic RuBP-binidng sites (Yokota 1991, Yokota et al. 1994). The sites also bind a transition state analogue 2-CABP (Yokota et al. 1991) and 6-phosphogluconate (Yokota et al. 1992), in addition to RuBP. On the other hand, the linear-type *Euglena* enzyme did not bind any extra 2-CABP (Fig. 2). The precise relationships between the protein structure, fallover and the non-catalytic binding sites is being examined in this laboratory.

Through the present study, we encountered interesting questions as to why and how the fallover sites were acquired by RuBisCOs of green land plants in the evolution from green microalgae. Why do all RuBisCOs of β-purple bacteria and non-green algae have the fallover-inducible sites and exactly show fallover? The phylogenetic tree

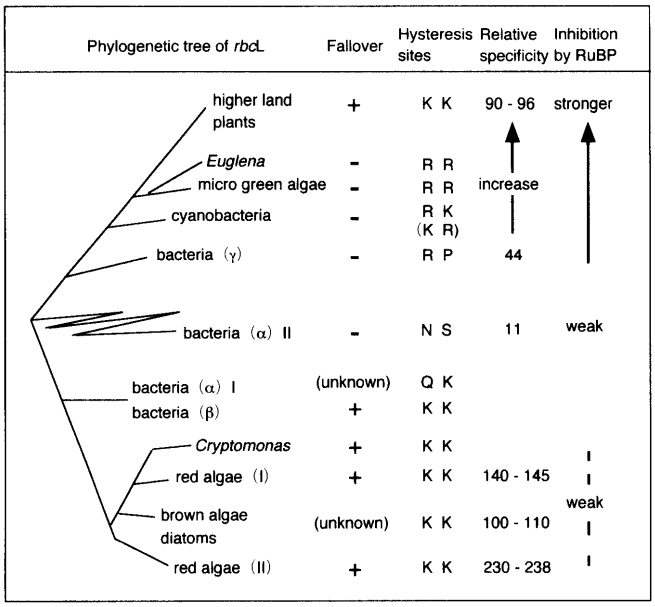

Fig. 7 Phylogenetic relationship between occurrence of fallover, amino acides of fallover-inducible sites and enzymatic properties of RuBisCOs. The lines of the tree do not depict the exact phylogentic distances between organisms, but only their phylogenetic relationships. Relative specificities for RuBisCOs of diatoms and α purple bacteria (type II) were from Read and Tabita (1994) and Andrews and Lorimer (1987), respectively. Those for the enzymes of other organisms were from Uemura et al. (1996, 1997). See the text for other details.

for the evolution of the gene for the large subunits of RuBisCO, *rbc*L, has been well accepted (Ueda and Shibuya 1992, Zetsche and Valentin 1993) (Fig. 7). The putative ancestral *rbc*L is thought to diverge into three main branches; (1) α-purple bacteria (type II), and (2) α-purple bacteria (type I), β-purple bacteria and non-green algae, and (3) γ-purple bacteria, cyanobacteria, green algae and plants. In the last branch, the large subunits of RuBisCOs have over 70% homology in the amino acid sequences (Kobayashi et al. 1991). Occurrence of fallover and the regulatory, non-catalytic RuBP-binding sites well followed the sequence of the adaptation of photosynthetic organisms to the terrestrial habitat or the increase in the relative specificity of RuBisCO. It might be conceivable that fallover is a consequence of the adaptation to the terresterial environment, at least in the evolution of green organisms. In this context, it is interesting to recall that RuBisCOs of the developed organisms in Conjugatae were the intermediate-type enzyme and the enzyme of the primitive one in this order did not show fallover. These organisms may have evolved from an ancestral aquatic organism which appeared when RuBisCO was evolved to adapt to the terrestrial environment. Exceptions for these considerations are RuBisCOs of green macroalgae, *Ulva, Entromorpha* and *Bryopsis*. These RuBisCOs resembled plant RuBisCO in the reaction course, although these organisms have been often thought to be close to green microalgae in the distributions of the glycolate-oxidizing enzymes and the superoxide dismutase isozymes (Schnarrenberger et al. 1992).

Another interesting point is the reason for occurence of the hysteresis-inducible sites in β-purple bacteria and non-green algae. The simplest idea is that when the ancestral *rbc*L was diverged into three major branches, the ancestral *rbc*L of this branch incorporated the sites by chance. The sites might have been transferred to the present organisms in this branch without any further mutation on these sites. Recent findings that the relative specificities of RuBisCOs of the non-green algae are larger than those of the C_3-plant enzymes (Read and Tabita 1994, Uemura et al. 1996, 1997) may support the above idea on the relationship between the relative specificity and fallover. Fallover may be an important function for the RuBisCO showing a high relative specificity.

The complex between RuBP and inactive RuBisCOs of *Rhodospillirum rubrum* (Jordan and Chollet 1983) and *Synechococcus* (Smrcka et al. 1990) is relatively easily converted into the active form of the enzyme in the presence of saturating CO_2 and Mg^{2+} compared with the plant enzyme. It is known that *Synechococcus* RuBisCO is much less susceptible to the inhibition by xylulose bisphosphate than the enzyme from plant sources (Smrcka et al. 1990). The severe inhibitions of the plant enzyme by RuBP and xylulose bisphosphate have been deduced to be the cause of fallover (Andrews et al. 1990, Edmondson et al. 1990a, b, McCurry

et al. 1981, Mott and Berry 1986, Robinson and Portis 1989, Zhu and Jensen 1991a, b). However, the reported much stronger inhibition for RuBisCO of *Chlamydomonas* (Roesler and Ogren 1990) is not consistent with this deduction, since this enzyme never showed fallover. RuBisCOs of non-green algae are also relatively insusceptible to the inhibition by RuBP (Newman et al. 1989, Read and Tabita 1994) but showed clear fallover in the present study (Fig. 5). It may be reasonable to conclude, from these considerations, that fallover is not simply due to the inhibition of the enzyme by RuBP or suicide inhibitors, but the existence of hysteresis-inducible sites including Lys-21 and Lys-305 in RuBisCO is indispensable for fallover. However, still we have to wait for further studies as to how these residues lower the activity during the reaction and why the green plants acquired the sites.

The authors thank Dr. H. Kobayashi, University of Shizuoka, for providing pCV23. We also thank Dr. M. Tanaka, Saibu Gas Co., Ltd., for gifting the cultured cells of *Alcaligenes eutrophus* by the courtesy of Professor A. Ishizaki, Kyushu University. This work was supported partly by Petroleum Energy Center (PEC) subsidized from Ministry of International Trade and Industry of Japan.

References

Allen, M.B. (1959) Studies with *Cyanidium caldarium*, an anomalously pigmented chlorophyte. *Arch. Mikrobiol.* 32: 270–277.

Andrews, T.J., Badger, M.R., Edmondson, D.L., Kane, H.J., Morell, M.K. and Paul, K. (1990) Rubisco: subunits and mechanism. *In* Current Research in Photosynthesis, Volume 3. Edited by Baltscheffsky, M. pp. 331–338. Kluwer, Dordrecht.

Andrews, T.J. and Ballment, B. (1984) Active-site carbamate formation and reaction-intermediate analog binding by ribulosebisphosphate carboxylase/oxygenase in the absence of its small subunits. *Proc. Natl. Acad. Sci. USA* 81: 3660–3664.

Andrews, T.J. and Lorimer, G.H. (1987) Rubisco: structure, mechanisms and prospects for improvement. *In* Biochemistry of Plants, Volume 10. Edited by Hatch, M.D. and Boardman, N.K. pp. 131–218. Academic Press, New York.

Asami, S., Takabe, T., Akazawa, T. and Codd, G.A. (1983) Ribulose 1,5-bisphosphate carboxylase from the halophilic cyanobacterium *Aphanothece halophytica*. *Arch. Biochem. Biophys.* 225: 713–721.

Curtis, S.F. and Haselkorn, R. (1983) Isolation and sequence of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase from the cyanobacterium *Anabaena* 7120. *Proc. Natl. Acad. Sci. USA* 80: 1835–1839.

Douglas, S.E., Durnford, D.G. and Morden, C.W. (1990) Nucleotide sequence of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from the chlorophyll *c*-containing alga *Cryptomonas* F: evidence supporting the polyphyletic origin of planstids. *J. Phycol.* 26: 500–508.

Dron, M., Rahire, M. and Rochaix, J.D. (1982) Sequence of the chloroplast DNA region of *Chlamydomonas reinhardtii* containing the gene of the large subunit of ribulose bisphosphate carboxylase and parts of its flanking genes. *J. Mol. Biol.* 162: 775–793.

Edmondson, D.L., Badger, M.R. and Andrews, T.J. (1990b) Slow inactiavtion of ribulosebisphosphate carboxylase during catalysis is caused by accumulation of a slow, tight-binding inhibitor at the catalytic site. *Plant Physiol.* 93: 1390–1397.

Edmondson, D.L., Kane, H.J. and Andrews, T.J. (1990a) Substrate isomerization inhibits ribulosebisphosphate carboxylase-oxygenase during catalysis. *FEBS Lett.* 260: 62–66.

Fujiwara, S., Iwahashi, H., Someya, J., Nishikawa, S. and Minaka, N. (1993) Structure and cotranscription of the plastid-encoded *rbc*L and *rbc*S genes of *Pleurochrysis carterae* (Prymnesiophyta). *J. Phycol.* 29: 347–355.

Gingrich, J.C. and Hallick, R.B. (1985) The *Euglena gracilis* chloroplast ribulose-1,5-bisphosphate carboxylase gene: II. The spliced mRNA and its product. *J. Biol. Chem.* 260: 16162–16168.

Graham, L.E. (1993) Origin of land plants. John Wiley & Sons. Inc., New York.

Hamada, J. (1978) Studies on several environmental fators for zygote formation and germination in *Closterium ehrenbergii*. *Bot. Mag. Tokyo* 91: 173–180.

Hartman, F.C. and Harpel, M.R. (1993) Chemical and genetic probes of the active site of D-ribulose-1,5-bisphosphate carboxylase/oxygenase: a petrospective based on the three-dimensional structure. *Adv. Enzymol.* 67: 1–75.

Hartman, F.C. and Harpel, M.R. (1994) Structure, function, regulation, and assembly of D-ribulose-1,5-bisphosphate carboxylase/oxygenase. *Annu. Rev. Biochem.* 63: 197–234.

Ichimura, T. (1971) Sexual cell division and conjugation-papilla formation in sexual reproduction of *Closterium stigosum*. *In* Proc. 7th Intn. Seaweed Symp. Edited by Nisizawa, K. pp. 208–214. University of Tokyo Press, Tokyo.

Jordan, D.B. and Chollet, R. (1983) Inhibition of ribulose bisphosphate carboxylase by substrate ribulose-1,5-bisphosphate. *J. Biol. Chem.* 258: 13752–13758.

Kobayashi, H., Viale, A.M., Takabe, T., Akazawa, T., Wada, K., Shinozaki, K., Kobayashi, K. and Sugiura, M. (1991) Sequence and expression of genes encoding the large and small subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase from *Chromatium vinosum*. *Gene* 97: 55–62.

Kono, M., Satoh, H., Okabe, Y., Abe, Y., Nakayama, K. and Okada, M. (1991) Nucleotide sequence of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from the green alga *Bryopsis maxima*. *Plant Mol. Biol.* 17: 505–508.

Lee, E.H., Harpel, M.R., Chen, Y-R. and Hartman, F.C. (1993) Perturbation of reaction intermediate partitioning by a site-directed mutant of ribulose-bisphosphate carboxylase/oxygenase. *J. Biol. Chem.* 268: 26583–26591.

Lorimer, G.H., Badger, M.R. and Andrews, T.J. (1976) The activation of ribulose-1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibra, kinetics, a suggested mechanism, and physiological implications. *Biochemistry* 15: 529–536.

McCurry, S.D., Pierce, J., Tolbert, N.E. and Orme-Johnson, W.H. (1981) On the mechanism of effector mediated activation of ribulose bisphosphate carboxylase/oxygenase. *J. Biol. Chem.* 256: 6623–6628.

McFadden, B.A. (1974) The oxygenase activity of ribulose-1,5-bisphosphate carboxylase from *Rhodospirillum rubrum*. *Biochem. Biophys. Res. Commun.* 60: 312–317.

Mott, K.A. and Berry, J.A. (1986) Effects of pH on activity and activation of ribulose 1,5-bisphosphate carboxylase at air level CO_2. *Plant Physiol.* 82: 77–82.

Neet, K.E. and Ainslie, G.R.J. (1980) Hysteretic enzymes. *Methods Enzymol.* 64: 192–226.

Newman, S.M., Derocher, J. and Cattolico, R.A. (1989) Analysis of chromophytic and rhodophytic ribulose-1,5-bisphosphate carboxylase indicates extensive structural and functional similarities among evolutionary diverse algae. *Plant Physiol.* 91: 939–946.

Ohyama, K., Fukuzawa, H., Kohchi, K., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H. and Ozeki, H. (1986) Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. *Nature* 322: 572–574.

Pickett-Heaps, J.D. (1975) *Green Algae. Structure, Reproduction and Evolution in Selected Genera.* Sinauer Associates Inc., Sunderland, Massachusetts.

Pierce, J., Tolbert, N.E. and Barker, R. (1980) Interaction intermediate partitioning by ribulose-bisphosphate carboxylases with differing substrate specificities. *Biochemistry* 19: 934–942.

Provasoli, L. and Pintner, I.J. (1959) Artificial media for fresh-water algae: problems and suggestions. *In* The Ecology of Algae, Special Edition No. 2. Edited by Tryon, C.A.J. and Hartmann, R.T. pp. 84–96. University of Pittsburgh Press, Pittsburgh.

Read, B.A. and Tabita, F.R. (1994) High substrate specificity factor ribulose bisphosphtae carboxylase/oxygenase from eukaryotic marine algae and properties of recombinant cyanobacterial rubisco containing algal residue modifications. *Arch. Biochem. Biophys.* 312: 210–218.

Robinson, S.P. and Portis, A.R.J. (1989) Ribulose 1,5-bisphosphate carboxylase/oxygenase activase protein prevents the in vitro decline in activity of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Plant Physiol.* 90: 968–971.

Roesler, K.R. and Ogren, W.L. (1990) Primary structure of *Chlamydomonas reinhardtii* ribulose 1,5-bisphosphate carboxylase/oxygenase activase and evidence for a single polypeptide. *Plant Physiol.* 94: 1837–1941.

Schloss, J.V. (1988) Comparative affinities of the epimeric reaction-intermediate analogs 2- and 4-carboxy-D-arabinitol 1,5-bisphosphate for spinach ribulose 1,5-bisphosphate carboxylase. *J. Biol. Chem.* 263: 4145–4150.

Schnarrenberger, C., Gross, W., Pelzer-Reith, Wiegand, S. and Jacobshagen, S. (1992) The evolution of isozymes of sugar phosphate metabolism in algae. *In* Phylogenetic Changes in Peroxisomes of Algae/Phylogeny of Plant Peroxisomes. Edited by Stabenau, H. pp. 310–329, University of Oldenburg Press, Oldenburg.

Sekimoto, H., Sone, Y. and Fujii, T. (1994) Regulation of expression of the genes for a sex pheromone by an inducer of the sex pheromone in the *Closterium peracerosum-stigosum-littorate* complex. *Planta* 193: 137–144.

Smrcka, A.V., Bohnert, H.J. and Jensen, R.G. (1990) Tight binding of inhibitors by rubiscos from evolutionary diverse sources. *Plant Physiol.* 93 Suppl.: 122.

Sueoka, N., Chiang, K-S. and Kates, J.R. (1967) Deoxyribonucleic acid replication in meiosis of *Chlamydomonas reinhardtii*. I. Isotopic transfer experiments with a strain producing eight zoospores. *J. Mol. Biol.* 25: 47–66.

Ueda, K. and Shibuya, H. (1992) Molecular phylogeny of *rbc*L and its bearing on the origin of plastids: bacteria or cyanophyta? *In* Endocytobiology V (5th International Colloquium on Endocytobiology and Symbiosis). Edited by Sato, S., Ishida, M. and Ishikawa, H. pp. 369–376. Tübingen University Press, Tübingen.

Uemura, K., Anwaruzzaman, Miyachi, S. and Yokota, A. (1997) Ribulose-1,5-bisphosphate carboxylase/oxygenase from thermophilic red algae with a strong specificity for CO_2 fixation. *Biochem. Biophys. Res. Commun.* 233: 568–571.

Uemura, K., Suzuki, Y., Shikanai, T., Wadano, A., Jensen, R.G., Chmara, W. and Yokota, A. (1996) A rapid and sensitive method for determination of relative specificity of RuBisCO from various species by anion-exchange chromatography. *Plant Cell Physiol.* 37: 325–331.

Viale, A.M., Kobayashi, H. and Akazawa, T. (1990) Distinct properties of *Escherichia coli* products of plant-type ribulose-1,5-bisphosphate carboxylase/oxygenase directed by two sets of genes from the photosynthetic bacterium *Chromatium vinosum*. *J. Biol. Chem.* 265: 18386–18392.

Watanabe, M., Kasai, F. and Sudo, R. (1988) NIES-Collection List of Strains, Second Edition 1988, Microalgae and Protozoa. pp. 163. The National Institute for Environmental Studies, Tsukuba.

Yokota, A. (1991) Ribulose bisphosphate-induced, slow conformational changes of spinach ribulose bisphosphate carboxylase cause the two types of inflections in the course of its carboxylase reaction. *J. Biochem.* 110: 246–252.

Yokota, A. and Canvin, D.T. (1985) Ribulose bisphosphate carboxylase/oxygenase content determined with [^{14}C]carboxypentitol bisphosphate in plants and algae. *Plant Physiol.* 77: 735–739.

Yokota, A., Higashioka, M., Taira, T., Usuda, H., Wadano, A. and Murayama, H. (1994) Binding of ribulose 1,5-bisphosphate to the noncatalytic sites of ribulose 1,5-bisphosphate carboxylase/oxygenase and its metabolic implications. *Plant Cell Physiol.* 35: 317–321.

Yokota, A., Higashioka, M. and Wadano, A. (1991) Cooperative binding of carboxyarabinitol bisphosphate to the regulatory sites of ribulose bisphosphate carboxylase/oxygenase from spinach. *J. Biochem.* 110: 253–256.

Yokota, A., Higashioka, M. and Wadano, A. (1992) Regulation of the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase through cooperative binding of 6-phosphogluconate to its regulatory sites. *Eur. J. Biochem.* 208: 721–727.

Yokota, A. and Kitaoka, S. (1989) Linearity and functioning forms in the carboxylase reaction of spinach ribulose 1,5-bisphosphate carboxylase/oxygenase. *Plant Cell Physiol.* 30: 183–191.

Yokota, A., Taira, T., Usuda, H. and Kitaoka, S. (1990) The in vivo functioning forms of ribulose1,5-bisphosphate carboxylase/oxygenase in plants. *In* Current Research in Photosynthesis. Volume 3. Edited by Baltscheffsky, M. pp. 199–202. Kluwer, Dordrecht.

Yokota, A. and Tokai, H. (1993) Lysine residues involved in the hysteresis and in the regulatory sites of spinach ribulose 1,5-bisphosphate carboxylase/oxygenase. *J. Biochem.* 114: 746–753.

Yokota, A. and Tsujimoto, N. (1992) Characterization of ribulose-1,5-bisphosphate carboxylase/oxygenase carrying ribulose 1,5-bisphosphate at its regulatory sites and the mechanism of interaction of this form of the enzyme with ribulose-1,5-bisphosphate carboxylase/oxygenase activase. *Eur. J. Biochem.* 204: 901–909.

Yokota, A., Wadano, A. and Murayama, H. (1996) Modeling of continuously and directly analyzed biphasic reaction courses of ribulose 1,5-bisphosphate carboxylase/oxygenase. *J. Biochem.* 119: 487–499.

Yoshinaga, K., Ohta, T., Suzuki, Y. and Sugiura, M. (1988) *Chlorella* chloroplast DNA sequence containing a gene for the large subunit of ribulose-1,5-bisphosphate carboxylase and a part of a possible gene for the beta subunit of RNA polymerase. *Plant Mol. Biol.* 10: 245–250.

Zetsche, K. and Valentin, K. (1993) Structure, coding capacity and gene sequences of the plastid genome from red algae. *Endocytobiosis Cell Res.* 10: 107–127.

Zhu, G. and Jensen, R.G. (1991a) Xylulose 1,5-bisphosphate synthesized by ribulose 1,5-bisphosphate carboxylase/oxygenase during catalysis binds to decrabamylated enzyme. *Plant Physiol.* 97: 1348–1353.

Zhu, G. and Jensen, R.G. (1991b) Fallover of ribulose 1,5-bisphosphate carboxylase/oxygenase activity. Decarbamylation of catalytic sites depends on pH. *Plant Physiol.* 97: 1354–1358.

(Received October 14, 1997; Accepted December 5, 1997)