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Engineering Rubisco to change its catalytic properties

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Abstract

The initial steps of carbon assimilation and photorespiration are catalysed by ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39). Natural variation in the kinetic properties of the enzyme suggest that it is possible to alter the enzyme to favour the carboxylation activity relative to oxygenation. Mutagenesis *in vitro* of the gene encoding the large subunit of the enzyme from *Anacystis nidulans* has been used to modify catalytic properties. Residues at the C-terminal end of loop 6 of the β/α barrel structure of the large subunit influence specificity towards the gaseous substrates, CO_2 and O_2. None of the residues altered by mutagenesis appear to interact directly with the transition state analogue and their effect on the reaction of the enediolate intermediate with the gaseous substrates and stabilization of the resulting transition state intermediates by lysine 334 must be indirect. Interactions with other parts of the enzyme must also be important in determining substrate specificity. Backbone carbonyl groups close to lysine 334 interact with lysine 128; mutation of lysine 128 to residues of less positive polarity reduces enzyme activity and favours oxygenation relative to carboxylation. The likely effects on assimilation rates of altering the kinetic properties of Rubisco have been modelled. A leaf with cyanobacterial Rubisco may out-perform a higher plant Rubisco at elevated CO_2 and cool temperatures.

Key words: *Anacystis nidulans*, Rubisco, specificity factor.

Introduction

In addition to catalysing the primary step of photosynthetic carbon metabolism by carboxylating ribulose-1,5-bisphosphate (RuBP), the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyses three other reactions, the most important of which is the oxygenation of RuBP. Consequently, the enzyme plays a pivotal role partitioning carbon between the Calvin cycle and the photorespiratory cycle. Photorespiration is wasteful because it consumes energy and results in the loss of fixed carbon. The oxygenase reaction is probably the most important metabolic constraint on plant productivity: it can result in a loss of up to 50% of the carbon fixed by a C_3 plant (Zelitch, 1973). Furthermore, Rubisco is a poor catalyst: the enzyme from higher plants has a turnover number of only $3\ s^{-1}$ at 25 °C. Plants thus invest heavily in Rubisco to sustain high photosynthetic rates, which represents a large investment of nitrogen (Rubisco accounts for up to 30% of total nitrogen in a typical C_3 leaf grown with adaquate nitrogen). One approach to optimizing nitrogen use and reducing fertilizer inputs as well as increasing production *per se* would be to engineer an enzyme with a higher turnover rate.

Catalysis

All the reactions catalysed by Rubisco occur at the same active site and have the same initial steps (see review, Hartman and Harpel, 1994), the binding of RuBP which is the substrate and the generation of the enediol intermediate (Gutteridge *et al.*, 1984; Pierce *et al.*, 1986). The enediolate is formed by the abstraction of the C3 proton by a group at the active site and its formation can be followed by NMR (Gutteridge *et al.*, 1984). In two side-reactions, this enediol intermediate is the subject of misprotonation either at C3 to generate xylulose

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Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; CABP, 2-carboxy-D-arabinitol-1,5-bisphosphate.

bisphosphate or at C2 to give 3-keto-arabinitol bisphosphate (Edmonson *et al.*, 1990). Both these compounds are thought to be tight-binding inhibitors of Rubisco and their release *in vivo* requires the influence of another protein, Rubisco activase.

The CO_2 and O_2 appear to compete directly for reaction with the enzyme-bound enediol intermediate since no formal binding sites for CO_2 and O_2 have been identified on the protein (Pierce *et al.*, 1986; van Dyke and Schloss, 1986). Once the enediol has reacted with either of the gases, the enzyme is committed to form products (Pierce, 1988). The carboxylation reaction is thought to proceed through as many as four enzyme-bound intermediates. Electrophillic attack by CO_2 at C2 of the enediol results in the formation of a 2-carboxy- 3-keto-intermediate (Lorimer *et al.*, 1986). This is hydrated at C3, perhaps simultaneously with the carboxylation, to form the *gem* diol (Schloss and Lorimer, 1982; Jaworowski *et al.*, 1984). Cleavage at the C2/C3 bond releases one molecule of 3-P-glycerate and a second enediol-like intermediate, the aci-carbanion form of 3-P-glycerate. Stereo-specific protonation of the latter produces a second 3-P-glycerate molecule (Saver and Knowles, 1982). The mechanism for the oxygenation reaction is probably formation of an intermediate caged radical pair, formally a superoxide radical anion and the C2 radical of the substrate, which combine to produce a 5-carbon hydroperoxy intermediate. This is attacked at position 3 by a hydroxyl ion to give one molecule of 2-P-glycolate and one of 3-P-glycerate (Hartman and Harpel, 1994).

Kinetic properties of Rubisco from different species

Irrespective of the source of the enzyme, in the absence of a formal binding site for the gaseous substrates on the enzyme, partitioning between the carboxylation and oxygenation reactions, might be expected to be immutable for any ratio of the substrates (Pierce, 1986). Comparisons of the relative specificity (τ) of the enzyme for CO_2 and O_2, $[\tau = (K_{\text{cat carboxylation}} \times K_{m(O_2)})/(K_{\text{cat oxygenation}} \times K_{m(CO_2)})]$ show that they differ considerably for Rubiscos isolated from diverse species (Jordan and Ogren, 1981, 1984; Parry *et al.*, 1989; Read and Tabita, 1994). Natural selection for specificity factor may still be continuing, as even the enzymes with the greatest τ have poor relative specificity when compared to other enzymes that also have to discriminate between substrate molecules where the differences are equally as subtle as those between CO_2 and O_2 (Kane *et al.*, 1994). A more extensive survey particularly amongst higher plant Rubiscos, may be worthwhile (Delgado *et al.*, 1995). Improved specificity may, however, have been achieved at some cost since there appears to be a negative correlation between specificity factor and maximum carboxylation rate, $K_{\text{cat carboxylation}}$ (Fig. 1). Whilst it is attractive to argue that the efficiency of carboxylation ($K_{\text{cat carboxylation}}/K_{m(CO_2)}$) has been improved in Rubiscos with a high specificity factor (Andrews and Lorimer, 1987), the apparent inverse relationship of specificity factor and turnover can not be ignored. However, these comparisons are difficult to establish with certainty because there is much variation in estimates of constants especially $K_{\text{cat carboxylation}}$.

The aim of increasing CO_2 assimilation rates in leaves *in vivo* must be achieved without an increase in the amount of Rubisco protein, particularly in those species where Rubisco already represents up to 60% of the soluble protein in a leaf and where further increases in Rubisco protein may not be possible. It is, therefore, important that any increase in specificity factor is not achieved at the expense of a decrease in turnover. Although the molecular basis for differences in specificity factor and turnover are not entirely clear, the goal of improving the efficiency of Rubisco in higher plants remains and appears feasible because of the relationship between kinetic properties and primary sequence.

Enzyme structure and engineering catalysis

In the last few years a number of Rubisco structures, resolved at the scale of electronic interactions, have been

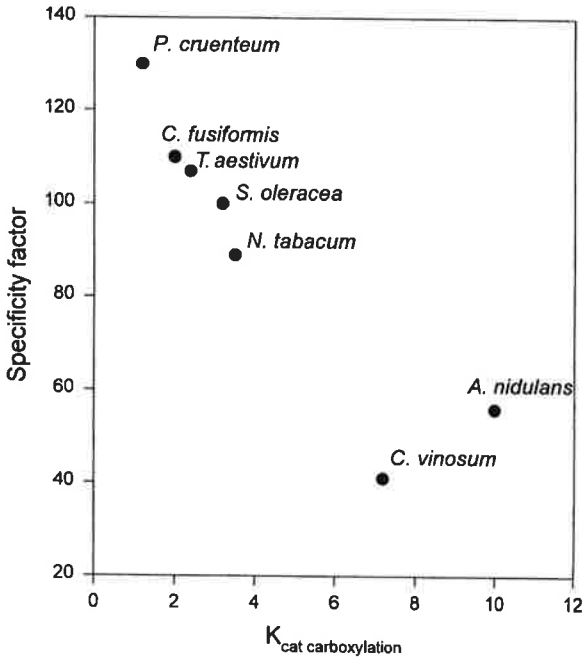

Fig. 1. The relative substrate specificity for the gaseous substrates and $K_{\text{cat carboxylation}}$ [mol CO_2 mol^{-1} Rubisco active site s^{-1}] of Rubiscos isolated from various sources. Data from Jordan and Chollet (1985); Jordan and Ogren (1981, 1984); Parry *et al.* (1989); Gutteridge *et al.* (1993); Read and Tabita (1994).

published. In most species the enzyme is a hexadecamer of eight large (M_r approximately 50 000) and eight small (M_r 12 000–14 000) subunit polypeptides. The small subunits are at the poles of the enzyme; their function is still unclear, but they appear to increase the rate of turnover at the active sites. The large subunit core of *Anacystis nidulans*, for example, catalyses both the oxygenase and carboxylase reactions, but at a much reduced turnover rate, of about 0.6% of the rate of the holoenzyme, however the specificity factor remains unchanged (Gutteridge, 1990). The large subunits are arranged as dimers and each has two major domains: an N-terminal domain and an eight stranded parallel β/α barrel. Each large subunit has an active site at the barrel's mouth, to which the N-terminal domain of the other subunit within the dimer contributes residues.

Although the large and small subunit genes of wheat and maize have been successfully cloned and expressed together in *E. coli*, the enzyme produced does not assemble and is insoluble (Gatenby *et al.*, 1981). Engineering studies have utilized the genes for Rubisco that can be expressed in *E. coli*, forming peptides that do assemble to produce active enzyme. Most research has focused on either the enzyme from *Rhodospirillum rubrum* or that from *A. nidulans*. The *R. rubrum* enzyme is a dimer of two identical large subunits, without any small subunits, but the three-dimensional structure of each subunit is strikingly similar to the large subunits of the hexadecameric enzyme (Schneider *et al.*, 1986; Chapman *et al.*, 1988; Knight *et al.*, 1990; Newman and Gutteridge, 1990). Nevertheless, it is difficult to relate engineered changes in structure of the *R. rubrum* enzyme, and the resulting subtle changes in kinetic characteristics, to Rubiscos with the more complex hexadecameric structure. The *A. nidulans* Rubisco has the hexadecameric structure and, although readily distinguished from the higher plant enzymes by its catalytic properties, it has 80% amino acid and nucleotide homology with the higher plant enzyme. In addition, there is no substantial variation in the number or disposition of structural elements in either subunit of the *A. nidulans* and *Spinacea oleracea* enzymes, crystallized with Mg^{2+}, CO_2 and 2-carboxy-D-arabinitol-1,5-bisphosphate (CABP), when structural models of these enzymes were superimposed (Newman and Gutteridge, 1993). Many of the loop residues that form the active site in the hexadecameric Rubisco are highly conserved. Several residues have been shown to influence specificity factor (Fig. 2). However, most of

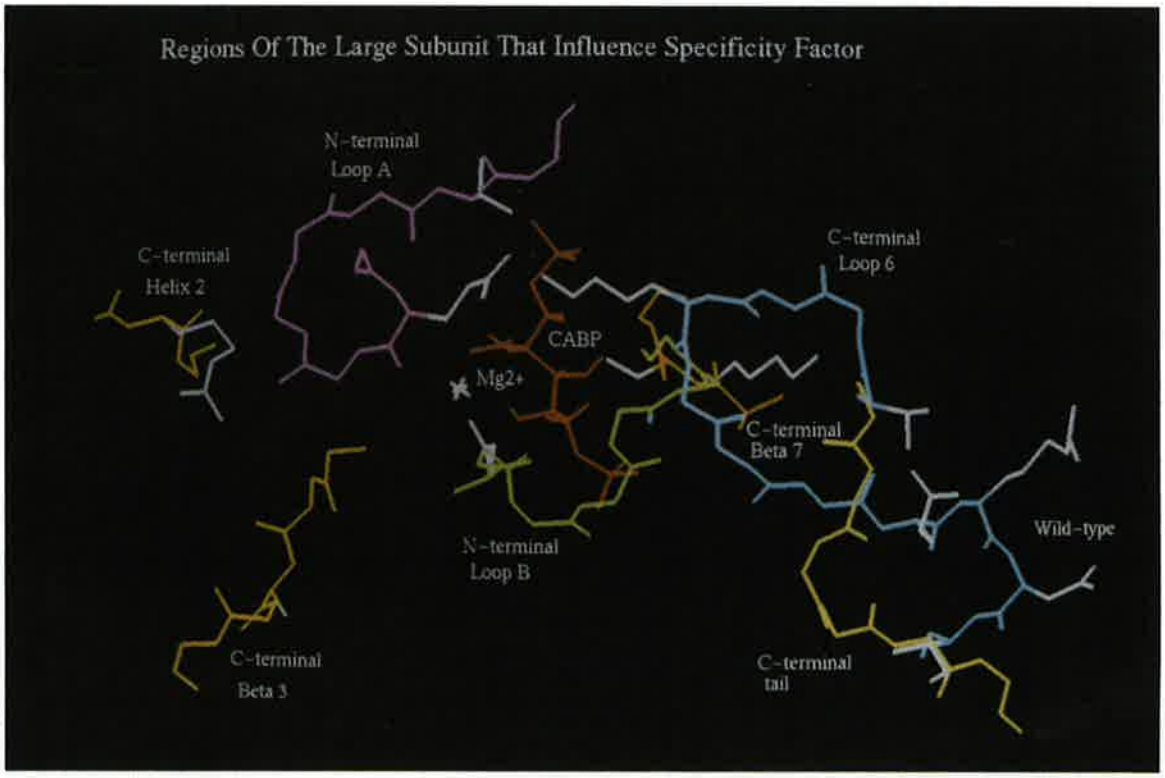

Fig. 2. Regions of the large subunit of Rubisco that alter τ revealed by site-directed and random mutagenesis (Chen and Spreitzer, 1989; Soper *et al.*, 1992; Chène *et al.*, 1992; Morel *et al.*, 1994; Thow *et al.*, 1994; Harpel and Hartman, 1992; Gutteridge *et al.*, 1993; Kane *et al.*, 1994). Structure based on the coordinates supplied by C.L. Branden for the spinach Rubisco.

these can not form ligands directly with the transition state analogue (CABP) in crystal structures and their effects on partitioning of the gaseous substrates must be indirect.

Attention has focused on the mobile, C-terminal loop 6 region at the mouth of the barrel since Chen and Spreitzer (1989) identified mutants with an altered loop 6 amino acid in the green alga *Chlamydomonas reinhardtii* which changed the specificity factor of Rubisco. Mutation of valine 331 to alanine reduced the specificity factor by almost 40% and provided the first clue to the identity of residues that are involved in substrate specificity. Analysis of various crystal forms of the enzyme suggests that the loop is flexible in the initial stages of carboxylation but then, early in catalysis, folds or slides over to close the active site. This conformation appears to be maintained by the residues of the C-terminal tail (Knight *et al.*, 1990) and the N-terminal loops (Newman and Gutteridge, 1993). Valine 331 is the 'spring' responsible for the movement of the loop (Newman and Gutteridge, 1993). Most of the residues in loop 6 are conserved, but only lysine 334 at the apex of the loop has ionic interactions with the transition state analogue CABP. In crystals of *S. oleracea* and *A. nidulans* Rubisco, lysine 334 has ionic interactions with one of the phosphates and the carboxyl group at C2 of CABP (Andersson *et al.*, 1989; Knight *et al.*, 1990). This residue appears to facilitate reaction of the enediolate with CO_2 or O_2 by stabilizing resulting intermediates in the reaction pathways.

Engineering loop 6

Site-directed mutants of lysine 334 catalysed enediolate formation, but were unable to catalyse the reaction of the enediolate with CO_2 or form a stable complex with CABP (Soper *et al.*, 1988; Hartman and Lee, 1989; Gutteridge *et al.*, 1993). Lysine 334 may play a specific role in stabilizing the transition state intermediates of the carboxylation and oxygenation reactions thereby facilitating the reactions of the gaseous substrates with the enediolate.

Only the C-terminal end of loop 6 differs between species. Although many of the differences do not radically alter the size or polarity of the amino acid side-chain, they could be responsible for the different catalytic properties of Rubiscos from different sources (Table 1). These residues have been mutated either individually or together in several studies (Table 2) and analysis of the resultant enzymes confirmed that loop 6 plays a role in determining the specificity factor of Rubisco. Changes to two residues, alanine 340 and threonine 342, significantly decreased specificity factor relative to the unaltered wild-type enzyme. In contrast, only those mutants where four residues were changed together demonstrated increased specificity factors. However, the increases in specificity factor in these mutants were small and did not fully account for the difference in kinetic properties between the *A. nidulans* and higher plant type Rubiscos. None of the altered residues interact directly with CABP and any effect on the reactivity of the enediolate intermediate by lysine 334 must be indirect. In addition, interactions with other parts of the enzyme must be important.

Structural studies have revealed that the conformation of loop 6 is maintained by the residues of the C-terminal tail (Knight *et al.*, 1990) and the N-terminal loops (Newman and Gutteridge, 1993). Portis (1990) demonstrated the importance of the C-terminus of the large subunit to catalytic function. Removal of the C-terminal residues of the *S. oleracea* or *C. reinhardtii* Rubisco large subunit by carboxypeptidase-A reduced carboxylase

Table 1. *The amino acid sequence of loop 6, with some preceding and following residues, of the Rubisco large subunit from different species; Anderson and Caton (1987); Curtis and Hazelkorn (1983); Dron* et al. *(1982); Gingrich and Hallick (1985); Hardison* et al. *(1992); Hwang and Tabita (1991); McIntosh* et al. *(1980); Shinozaki and Sigiura (1982); Shinozaki* et al. *(1983); Valentin and Zetsche (1989); Viale* et al. *(1990); Zurawski* et al. *(1981,1986). Residues identical to those of* A. nidulans *are shown as '.'*

	β6			Loop 6								Helix 6							
	330											340							
A. nidulans	G	T	V	V	G	K	L	E	G	D	K	A	S	T	L	G	F	V	D
Zea mays	.	.	.	.	.	.	.	.	.	E	R	E	I	.	.	.	.	.	.
Triticum aestivum	.	.	.	.	.	.	.	.	.	E	R	E	M	.	.	.	.	.	.
Spinacea oleracea	.	.	.	.	.	.	.	.	.	E	R	D	I	.	.	.	.	.	.
Pisum sativum	.	.	.	.	.	.	.	.	.	E	R	E	I	.	.	.	.	.	.
Nicotiana tabacum	.	.	.	.	.	.	.	.	.	E	R	D	I	.	.	.	.	.	.
Chlamydomonas reinhardtii	.	.	.	.	.	.	.	.	.	E	R	E	V	.	.	.	.	.	.
Euglena gracilis	.	.	.	.	.	.	.	.	.	E	R	E	V	.	.	.	.	.	.
Anabaena	.	.	.	.	.	.	.	.	.	E	R	E	I	.	M	.	.	.	.
Chromatium vinosum	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	W	I	.
Alcaligenes eutrophus	A	.	.	.	.	.	.	.	.	.	P	L	T	V	Q	.	Y	Y	N
Porphyridium aerugineum	.	.	.	.	.	.	.	.	.	.	P	L	M	I	K	.	.	Y	N
Cylindrotheca fusiformis	.	.	.	.	.	.	.	.	.	.	P	L	M	I	K	.	.	Y	.
Olisthodiscus	.	.	.	.	.	.	.	.	.	.	P	L	M	V	K	.	.	Y	.

Table 2. *The per cent increase or decrease (relative to the wild type) in substrate specificity for the gaseous substrates and* $K_{cat\ carboxylation}$ *for Rubisco from* Anacystis nidulans *Rubisco in which residues within loop 6 and helix 6 have been substituted by site directed mutagenesis*

The sequence number preceeded by the single-letter representation for the wild-type residue at that position is followed by the single letter representation for the replacement. *Significant differences from wild type ($P=0.05$).

Mutant	Authors	K_{cat}(%)	τ(% wt.)
D338E	Parry *et al.*, 1992	94	+4
K339R	Parry *et al.*, 1992	100	0
K339P	Read and Tabita, 1994	14	−3
A340E	Parry *et al.*, 1992	88	−18*
A340L	Read and Tabita, 1994	65	−8
S341I	Parry *et al.*, 1992	90	−1
S341M	Read and Tabita, 1994	99	+7
T342A	Gutteridge *et al.*, 1993	2	nd
T342I	Read and Tabita, 1994	54	−18*
	Gutteridge *et al.*, 1993	30	−7
T342L	Gutteridge *et al.*, 1993	40	−7
T342M	Gutteridge *et al.*, 1993	60	−11
T342V	Read and Tabita, 1994	51	−23*
DKAS338-341EREI	Parry *et al.*, 1992	92	+7*
DKAS338-341ERDI	Gutteridge *et al.*, 1993	60	+3
	Kane *et al.*, 1994	nd	+5*

activity by 60–70%. Similarly, mutants in which the large subunit was truncated lost catalytic activity and were no longer able to trap CABP (Gutteridge *et al.*, 1993).

In the absence of X-ray structural information for the mutants, evidence for altered interactions has been gathered from biochemical studies. Activity of one mutant was lost more slowly than that of the wild type following exposure to carboxypeptidase-A in the presence of RuBP. This is consistent with modelling studies suggesting that hydrogen bonds may be formed between some of the mutated residues and the C-terminal tail (Parry *et al.*, 1992).

Sandwiched between loop 6 and the C-terminal tail is the side chain of lysine 128, on an N-terminal loop from the opposing subunit in the dimer (Fig. 3). The side-chain of lysine 128 is fully extended over loop 6 and the ϵ-amino group is within hydrogen bonding distance of the main chain carbonyls of valine 331 and glycine 333, close to the apex of loop 6, and within 0.3 nm of the carbonyl of phenylalanine 467 close to the C-terminus. In addition, asparagine 123 on the same loop interacts directly with CABP; mutation of the equivalent residue in *R. rubrum* (asparagine 111) to glycine decreased specificity factor more than 10-fold and drastically reduced $K_{cat\ carboxylation}$ to 1% of wild type (Chène *et al.*, 1992;

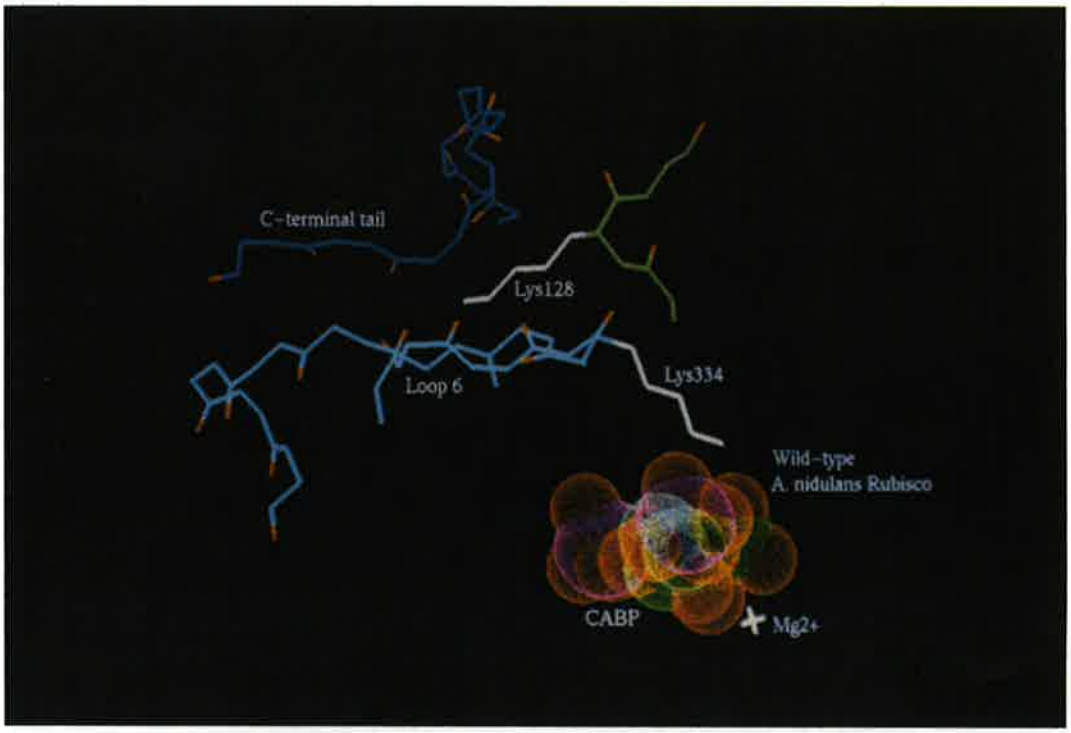

Fig. 3. The position of the lysine 128 side chain sandwiched between the C-terminal loop 6 and the C-terminal tail of the opposing large subunit within the dimer and above the transition state intermediate CABP. Structure based on the coordinates supplied by C.I. Branden for spinach Rubisco.

Soper *et al.*, 1992). The region around lysine 128 is highly conserved; phenylalanine 127 and lysine 128 are absolutely conserved in all sequences so far examined. Lysine 128 was changed to arginine, glycine, asparagine, histidine, and glutamine to determine whether its contribution was steric or due to the functional side chain. When the genes for the mutant enzymes were expressed in *E. coli*, all of them produced assembled, active Rubisco which had the activation characteristics of the wild type. This was surprising given the absolute conservation of the residue. Only the K128R was capable of catalysing the carboxylation of RuBP at a reasonable rate, and the $K_{cat\ carboxylation}$ was about 30% of the wildtype (Table 3). The $K_{cat\ carboxylation}$ of the other enzymes were very low, less than 5% of the wild type. Specificity factors were determined using [1-^{14}C] RuBP, as substrate. The reaction products were separated by HPLC. In the mutant with similar positive polarity (K128R) to the wild type Rubisco, τ was unchanged. Whilst removal of the side chain (K128G) only slightly decreased τ, the presence of less positively charged (K128H), bulky (K128H; K128Q) or negatively charged (K128D) side-chain drastically reduced τ and enzyme activity. Modelling studies indicate that lysine 128 can not play a direct role in the reaction of the enediolate with the gaseous substrates, but mutation of the residue may interfere with the ability of lysine 334 to achieve this. However, confirmation of this requires structural information.

Table 3. *The substrate specificity factor for the gaseous substrates and* $K_{cat\ carboxylation}$ *of* Anacystis nidulans *Rubisco in which Lysine residue 128 has been substituted by site directed mutagenesis*

The sequence number preceded by the single-letter representation for the wild-type residue at that position is followed by the single letter representation for the replacement. The figures in parenthesis are the standard errors for each curve.

Rubisco	$K_{cat\ carboxylation}$[a]	τ
Wild type	7.2 (0.15)	46.3 (0.9)
K128R	2.9 (0.30)	42.5 (1.4)
K128G	0.15 (0.01)	38.3 (1.7)
K128Q	0.23 (0.05)	6.7 (0.7)
K128H	0.22 (0.01)	6.1 (0.9)
K128D	0.13 (0.08)	5.1 (1.1)

[a] Mol CO_2 mol^{-1} Rubisco active site s^{-1}.

Conclusions

The use of X-ray structural analysis and mutagenesis has enhanced understanding of the structure and mechanism of Rubisco. The promise of a less promiscuous and more active Rubisco appears feasible. In just a few years, residues that alter τ have been identified and manipulation

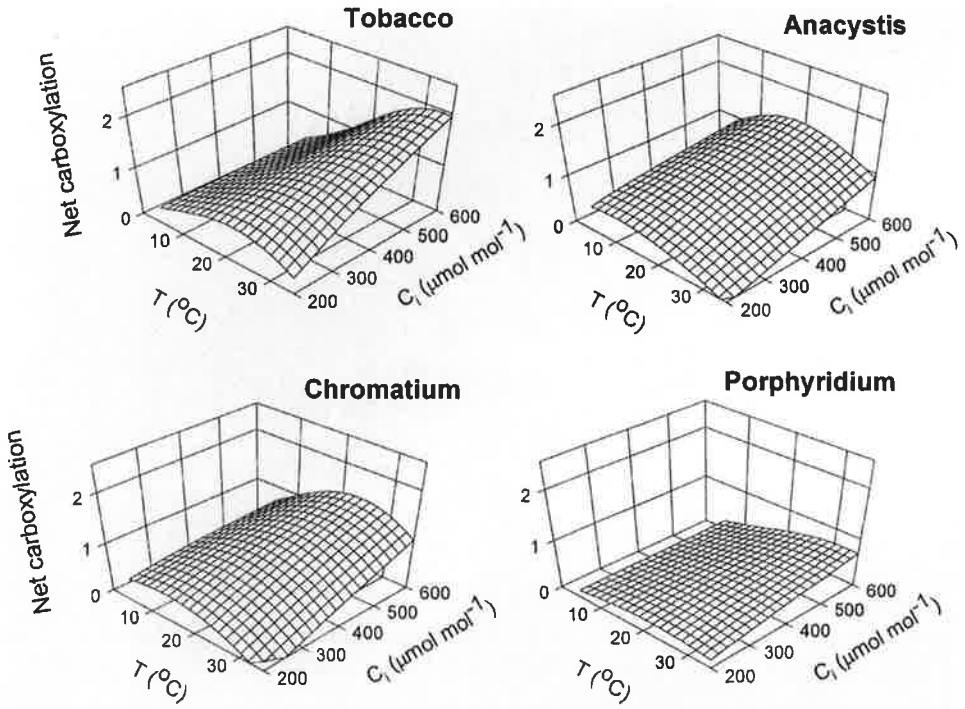

Fig. 4. Model predictions of the net carboxylation rates [mol CO_2 mol^{-1} Rubisco active site s^{-1}] in leaves containing different Rubiscos. The rates are calculated for a range of intercellular CO_2 concentrations (Ci; typically 0.7–0.8 of external air CO_2 concentration in unstressed leaves) with 0.21 mol mol^{-1} O_2 and a range of temperatures.

of some of these has increased τ without always adversely affecting turnover. However, many further mutants and the atomic structures of existing and new mutant Rubiscos are needed to understand better the structural and electronic components determining specificity factor and how to improve it further without reducing turnover. The production of more mutants will also increase the chance of finding better enzymes. The enabling technologies are available and the goal of a better enzyme remains a target of great agronomic importance.

Whilst to most protein engineers even the differences in kinetic properties between Rubisco from various species are rather small, such small differences in properties would have major effects on photosynthetic rates. It is possible to model the likely effects of these differences. Figure 4 compares predicted performance of such hypothetical leaves in which the native Rubisco has been replaced with Rubisco from *Porphyridium aerugineum*, *Chromatium vinosum* and *A. nidulans* with those of *Nicotiana tabacum* containing its own Rubisco. The calculations assume that photorespiratory metabolism is adequate to deal with the changed oxygenase activities introduced. Surfaces were generated assuming that (1) the solubilities of O_2 and CO_2 in the stroma are similar to those in pure water; (2) the temperature dependency of the kinetic parameters of the different Rubiscos is the same as that of *S. oleracea* Rubisco (Jordan and Ogren, 1984); (3) RuBP concentrations are saturating, and (4) all the Rubisco is fully activated. At current air concentrations of CO_2 and temperatures above 15 °C none of the alternative Rubiscos are predicted to be as efficient *in vivo* as that from tobacco (Fig. 4). However at elevated CO_2 concentrations and lower temperatures, Rubisco from *C. vinosum* and *A. nidulans* should out-perform the tobacco Rubisco. Thus, even comparatively modest changes in catalytic properties, achieved by genetic manipulation of Rubisco genes, may be exploited by using chloroplast transformation or by targeting large subunits to the chloroplast after nuclear transformation. Both procedures could markedly improve the efficiency of crop production in given environments.

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