Invited Trends Article:

Rubisco*, an Old Challenge with New Perspectives

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I. Introduction

The importance of Rubisco derives from its special role as the main catalyst for the flow of carbon dioxide from the inorganic sphere to the plant kingdom. Since its discovery as fraction I protein fifty years ago (Wildman and Bonner, 1947) and its role as the key enzyme in the Calvin cycle forty years ago (Quaile *et al.*, 1954; Weissbach *et al.*, 1954) it attracted attention for a series of specific reasons concerning its enzyme mechanism, its regulation and its structural features. Twentyfive years ago the bifunctionality of this enzyme was discovered by the fact that it catalyses not only CO_2 fixation but also the RuBP oxygenase reaction, and therefore, Rubisco is also the key enzyme for the photorespiration (Bowes *et al.*, 1971). Since photorespiration is generally considered a wasteful process due to the loss of fixed carbon and the additional consumption of energy, partitioning of carbon between Calvin cycle and the photorespiratory pathways plays a crucial role in the efficiency of photosynthesis (Zelitch, 1973).

The predominant role of this enzyme as an important research area in plant biochemistry can be illustrated by the vast and still growing number of communications of sequence data (above 1500) and of twelve reports of X-ray structures (enzymes from photosynthetic bacteria, cyanobacteria and higher plants). The recent renaissance of Rubisco research should lead to a better understanding of its mechanisms of function by a combined effort of site-specific mutagenesis, structural analysis and chemical analysis of side and abortive reaction products. Recent advances in comprehending the mechanisms of catalysis of both reactions make it feasible to develop strategies for „improvement" of the enzyme with respect to its relative specificity for CO_2 as opposed to O_2, and its catalytic turnover rates in organisms with low specificity or low turnover rates. Genetic engineering can be used to manipulate partitioning of carbon flow between photosynthesis and photorespiration in those organisms.

The key to these challenges is the evolutionary variety of Rubiscos with different properties. The enzyme from the nonsulfur purple bacteria *Rhodospirillum rubrum* consists of only two large subunits (type II Rubisco) whereas the enzyme of cyanobacteria, green algae and higher plants are assembled of eight large subunits and eight small subunits (type I Rubisco). The bifunctional enzyme changed its specificity toward a better carboxylase during the evolution of photosynthetic organisms. The lowest values for the ratio of $V_c.K_o$ / $V_o.K_c$ (specificity factor, abbreviated: SF; see Scheme 1) is found with the enzyme isolated from *R. rubrum* (SF values of 10 to 15), whereas high values (SF of about 80) were obtained with enzymes isolated from higher plants (Jordan and Ogren, 1981). The price for the better carboxylase / oxygenase ratio of enzyme activities is paid for by a parallel drop in the k_{cat} of the carboxylase reaction (Bainbridge *et al.*, 1995). Negative correlation between specificity and turnover rates must be seen, it seems, in conjunction with both enzyme mechanisms.

The question why the enzyme of unicellular organisms has a lower specificity factor compared to multicellular organisms, still needs to be answered.

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* Rubisco: ribulose 1,5-bisphosphate carboxylase/oxygenase; E.C. 4.1.1.39.

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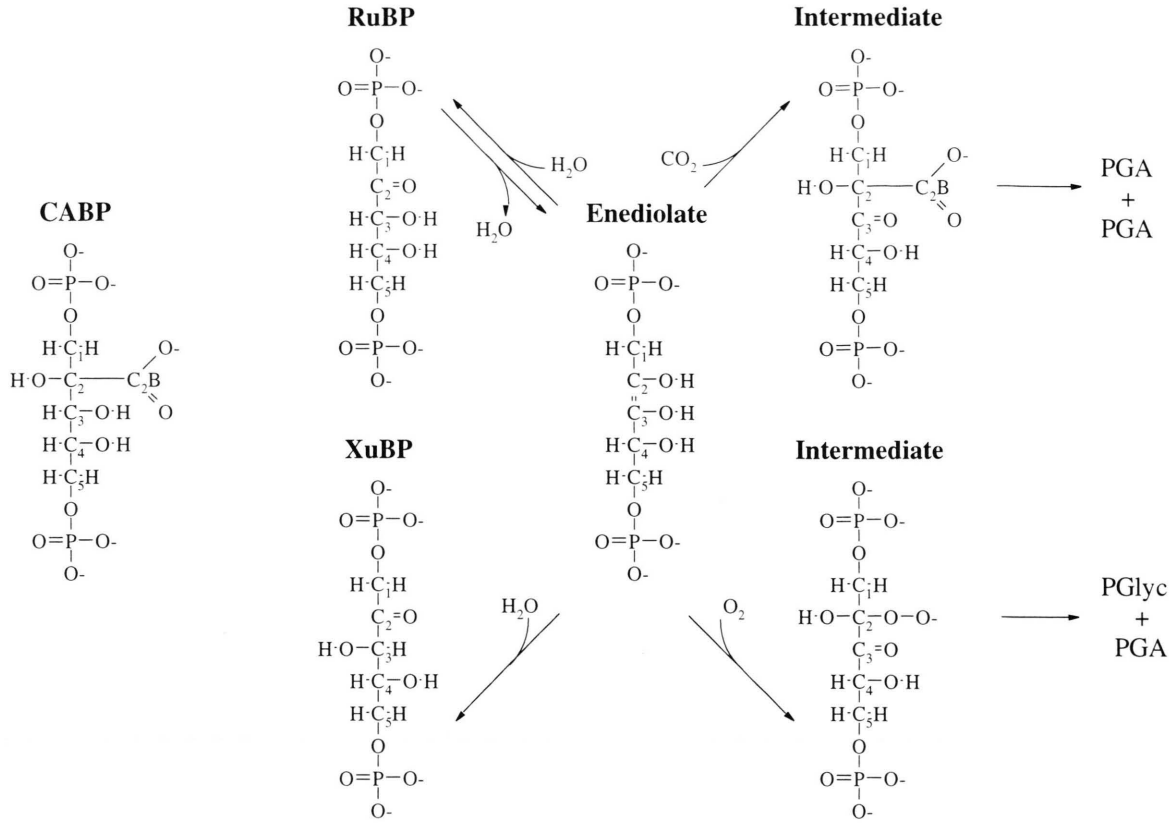


Scheme 1.

In order to elucidate the crucial differences between Rubisco enzymes of these two groups we have compared the structure of enzymes with different specificity factor. Our analysis includes the amino acid residues of the active center and the positions of the atoms of the transition state analog, CABP (2-carboxy-D-arabinitol 1,5-bisphosphate), in relation to the active center. The active center is located in the C-terminal α/β barrel domain of the large subunit and residues from the N-terminal domain of the other large subunit of the functional dimer are also part of the active center (Schneider *et al.*, 1986; Chapman *et al.*, 1988; Andersson *et al.*, 1989). The comparison of the primary sequences of Rubiscos from different organisms is not sufficient and has to be supplemented by structural comparison as achieved by superimposition of the x-ray structure data sets (presented in Section IV). This analysis will lead to stereotypic characterization of the interaction network between CABP and the protein shell for a Rubisco with high specificity and an enzyme molecule with low specificity.

II. How to Change Enzyme Specificity

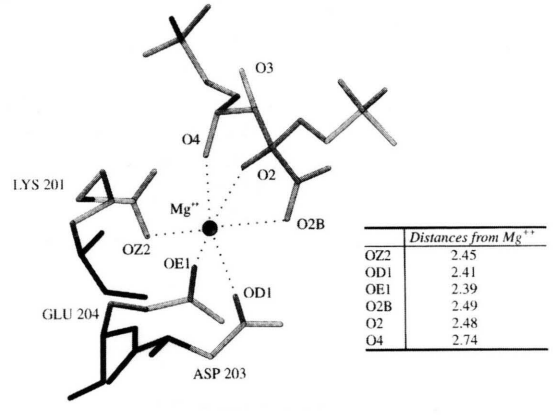
Any explanation for the Rubisco specificity has to take into account that the specificity factor of a pertinent enzyme is not an invariant property of the molecule but can be altered. For example, it can be altered by replacement of the active-site metal ion, Mn^{2+}, instead of Mg^{2+} (Wildner and Henkel, 1978; Jordan and Ogren, 1983) or by substitution of amino acid residues (see for a review Spreitzer, 1993), or simply by changing the reaction temperature (Jordan and Ogren, 1984).

II.1 Rubisco-metal ion complex and specificity

Rubisco is activated by pretreatment with bicarbonate and Mg^{2+} ions forming an enzyme-carbamyl Mg^{2+} complex. The carbamylation of the ε-amino group of K 201 residue (respectively K 191

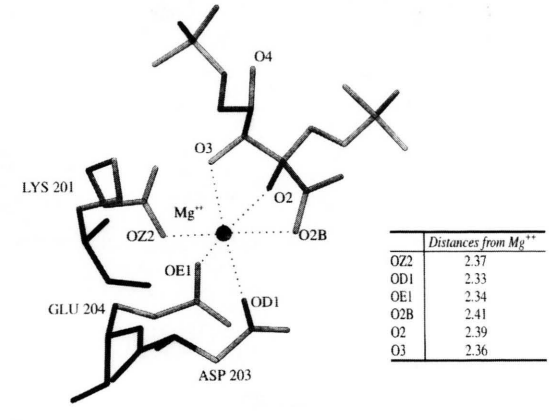
in *R.rubrum*) is stabilized by the binding of Mg^{2+} (Lorimer *et al.*, 1976). The environment of the Me^{2+} in the ternary complex of spinach Rubisco (Knight *et al.*, 1990) is determined by a coordinated octahedral complex with the following ligand residues (Fig. 1a): the hydroxyl groups of C-2 and C-4 of the transition state analogue CABP, the carbamate of K 201, the residues D 203 and E 204 and an additional ligand position for the reaction intermediate (-COO^- or -O-O^-). The ligand field is similar for the *Synechococcus* enzyme (Newman and Gutteridge, 1993), except for the participation of the hydroxyl group of C-3 instead of C-4 as observed with spinach Rubisco (Fig. 1b). It seems that the Mg^{2+} ion has at least a double role: it participates in both the binding and the conversion of substrate. Mg^{2+} directs the substrate into the correct binding position, because non-activated enzyme molecules bind the substrate analog (CABP) in an inverted fashion (Lundqvist and Schneider, 1989). Beside the positioning effect, Mg^{2+} serves also in the polarization of the carbonyl group at C-2 to facilitate the enolization, and furthermore, stabilizes the reaction intermediate by complexing either the carboxyl group or the hydroperoxide.

a



	Distances from Mg^{++}
OZ2	2.45
OD1	2.41
OE1	2.39
O2B	2.49
O2	2.48
O4	2.74

b



	Distances from Mg^{++}
OZ2	2.37
OD1	2.33
OE1	2.34
O2B	2.41
O2	2.39
O3	2.36

Fig. 1. Octahedral coordination complex of the active site Mg^{2+} with CABP and amino acid residues of spinach (Fig. 1a) and *Synechococcus* (Fig. 1b) Rubisco. In the insert the distances (in Å) of the ligand oxygen atoms from the central Mg^{++} ion were computed by Insight 95.0 with the 8RUB.pdb and the 1RBL.pdb files. The numbers of the amino acid residues correspond to the spinach sequence.

The metal ion can be replaced by other divalent cations. The enzyme can accomodate in addition to Mg^{2+} also Ca^{2+}, Ni^{2+}, Co^{2+}, Cr^{2+}, Fe^{2+}, Cu^{2+}, and Mn^{2+} (Andrews and Lorimer, 1987). The question whether the replacement of Mg^{2+} by redox-active metal ions such as Mn^{2+} or Co^{2+} could change the specificity of the Rubisco has been addressed. The E-CO_2-Mn^{2+} complex of higher plants has a specificity factor value which is twenty times lower as the one for the appropriate E-CO_2-Mg^{2+} complex from the same Rubisco (Jordan and Ogren, 1983).

The E-CO_2-Co^{2+} complex in *R.rubrum* functions only as an oxygenase without detectable carboxylase activity (Christeller, 1981). The interpretation of the EPR spectra of the E-CO_2-Me^{2+}-CABP complexes with Mn^{2+}, Co^{2+}, and Cu^{2+} focussed on the highly distorted coordination system of the metal ion (Styring and Bränden, 1985). This structural distortion would be evident in X-ray studies of the activated enzymes, complexed with the substrate analog, CABP. Such investigations have not been carried out so far.

Another explanation was offered (Chen and Spreitzer, 1992) for the Mn^{2+} effect on the specificity, proposing the involvement of Mg^{2+} preferably in the carboxylation reaction (higher effective cationic charge) while the involvement of Mn^{2+} was preferred in the oxygenase reaction (facilitation of the activation of triplet O_2 into singlet O_2). A switch from a free-radical mechanism in presence of Mg^{2+} to a more favorable ionic mechanism in presence of Mn^{2+} was assumed.

II.2 Site-specific mutations and specificity

The search for Rubisco with improved kinetic properties which overcome the rate-limiting step

in CO_2 assimilation triggered a series of site-specific mutation experiments. Since the determinants of the enzyme for the partitioning are not known single amino acid residues have been picked either randomly or by selection, utilizing the experiences of chemical modification experiments. In course of time when increasing informations about the structures of Rubisco were available it was possible to pick out not only residues with a functional role in enzyme catalysis but also residues on the basis of steric considerations. Several structural elements of Rubisco have been selected for amino acid residue substitutions: N-terminal region, loop 6, and loop 7 which are in close proximity to the active center. The substitutions and their impact on the specificity factor are listed in Table I and the location of the mutated amino acid residues are presented in Fig. 2.

The catalytic center is located at the interface between the large subunits of the L_A L_B dimer. Despite the fact that most of the amino acid residues of the active center belong to the C-terminal α/β barrel of large subunit A, there are a few residues of the N-terminal domain of large subunit B which play a significant role in substrate binding as well as in interaction with loop 6 (Knight *et al.*, 1990). A highly conserved region of the N-terminus forms a loop (residues 58 to 68) which is very close to the active center. This region is disordered in non-activated enzyme but shows a definite structure in the activated E-CO_2-Mg^{2+} form

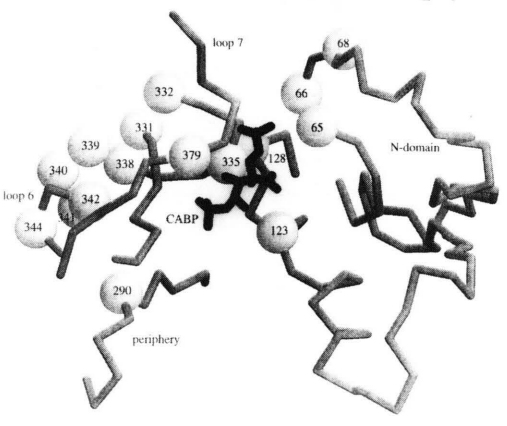


Fig. 2. Mutation-sites of Rubisco with altered specificity.The protein with the CABP molecule is presented with the N-terminal domain, C-terminal periphery, loop 6 and loop 7. The sites listed in Table I were documented by Rasmol, version 2.5 using the file 8 RUB.pdb.

(Schneider *et al.*, 1986). Residues T 53 and N 54, in combination with residues G 370,G 393 and G 394 participate in the binding of the P_A – phosphate group of CABP via hydrogen bonds in *R.rubrum* (Hartman and Harpel, 1994). Amino acid substitutions (T53A, N54A and N54W) have caused a decrease in the enzyme specificity. The same threonine residue in the L_8S_8 enzyme of *Synechococcus* has been changed and all substitutions of T 65 are linked to a decrease in specificity; the change to Ser (Table I, line 4) has the least pronounced effect. It can be concluded that the firm anchoring of the P_A-phosphate group of RuBP and the substrate analog CABP in the binding niche prevents greater flexibility of the substrate and, by that, a drop in SF value. An even more drastic effect is obtained by severely weakening the binding forces for the P_C-phosphate group resulting in the exchange of R 292 to Leu or Lys. This substitution is accompagnied by a complete loss of enzymatic functions (Haining and McFadden, 1990).

Amino acid residue N 111 (L2), respectively N 123 (L8S8) is located in the second conserved stretch of the N-terminus which is envolved in enzyme catalysis. The side chain of this residue was identified as part of the ligand field surrounding the Mg^{2+} ion in the active ternary complex (Lundqvist and Schneider, 1991; Schneider *et al.*, 1992). Upon binding of CABP (or RuBP) to the enzyme this position is taken by a hydroxylgroup of CABP (or RuBP) and in exchange a hydrogen bond is formed between the amide group of asparagine and the O-4 group of CABP (or RuBP) as observed for the spinach Rubisco or the O-3 group of CABP for the *Synechococcus* Rubisco. Substitutions of this residue caused a significant decrease in specificity (Chene *et al.*, 1992; Zhu and Spreitzer, 1994).

Residue K 128 in *Synechococcus* (corresponding to K 125 in higher plant large subunit) is strictly conserved, nevertheless, it is not envolved directly in either the carboxylase or oxygenase reaction mechanism. The residue is situated in a N-terminal domain which forms a loop with the α-helix G followed by a β-sheet stretch. It is in close proximity to both the C-terminal tail and the loop 6 region because the side chain of K 128 is fully extended over this loop 6. A conservative substitution to Arg has little effect on the speci-

Table I. Site-specific mutations and Rubisco specificity.

Mutant	Organism	Segment	Specificity change WT	Mutant	Reference
T 53 A (65)	R.r.	N-domain	11	7	a)
N 54 A (66)	R.r.	N-domain	11	9	a)
N 54 W (66)	R.r.	N-domain	11	6	a)
T 65 S (68)	S.	N-domain	43	37	b)
T 65 A (68)	S.	N-domain	43	22	b)
T 65 V (68)	S.	N-domain	43	19	b)
N 111 G (123)	R.r.	N-domain	6.7	0.6	c)
N 123 G	C.r.	N-domain	64	12	d)
K 125 R (128)	S.	N-domain	46	42.5	e)
K 125 G (128)	S.	N-domain	46	38	e)
K 125 Q (128)	S.	N-domain	46	7	e)
K 125 H (128)	S.	N-domain	46	6	e)
K 125 D (128)	S.	N-domain	46	5	e)
L 290 F	C.r.	C-domain	62	54	n)
V 331 A	C.r.	loop 6	62	39	f)
V 328 A (331)	S.	loop 6	46	45	g)
V331 A T342 I	C.r.	loop 6	62	52	f)
V331A G 344S	C.r.	loop 6	62	45	h)
V 328 G (331)	S.	loop 6	56	20	i)
V 328 A (331)	S.	loop 6	56	30	i)
V 328 L (331)	S.	loop 6	56	49	i)
V 328 M (331)	S.	loop 6	56	16	i)
L 329 M (332)	S.	loop 6	43	20	j)
L 329 I (332)	S.	loop 6	43	14	j)
L 329 T (332)	S.	loop 6	43	17	j)
L 329 T (332)	S.	loop 6	43	28	j)
L 329 A (332)	S.	loop 6	43	19	j)
M 330 L (335)	R.r.	loop 6	7.2	7.7	k)
M 330 L (335)	R.r.	loop 6	7.4	6.3	k)
D 335 E (338)	S.	loop 6	46	48	g)
K 336 P (339)	S.	loop 6	39	38	l)
A 337 E (340)	S.	loop 6	46	38	g)
A 337 L (340)	S.	loop 6	39	36	l)
S 338 I (341)	S.	loop 6	46	45	g)
S 338 M (341)	S.	loop 6	39	42	l)
T 339 I (342)	S.	loop 6	39	32	l)
T 339 V (342)	S.	loop 6	39	30	l)
T 339 L (342)	S.	loop 6	56	50	i)
T 339 M (342)	S.	loop 6	56	52	i)
S 379 A	C.r.	loop 7	64	18	d)
S 368 A (379)	R.r	loop 7	11	17	m)

The mutations were put in order by their sequence number and the number in parenthesis refer to the spinach sequence number.
Abbreviations for organisms: R.r. – *Rhodospirillum rubrum*, S. – *Synechococcus*, and C.r. – *Chlamydomonas reinhardtii*.
The index for the references as listed: a) Larimer *et al.*, 1994; b) Morell *et al.*, 1994; c) Chene *et al.*, 1992; d) Zhu and Spreitzer, 1994; e) Bainbridge *et al.*, 1995; f) Chen and Spreitzer, 1989; g) Parry *et al.*, 1992; h) Chen *et al.*, 1991; i) Gutteridge *et al.*, 1993; j) Lee *et al.*, 1993; k) Terzaghi *et al.*, 1986; l) Read and Tabita, 1994; m) Harpel and Hartman, 1992; n) Chen *et al.*, 1988).

ficity but causes a 70% reduction of k_{cat}(carboxylase). This is an example indicating that mutations can effect the turnover rate but not partitioning. A change from a positively charged amino acid residue to a negatively charged residue (for example K 125 D, Table I, line 13) re-

sults in the decrease of both specificity and turnover rate (Bainbridge *et al.*, 1995).

An interesting „low-specificity" mutant (L290F) has been isolated following random mutagenesis of *C.reinhardtii* cells (Chen *et al.*, 1988). This mutant showed temperature-sensitivity, i.e. no photosynthetic growth was observed at temperatures above 32°. The site of the substitution was located at the periphery of the large subunit, distant to the active center. This mutation has been reproduced by chloroplast transformation to exclude pleiotropic effects and to investigate the cause of temperature sensitivity (Wildner *et al.*, 1991). Analysis of the reaction products of the carboxylase reaction documented increased levels of XuBP which is the product of the „misfire"-reaction, the misprotonation of the enediol at C-3 in the backreaction (Scheme 1). The steric disturbance of the larger phenyl residue at the periphery has a long range effect on the geometry of the active center causing apart from low specificity also the „misfire" reaction.

The quest for an enzyme with higher carboxylase activity focussed mainly on the mobile loop 6 region.since this loop 6 plays a fundamental role in Rubisco catalysis. The functional role of loop 6 during the catalytic cycle can be assigned to the seclusion of the active center. This flexible loop 6 is in an open position at the beginning of the reaction cycle and closes following the binding of RuBP. Loop 6 covers as a lid the barrel and prohibits access to the catalytic center (Knight *et al.*, 1990). The amino acid residue K 334 at the apex of loop 6 participates in the stabilization of the transition state intermediate for both reations (Harpel *et al.*, 1995). Mutations in this region of *R.rubrum, Synechococcus* or *C. reinhardtii* have been reported and are summerized in Table I. The list in Table I includes those mutants which show substantial carboxylase activity.

The loop 6 region with the two hinges opens and closes like a flap during the catalytic cycle. The isolation and the characterization of the *C.reinhardtii* mutant V 331 A (Chen and Spreitzer, 1989) stimulated the search for novel carboxylase mutants in this field because a decline in specificity due to the change of Val to Ala could be partially compensated by an additional mutation, V331A plus T342I (Chen and Spreitzer, 1989). The partial restoration was interpreted by structural arguments, the gap between the Ala-Thr contact might be offset by a larger Ile residue side chain. This example of steric influence of protein changes on Rubisco catalysis shows how drastic enzyme activities may change in response to substitutions of amino acid residues which do not even participate in the reaction catalysis.

The same mutation in *Synechococcus* has almost no effect on the specificity (Table I, line 15) according to one group (Parry *et al.*, 1992) but experiments from another group (Gutteridge *et al.*, 1993) showed similar results as in *C.reinhardtii* with a twentyfold decline in k_{cat} of the carboxylase reaction.

Substitution experiments with L 332, D 338, K 339, A 340, S 341, and T 342 reveal that single changes in the environment of the active center can have a variable effect at the enzyme specificity. A small increase of the specificity factor value in the 5% range is difficult to assess since the different methods to determine this value are incriminated by higher error margins (Zhu *et al.*, 1992; Kostov and McFadden,1995).

The move from single site-specific mutations experiments to the exchange of segments was considered a step further in the search for determinants of higher specificity. The multiple substitution of four amino acid residues at the loop 6 region (DKAS in *Synechococcus*, Parry *et al.*, 1992) was carried out by replacement with the stretch of four amino acids from higher plants (ERDI spinach 336–340; EREI pea or maize 336–340). The substitution of the *Synechococcus* segment caused only a 3–7% increase in specificity for both, but a 40% reduction of k_{cat} in the case of ERDI (Gutteridge *et al.*, 1993), respectively a 8% decrease in k_{cat} for EREI (Parry *et al.*, 1992).

The loop 7 stretch contains a S 379 (Fig. 2) residue which participates in the binding of CABP (or RuBP) at the P_C-phosphate binding site in *R.rubrum* (Lundqvist and Schneider, 1991) and also in Rubisco of higher plants (Schneider *et al.*, 1992). An additional role of the γ–hydroxyl group of Ser may be assigned to the formation of a hydrogen bond to a hydroxyl group of CABP (Schneider *et al.*, 1992). This strictly conserved amino acid residue in the catalytic center was of special interest for site-specific mutation experiments due to its interaction with the substrate.

The S 368 A mutant exhibited an 60% increase in the specificity factor in the experiment with *R.rubrum* (Harpel and Hartman, 1992) but a contradictory 70% decrease in *C.reinhardtii* (Zhu and Spreitzer, 1994). The change of specificity is linked for both organisms to an impaired carboxylase activity (50 fold lower in *R.r* and 25 fold lower in *C.r.*). The obvious discrepancy has to be resolved and warns for simplified transference of experimental evidence from the L2 (type II) Rubisco to the L8S8 (type I) enzyme.

Site-specific mutation experiments yielding a S 376 A mutant have been carried out in *Synechococcus* (Lee and McFadden, 1992). The cells have been almost devoid of carboxylase activity, but retained 16% of their original oxygenase activity and showed a drastically lowered specificity factor (value not published).

The experiments with mutagenized small subunits have not been included in Table I. The problem if and how the small subunits can influence the specificity of Rubisco is still in question. The impact of the stretch (87–92) of the small subunit on the specificity was investigated by site specific mutations in *Synechococcus* (Read and Tabita, 1992). The mutations I87V, R88K, G91V and F92L (not listed in Table I) did not significantly alter the specificity (in the margin of more or less 10% of the wild-type value) but showed drastic losses (90 to 50%) of the V_{max} values for the carboxylase and the oxygenase reaction.

Drastic changes of amino acid residues at the interface between small and large subunits can have disadvantageous effects on the enzyme structure causing also a decrease in specificity. So far no mutation of small subunits have ben described with a positive effect on the carboxyylase *versus* oxygenase reaction.

All the mutation experiments in Rubisco yielded a vast wealth of informations concerning the role of the altered amino acid residues and their influence on enzyme catalysis for both reactions. Also „lethal" mutations which knocked out completely the overall reactions showed interesting details of partial enzyme reactions.

In summary it can be stated that the best model systems for genetic engineering of Rubisco turned out to be *R.rubrum*, *Synechococcus* and *C.reinhardtii*. Plasmids carrying the rbcL and rbcS genes of *R.rubrum* and *Synechococcus* can be easily expressed together in *E.coli*, and both subunits are properly assembled to yield a catalytic functional enzyme (Gutteridge and Gatenby, 1995). The advantage to employ *C.reinhardtii* as a model system for higher plant enzyme is connected to the use of the biolistic transformation procedure which allows the genetic manipulation of the chloroplast (see for a review Boynton and Gillham,1993). Plasmids carrying site-specific mutations in the rbcL gene can be incorporated into the plastom by homologous recombination. Furthermore, the isolation of intragenic suppressor mutants is an additional instrument to study genotype – phenotype relations (Chen and Spreitzer, 1989).

II.3 Reaction temperature and specificity

The specificity of Rubisco is dependent on the reaction temperature, i.e. with increasing temperature the enzyme specificity decreases significantly (Jordan and Ogren, 1984). Their experimental data with Rubisco isolated from spinach showed that the value of V_O / K_O increased more rapidly than the value for V_C / K_C with increasing temperature in the range from 7° to 35°. The specificity factor is determined by the ratio of the second order rate constants k_C and k_O ($SF=k_C / k_O$) for the irreversible carboxylase and oxygenase reaction (Pierce *et al.*, 1986), and therefore, the specificity factor can be related to their activation energies of the carboxylase and the oxygenase reactions: $RT \ln SF = \Delta G_C^{\neq} - \Delta G_O^{\neq}$, the free activation energy difference between both transition states (Chen and Spreitzer, 1991). The plot ln SF *versus* 1/T yields a straight line and from the slope a value of -21 kJ/mol can be determined for the difference in activation enthalpies between the carboxylase and the oxygenase reaction using the published data set of spinach Rubisco (Jordan and Ogren, 1984). The difference of free activation energies was calculated from the same data set as -11 kJ/mol and the difference in activation entropies as -34 J/mol. K°. Those differences are usually related to differential stabilization of the transition state intermediates. The X-ray structural analysis suggests that the carboxyl group of CABP as the transition state analogue is oriented toward the K 334 residue and the central Mg^{2+}. The orientation of the peroxy transition state intermediate is unknown but on the basis of ESR studies it is likely

that the peroxy group participates in the ligand field of Me^{2+} (Miziorko and Sealy, 1984).

Comparing the specificity from organisms with the lowest value (*R.rubrum* SF=10) and the highest values for higher plants (wheat, rice SF=100) the free energy difference is only 5.7 kJ/mol, which is in the order of a single hydrogen bond (Lorimer *et al.*, 1993).

The enigma of structural similarities in the active center of all Rubisco molecules and nevertheless, the significant differences in catalytic rate constants awaits to be solved.

III. Mechanistic Views

A sequence of five partial reactions can be depicted for the carboxylase reaction: deprotonation of C-3 and enolization of RuBP, carboxylation, hydration, C-2 - C-3 cleavage, and protonation of C-2, to yield two molecules of 3-phosphoglycerate (Andrews and Lorimer, 1987). It is well accepted that the transition intermediate for the carboxylase and for the oxygenase reaction is the enolized form of RuBP. After deprotonation of the C3 – H bond, a C-2,C-3 – *cis* enediol is formed whereby the oxygen atoms participate in the ligand field of the central Mg^{2+} ion (Gutteridge and Lundqvist, 1994).

The enediol can react in four different ways: two reactions at the same C-2 atom, the carboxylation with CO_2 and the oxygenation with O_2, and two backreactions at the atom C-3, the reprotonation yielding RuBP or XuBP depending from which side the proton is added (Scheme 1).

The structural requirements for the enolization reaction were investigated by altering Rubisco: a loop 6 deletion mutant of *R.rubrum* was constructed (Larson *et al.*, 1995). This mutant was still able to catalyze the enolization despite its total impairment of the carboxylase activity. It can be concluded, that the binding and deprotonation reaction occurs before the loop 6 flap closes and entraps the enediol-RuBP.

The deprotonation of C-3 – RuBP was verified by isotope exchange studies very early (Fiedler *et al.*, 1967; Saver and Knowles, 1982) but the deprotonating agent is still not identified. Several candidates could serve as an acceptor for the proton of C-3: P_A-phosphate group, His-294 plus bound H_2O near C-3, or the carbamino oxygen of the active site K-201 residue (Schneider *et al.*, 1992; Gutteridge *et al.*, 1993).

The addition of the gaseous substrates CO_2 or O_2 occurs as a chemical reaction and they do not form a Michaelis complex. However, a transient Mg^{2+} – CO_2 complex close to the C-2 atom of the enediol – RuBP is discussed (Schneider *et al.*, 1992) but no formal binding sites for carbon dioxide and oxygen have been identified on the protein (Pierce *et al.*, 1986). In contrast to the enolization reaction the addition of CO_2 or O_2 to the enediol of RuBP occurs irreversibily. The transition state adduct (2-carboxy or 2-peroxy RuBP)is stabilized by ionic interaction with the positive charge on the ε-NH_3^+-group of K-334 at the apex of loop 6 and the central Mg^{2+} (Schneider *et al.*, 1992). This residue is absolutely essential for the catalytic activity of both reactions. Even a conservative substitution of K-334 to Arg in *Synechococcus* reduced drastically the carboxylase activity (99.5%) and the specificity (200fold) (Gutteridge *et al.*, 1993). The exchange of K-334 to Gly in *R.rubrum* showed also no carboxylase activity but the catalytic function of the enzyme in the enolization process and the final step of the cleavage of the 3-keto-CABP to PGA was still observed (Hartman and Lee, 1989; Lorimer *et al.*, 1993).

Besides the rapidly growing information from site-specific mutants in organisms with different specificity an additional avenue opens for better understanding the enzyme mechanism by studying side reactions. Significant progress has been achieved in the chemical analysis of the reaction products by modern methods of anion separation and detection techniques to enable the detection of minor side reaction products. The „Fallover" phenomenon, a decline of the carboxylase activity despite sufficient substrate concentrations, was linked to the phenomenon of „misfiring", the rise of XuBP due to misprotonation of the reaction intermediate (Edmondson *et al.*, 1990; Zhu and Jensen, 1991). The combination of site-specific mutagenesis and reaction product analysis appears to be a new powerful tool to shed light on the unsolved puzzle of the structural basis for the diversities of Rubisco molecules concerning carboxylase, oxygenase and epimerase.

IV. Steric Insights

We chose the approach to compare three dimensional structures of activated Rubisco with

bound CABP from a low specificity enzyme (*Synechococcus*, Newman and Gutteridge, 1993) and a high specificity enzyme (spinach, Knight *et al.*, 1990) to deduce significant features of structural differences. The goal of those studies is to learn more about structural requirements for the orientation of CABP as a model for the substrate intermediate in the protein shell of the active center. The analysis focussed especially on the position of the atoms of the CABP molecules and the amino acid residues of the active center (Fig. 3). The modelling studies were performed with the Biosym Insight 95.0 software tool. After construction of the functional dimers from the X-ray data (8RUB and 1RBL, Protein Data Bank) one CABP molecule and a 10 Å environment shell was selected for further investigation. This environment comprises not only parts of the C-terminal α/β–barrel of the large subunit where the CABP molecule is embedded, but also few amino acid residues of the N-terminal part of the adjecent large subunit forming the functional dimer. A total of 109 amino acid residues was selected consisting of 1544 atoms for *Synechococcus*, and 95 residues with 1473 atoms for spinach. While the substrate conformations differ clearly, the protein environments exhibit a surprising similarity (Fig. 3). It turned out that it was sufficient to superimpose the phosphate P_A and P_C – groups and the Mg^{2+}-ion of both structures (*Synechococcus* and spinach) in order to reach an almost perfect agreement of the position of all amino acid residues which are in direct contact to the substrate molecule. Like for the P_A and P_C-phosphate groups, also the carboxyl group at the C-2 atom were found situated in identical positions (Fig. 4). The major discrepancies between both molecules were discovered in the positions for the C-3 and the O-3 and for the C-4 and O-4 atoms which look in diametral (opposite)directions as seen in the Fig. 4. The deviation of the O-3 atoms, as measured as dihedral angle O3-$C3_{spin}$- $C3_{syn}$-O3 corresponds to an angle of 114.6°, respectively -152.2 ° for the O-4 atoms (Fig. 4). The different pyramidalization of the C-3 atoms can be recognized by measuring the dihedral angle O2 – C2 – C3 – O3 which is equal to 67° for CABP in the spinach structure and -49° for CABP in the *Synechococcus* structure. A similar twist can be recorded for the dihedral angle O3 – C3 – C4 – O4, for spinach CABP -142°, respectively for *Synechococcus* CABP 131.6°(Fig. 4).

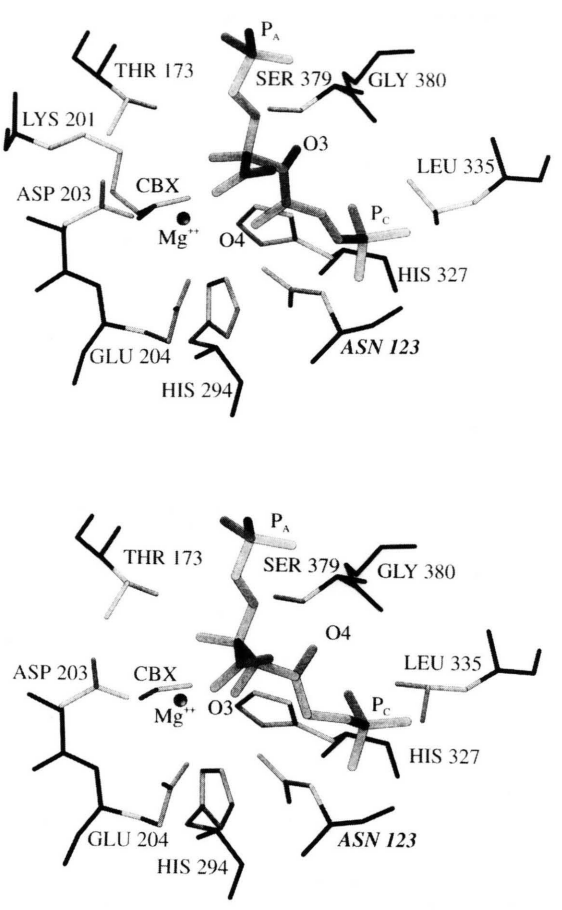


Fig. 3. View of the Rubisco active center with hetero atoms (Mg^{2+}, CBX and CABP) for spinach (Fig. 3a) and *Synechococcus* (Fig. 3b). The residues were presented with the backbone in black ink and the side chains in gray colors. All residues belong to the C-terminal α/β–barrel, except *Asn 123* of the N-terminal domain of the other large subunit.CBX is the abbreviation for the carbamyl-group of Lys 201; P_A and P_C are the phosphate groups of CABP (2-carboxy-D-arabinitol-1,5-bisphosphate). Despite the separate presentation of the active site of both organisms, the molecules were documented by the same view after superimposition of the crystal structures.

The nearest neighbour analysis for the atoms O-3 and O-4 of both Rubisco molecules were carried out with a radius of 5 Å and the results are presented in Table II. At first glance, the analysis of Table II indicates that the binding niche of both O-3 atoms are different and the one for the O-3

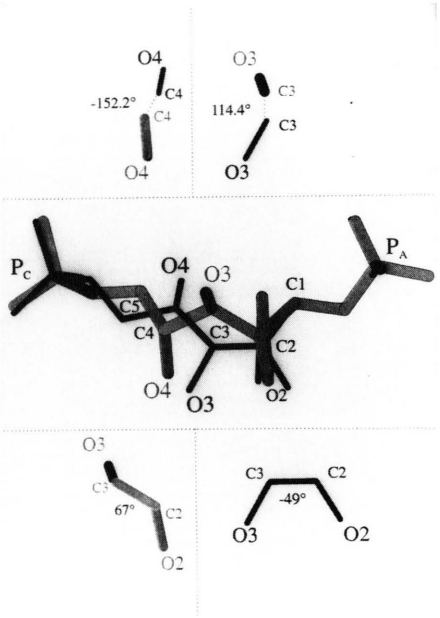


Fig. 4. Superimposition of the CABP molecules, analysis of their structure and determination of the dihedral angles in spinach and *Synechococcus*. The positions of the atoms were derived from the pdb files 8RUB (spinach) and 1RBL (Synechococcus), the carbon and oxygen atoms were labeled (spinach, black; *Synechococcus* gray). The hypothetic cross sections at the atoms C3–O3, respectively at the atoms C4 and O4 of both CABP molecules were shown in the insert above and the hypothetic dihedral angle ($O3_{spin}$-$C3_{spin}$–$C3_{Syn}$-$O3_{Syn}$) was determined. The insert at the bottom shows a molecule section of both CABP with the atoms O2–C2–C3–O3 and the dihedral angles.

(Spinach) is similar to the one of O-4(*Synechococcus*) and *vice versa*. Furthermore, the central atom O-3 (*Synechococcus*) has in the nearest environment three additional amino acid residues: T 173, K 201 and D 203 which belong to the C-terminal α/β barrel domain. The additional number of amino acid residues in the binding niche of O-3 (*Synechococcus*) reflect that more interactions between the protein shell and the CABP molecule can occur. The relatively low temperature factor of CABP in the structure of *Synechococcus* (Newman and Gutteridge, 1993) compared to the spinach structure (Knight *et al.*, 1990) is a further indication that the CABP molecule is more fixed in the *Synechococcus* structure.

As already discussed above (Section II.2) the N-terminal domain also participates in the binding of CABP (or RuBP). The amino acid residue N 123 bridges via a hydrogen bond to the O4 atom in the spinach structure (distance of 3.4 Å to ND2 and 3.5 Å to OD1) and to the O3 atom in the *Synechococcus* structure (distance of 3.9 Å to ND2 and 4.8 Å to OD1). In this connection it is interesting to note that the substitution of this amino acid residue N111G (Table I, line 7 and 8) for *R.rubrum* and N 123 G for *C. reinhardtii* causes a drastic reduction of the enzyme specificity.

We assume that the analysis of the superimposed structures can tell us about differences in the network of intermolecular interactions, and guides us to the understanding of the structure-function (specificity) relation in Rubisco. Furthermore, we can develop procedures to simulate the movement of CABP in the protein shells of the *Synechococcus* structure and of the spinach structure.

V. The „Inevitability" Hypothesis

The riddle that evolution did not correct the wasteful phenomenon of RuBP oxygenation and photorespiration is puzzling. The hope was raised in several publications that with the help of protein-engineering mutant forms of Rubisco could be constructed with a diminished or even without RuBP oxygenase activity. Contrary to these efforts the „inevitability" hypothesis was postulated relatively early (Somerville *et al.*, 1984). This idea of „inherent architectural constraints" was adopted because of the lack of accomodation of further changes of Rubisco in the time course of evolution. The constraint which is not totaly fixed allowed the selection pressure to modify the specificity but prohibits the elimination (Andrews and Lorimer, 1987). The confirmation of the „inevitability" hypothesis on the basis of theoretical arguments was established by acknowledging that „the substrate deformation is intrinsically built into the transition vector of the carboxylase" (Tapia and Andres, 1992).

Two factors determine the oxygenase activity: the accessibility of the carbanion intermediate to molecular oxygen and the stabilization of the initially formed peroxide anion. Since the discovery of the oxygenase activity of RuBP carboxylase the list of enzymes which produce a carbanionic intermediate and show oxygen consumption in the reaction assay is growing larger, among them are

Table II. Nearest neighbour analysis of O-3 and O-4 atoms of *Synechococcus* and spinach Rubisco.

O-3 *Synechococcus*	**O-3** Spinach	**O-4** *Synechococcus*	**O-4** Spinach
N-terminal domain (subunit B)			
N 123			N 123
C α/β barrel domain (subunit A)			
	L 335	L 335	
	G 380	G 380	
H 327	H 327	H 327	H 327
S 379	S 379	S 379	S 379
CBX	CBX	CBX	CBX
T 173			
K 201			
D 203			
E 204			E 204
H 294			H 294
Mg^{++}			Mg^{++}
H_2O	(H_2O)	H_2O	(H_2O)

acetolactate synthase, pyruvate decarboxylase and class II aldolase (Abell and Schloss, 1991). The reaction of the singlet enediolate of RuBP with the molecular triplet oxygen is linked to an intersystem crossing process. A geometric distortion of the planar O2 – C2 – C3 – O3 structure, like the rotation around the C2 – C3 bond, could disturb the π-bond system profoundly to make this intersystem crossing possible, i.e. the singlet state would be destabilized and the triplet state more stabilized (Andres *et al.*, 1992). The extent of the pyramidalization of the C3 atom would be directly linked to the energy level of the triplet state, i.e. as more the C3 atom is in an out of plane conformation the lower is the energy of the triplet state of the RuBP enediolate. Rubisco with the bound and twisted enediolate of RuBP – the energy gap between the singlet and the triplet state will be narrowed – will be afflicted by a high oxygenase activity and a low specificity.

With this view of geometric changes of the bound substrate RuBP in mind the causal connection between the deforming forces and oxygenase activity is becoming clear. Preliminary data of modeling experiments with the protein shell of *Synechococcus* and CABP of spinach reveal the dynamic interchanges, the interconversion of CABP from the spinach to the *Synechococcus* form. Therefore, this model system is best suited to study the impact of amino acid substitutions on the substrate dynamics.

The inevitability of the oxygenase activity of Rubisco is a realistic constraint for attempts to engineer an oxygenase-free RuBP carboxylase. However, the explanation of the cause of differences in enzyme specificity is still a rewarding research goal. The model of the „twisted bond" could be helpful to develop a biomechanical approach. The basic questions which have to be answered should focus on the revolving forces on the substrate RuBP and the amino acid residues participating in this process.

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