Eur. J. Biochem. *253*, 476−479 (1998)

Effect of mutations of residue 340 in the large subunit polypeptide of Rubisco from *Anacystis nidulans*

Pippa J. MADGWICK, Saroj PARMAR and Martin A. J. PARRY

Biochemistry and Physiology Department, IACR-Rothamsted, Harpenden, UK

(Received 22 December 1997) − EJB 97 1804/4

Residues 338−342 at the C-terminal end of loop 6 in the large subunit β/α barrel structure of Rubisco influence specificity towards CO_2 and O_2. In *Anacystis nidulans* Rubisco, replacement of alanine 340 by tyrosine or histidine increased the specificity factor by 12−13%, accompanied by a 25−33% fall in V_c, the rate of carboxylation, while replacement by asparagine increased the specificity factor by 9% and V_c by 19%. Other mutations did not significantly alter specificity. Alanine 340 does not interact directly with the bisphosphate substrate, thus replacing it with other residues must have indirect effects on the specificity factor and rate of carboxylation.

Keywords: rubisco; large subunit; loop 6; ribulose 1,5-bisphosphate; specificity factor.

The enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) from higher plants is inefficient (Hartman and Harpel, 1994; Wildner et al., 1996): they have a low turnover number of about 3 s^{-1} and catalyses not only the reaction of CO_2 with ribulose 1,5-bisphosphate, but also a number of side reactions including the wasteful oxygenation of ribulose 1,5-bisphosphate, leading to the loss of fixed CO_2 and consumption of ATP in photorespiration. Plants with an improved Rubisco would need to invest fewer resources in this enzyme and may use water and nitrogen more efficiently.

The specificity factors, τ, of Rubiscos from different species vary from 10 in the purple sulphur bacteria to over 200 in some marine algae (Read and Tabita, 1994; Uemura et al., 1997) and many attempts have been made to manipulate τ. This is a difficult goal since neither CO_2 or O_2 bind directly to the active site (Pierce, 1986). However, it has been possible to alter τ in several ways including oligonucleotide-directed mutagenesis of Rubisco genes cloned in the bacterium *Escherichia coli*. Studies have largely focused on Rubiscos from *Rhodospirillum rubrum* or the cyanobacterium *Anacystis nidulans*. The *A. nidulans* Rubisco has the higher plant structure and both subunits will fold and assemble correctly in *E. coli* to produce active holoenzyme. The *A. nidulans* Rubisco large subunit polypeptides and the corresponding gene transcripts have, respectively, over 80% amino acid and nucleotide identity with the higher plant enzymes (Knight et al., 1990).

High-resolution three-dimensional structures have been published for Rubisco from tobacco (Schreuder et al., 1993; Zhang et al., 1994) spinach (Andersson et al., 1989; Andersson, 1996; Knight et al., 1990), *A. nidulans* (Newman and Gutteridge, 1990, 1993) and *R. rubrum* (Schneider et al., 1990). Comparison of non-activated Rubisco structures with activated, enzyme-bound inhibitor complexes (Knight et al., 1990; Curmi et al., 1992; Newman and Gutteridge, 1993) show that there are several regions of the large subunit that are mobile during catalysis including loops in the N-terminal domain, loop 6 (V331−S341, the loop between β-sheet strand 6 and α-helix 6 of the β/α barrel) and the C-terminus of the polypeptide. τ is thought to be determined by both the exact position of the ε-amino group of Lys334, in loop 6 and the presence of the essential metal ion.

Most attempts to engineer Rubisco's specificity for CO_2 have not produced more efficient enzymes (Chène et al., 1992; Harpel and Hartman, 1992; Thow et al., 1994; Zhu and Spreitzer, 1994, 1996). However, some mutations in loop 6 and at the C-terminus of the protein have achieved small increases in τ without large decreases in V_c. Most of the amino acid residues in loop 6 are conserved between different species but the four residues (positions 338−341 in the spinach sequence) at the C-terminal hinge of the loop vary. Mutation of these four residues in *A. nidulans* Rubisco to the residues found in maize or tobacco resulted in 3−7% increase in τ, accompanied by a fall in V_c of less than 25%, compared with the wild-type enzyme (Parry et al., 1992; Kane et al., 1994). Mutation to the spinach sequence did not significantly alter τ (Gutteridge et al., 1993). A single mutation, A340E, led to a 17% decrease in τ with a fall in V_c of just over 10% (Parry et al., 1992) while another mutation, A340L, led to a 7% fall in τ, with a fall in V_c of 33% (Read and Tabita, 1994). The only other region of the polypeptide where mutation has resulted in an increased τ is the C-terminal tail (Gutteridge et al., 1993).

Since most mutations at position 340 affected τ without decreasing V_c by more than 10%, we have made six further mutations at position 340 (A340D, A340G, A340H, A340N, A340R and A340Y) to explore the role of size and charge of different amino acid side chains at this position.

MATERIALS AND METHODS

Materials. The plasmid pAn92 (Kettleborough et al., 1987) was used as a source of the *A. nidulans* Rubisco genes. The

Correspondence to P. J. Madgwick, Biochemistry and Physiology Department, IACR-Rothamsted, Harpenden, Herts, AL5 2JQ, UK

Fax: +44 1582 760981.

E-mail: pippa.madgwick@bbsrc.ac.uk

Abbreviations. τ, specificity factor; V_c, maximum rate of carboxylation; V_o, maximum rate of oxygenation; K_o, Michaelis constant for O_2.

Enzyme. Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39).

Table 1. Relative specificities and kinetic properties of wild-type and mutant Rubisco. A340 is the wild-type enzyme; the data for the wild-type enzyme was used on each occasion to normalise the mutant data; (*), significantly different from the wild-type enzyme $P = 0.05$; (**), significantly different from the wild-type enzyme $P = 0.01$; specificity factor, K_c, K_{RuBP} and V_c are shown with their standard errors.

Type	Specificity factor	Change in specificity	K_c	K_{RuBP}	V_c	Change in V_c	V_c/K_c
		%	μM		μmol CO_2 · μmol catalytic site · s^{-1}	%	
A340	46.30 ± 0.38		85.5 ± 3.7	216.9 ± 19.3	6.0 ± 0.08	−	.070 ± .022
A340D	48.44 ± 0.52	5	79.5 ± 4.8	244.9 ± 24.1	4.0 ± 0.07	−33	.051 ± .015
A340G	43.68 ± 1.70	−6	74.0 ± 7.9	232.3 ± 31.9	4.1 ± 0.10	−31	.056 ± .013
A340H	52.31** ± 0.99	13	55.1 ± 3.9	249.0 ± 10.6	4.0 ± 0.06	−33	.073 ± .016
A340N	50.26* ± 1.98	9	74.9 ± 3.0	155.4 ± 10.8	7.1 ± 0.07	+19	.095 ± .023
A340R	47.70 ± 1.71	3	91.6 ± 6.6	271.2 ± 11.6	5.7 ± 0.14	−4	.063 ± .021
A340Y	51.70** ± 1.00	12	72.9 ± 4.7	248.2 ± 20.7	4.5 ± 0.07	−25	.061 ± .015

plasmid was propagated in *E. coli* strain JM83 [*ara*, *Δ*(*lac-pro*), *str*A, *thi*, *Φ80dlacZΔ*M15]. The bacteriophage M13mp9 and derivatives containing the Rubisco genes were grown in *E. coli* strain TG1 [*Δ*(*lac-pro*AB), *supE*, *thi*, *hsd*D5 F' : *tra*D36, *pro*AB, *lac*I^q, *lacZΔ*M15].

Restriction and modification enzymes were obtained from Gibco-BRL Life Technologies and Boehringer Mannheim UK. Sequencing was carried out using Sequenase version 2.0 from United States Biochemicals (USB). Site-directed mutagenesis was carried out with the T7-Gen *in vitro* Mutagenesis kit, obtained from USB. Oligonucleotides were purchased from Pharmacia Biotech. They were used according to the manufacturer's instructions. Routine DNA preparations and manipulations were carried out as described in Sambrook et al. (1989).

General laboratory chemicals were obtained from Fisons, Sigma-Aldrich or BDH. [2′-^{14}C]Carboxyarabinitol bisphosphate, a transition-state analogue, was made according to Pierce et al. (1980). [1-^{14}C]Ribulose 1,5-bisphosphate (12.5 Ci/mol.) was synthesized enzymatically from erythrose 4-phosphate (Serianni et al., 1982a,b) and purified using a 1.7 cm×51 cm Dowex 1 column in 3 mM HCl and developed with a 0.0 to 0.4 M gradient. Ultima flow scintillation fluid was from Packard.

Site-directed mutagenesis. Site-directed mutagenesis carried out with the T7-Gen kit requires a single-stranded template. The *A. nidulans* Rubisco genes (*rbc*L and *rbc*S) from the plasmid pAn92 were excised on a 2.2-kb *Pst*I fragment and cloned into the bacteriophage M13mp9 which had been linerised with *Pst*I. A 28-base degenerate oligonucleotide GGAAGGCGACA-AANNSTCGACCTTGGGC was designed to direct the replacement of alanine at position 340 by every other amino acid; the initial mutagenesis did not yield all the 19 possible amino acid replacements so two further oligonucleotides GGAAGGCGAC-AAANDGTCGACCTTGGGC and GGAAGGCGACAAAHH-CTCGACCTTGGGC were synthesised and used to generate further mutations at this position. The oligonucleotides were phosphorylated and used without further purification. Site-directed mutagenesis was carried out and the introduction of specific mutations was confirmed by sequencing.

Production of mutant proteins. The mutated Rubisco genes were subcloned into the plasmid pUC19 on a 2.2-kb *Pst*I fragment. Clones with the insert in the same orientation as the *lac* promoter of pUC19 were identified and the A340 region was sequenced to confirm their identity. Cultures of *E. coli* strain JM83 expressing wild-type or mutant genes were grown, and Rubisco was purified from them as described previously (Parry et al., 1992).

Determination of kinetic constants, carboxyarabinitol 1,5-bisphosphate binding and specificity factor. K_m determinations were carried out as described previously (Kettleborough et al., 1987). The concentration of Rubisco active sites which is needed to calculate V_c, was determined from the binding of [^{14}C]carboxyarabinitol 1,5-bisphosphate (1.0 Ci/mol) to mutant and wild-type Rubiscos (Gutteridge et al., 1993). [^{14}C]Carboxyarabinitol 1,5-bisphosphate was added to desalted enzyme that had first been incubated, for 10 min at 25°C, with unlabelled $NaHCO_3$ to generate the activated quaternary complex. After a further 30-min incubation, the enzyme complex was freed of excess inhibitor by gel filtration through a column (30 cm×1 cm) of Superdex 75 (Pharmacia). Changes in τ were analyzed using [^{14}C]ribulose 1,5-bisphosphate (Bainbridge, 1995).

To determine whether the introduced mutations altered the rate of dissociation of Rubisco and carboxyarabinitol 1,5-bisphosphate, the complex was challenged with a 10-fold molar excess of unlabelled carboxyarabinitol 1,5-bisphosphate and the amount of radioactivity remaining with the enzyme fraction was measured at different times (Gutteridge et al., 1993).

RESULTS

Construction of the mutant genes and production of mutant Rubiscos. Six mutant genes for the *A. nidulans* Rubisco large subunit were constructed, to explore the effects of changing the size and charge of the residue at position 340. Each mutation was introduced into the Rubisco large-subunit gene by site-directed mutagenesis. *E. coli* was grown harbouring the wild-type small-subunit gene, *rbc*S, together with either the wild-type or a mutant large-subunit gene, *rbc*L. The Rubisco polypeptides were expressed and purified.

Kinetic parameters for wild-type and mutant Rubiscos. For each mutant Rubisco, τ was determined and compared with the value for the wild-type enzyme. The measurements were repeated at least 15 times for each mutant, using at least two different preparations of the enzyme and on at least three different days. The mutants A340D, A340G and A340R had specificities which were not significantly different from the wild-type value (Table 1). However significant increases (at 99% confidence levels) occurred when a residue with a large bulky polar sidechains was introduced: τ was 13% higher in the A340H and 12% in the A340Y mutants. A smaller increase of 9% (significant at 95% confidence levels) was recorded with the mutant A340N.

Increasing the specificity of Rubisco would be of little benefit to a plant if this increase were at the expense of either

decreasing the V_c of the enzyme or of lowering the affinity of the enzyme for its substrates. The Michaelis constants for ribulose 1,5-bisphosphate and CO_2 were estimated and the V_c values for each enzyme were determined (Table 1). All of the mutant enzymes bind both CO_2 and ribulose 1,5-bisphosphate approximately as tightly as the wild-type enzyme. The V_c values range from 67% to 119% of the wild-type values.

While the specificty factor can be determined precisely for different mutants, measurements of some other kinetic parameters are more difficult. For example, the apparent V_c is dependent on purity, complete activation, assay conditions and assumes that all of the enzyme is catalytically active. As a consequence, the values reported for the apparent V_c for Rubisco for different groups and on different occasions vary fourfold, from 2.4 s^{-1} (calculated from Andrews and Abel, 1981) to 13.3 s^{-1} (Morell et al., 1992). Other parameters also vary. The lower and variable values may reflect damage caused to Rubisco during isolation and purification.

The parameter V_c/K_c gives a measure of the catalytic efficiency of the enzyme (Table 1). Only A340N can be described as an improved enzyme, achieved by small increases in both τ and V_c. In the other enzymes any improvement in τ is offset by decreases in the rate of reaction, for example, by 33% for A340H and by 25% for A340Y.

[^{14}C]Carboxyarabinitol 1,5-bisphosphate is a tight binding inhibitor of Rubisco. The stability of the quaternary complex between two mutant enzymes and [^{14}C]carboxyarabinitol 1,5-bisphosphate was compared to the stability of the equivalent wild-type complex. The mutant enzymes did not behave differently from the wild-type enzyme.

DISCUSSION

These results confirm the importance of loop 6 in determining the ability of Rubisco to discriminate between CO_2 and O_2. Mutations have been made at position 340 in *A. nidulans* Rubisco, based on the earlier observation that changes at this position produced changes in the specificity factor without substantially decreasing the rate of reaction (Parry et al., 1992; Read and Tabita, 1994). The amino acid found at position 340 varies in different species and position 340 is remote from the active site of Rubisco at the C-terminal end of loop 6, thus effects on specificity of mutations at this position must be indirect. For example they may alter the flexibility of the loop or the final conformation which the loop can adopt prior to the addition of gaseous substrates. In particular, the exact position of the essential lysine residue at position 334 is important and any change in the orientation of the lysyl side-chain can affect activity (Lorimer et al., 1993).

The positive effect on specificity factor in A340H and A340Y mutants of replacing a relatively small non-polar side-chain, alanine, with bulkier side-chains that can form hydrogen bonds is intriguing. By analogy with triose phosphate isomerase, in which catalysis also involves a flexible loop from a β/α barrel, the tip of the loop is expected to move as a rigid body with large movements only in the two hinge regions (Wierenga et al., 1992). An earlier model of the region around A340E, based on the spinach Rubisco structure (Parry et al., 1992), suggested a novel hydrogen bond between the introduced glutamic acid and Lys474 but modelling A340E in the *A. nidulans* Rubisco structure suggests that this is unlikely. Modelling also suggests that both A340H and A340Y could form hydrogen bonds with Ser359 whereas A340N is unable to hydrogen bond with Ser359; none of these residues could interact with the C-terminal tail. Extra rigidity caused by the formation of a new hydrogen bond might be responsible for reducing reducing V_c in the A340H and A340Y mutants but not in A340N. The effects of the mutations on τ and V_c must be due to subtle effects on the positioning of Lys334 or other residues involved in catalysis. The movement of loop 6 and other loops closes the active site, which results in the almost irreversible association of the activated enzyme with carboxyarabinitol 1,5-bisphosphate. Dissociation of carboxyarabinitol 1,5-bisphosphate from the A340H and A340Y mutant enzymes was similar to the wild-type enzyme, suggesting that the ability of loop 6 and other loops to close over the active site is not impaired by these changes.

The A340G mutant shows a reduction in reaction rate, while the specificity factor is not altered significantly. This change could allow the essential Lys334 to be held in a satisfactory position for discriminating between CO_2 and O_2, but with too much flexibility to allow the reaction rate to be maintained.

In general, plants with higher specificity factors tend to have lower V_c (see Bainbridge et al., 1995) and it is possible that any increase in τ will be at the expense of decreasing the rate of reaction. The maximum rates and specificity factors for these A340 mutants were not sufficiently different from the wild-type enzyme to see whether they fit this trend. However, the A340N mutant appears to have both increased τ and V_c, hence showing a small increase in efficiency. The recent discovery of Rubisco from red alga with a specificity factor three-times that of crop plants suggests that the reduction of photorespiration in crop plants should become a reality (Shibata et al., 1996; Uemera et al., 1997).

The help of Jim Pitts and Glen Wright (Birkbeck College, London) in modelling the mutations is gratefully acknowledged. We would also like to thank A. J. Keys and P. J. Andralojc for useful discussions. IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

REFERENCES

Andersson, I., Knight, S., Schneider, G., Lindqvist, Y., Lundqvist, T., Branden, C.-I. & Lorimer, G. H. (1989) Crystal structure of the active site of ribulose-bisphosphate carboxylase, *Nature 337*, 229–234.

Andersson, I. (1996) Large structures at high resolution: the 1.6 Å crystal structure of spinach ribulose-1,5-bisphosphate carboxylase/oxygenase complexed with 2-carboxyarabinitol bisphosphate, *J. Mol. Biol. 259*, 160–174.

Andrews, T. J. & Abel, K. M. (1981) Kinetics and subunit interactions of Ribulose bisphosphate carboxylase-oxygenase from the cyanobacterium, *Synechococcus* sp., *J. Biol. Chem. 256*, 8445–8451.

Bainbridge, G. (1995) *Investigation into the structure and function relationships in Ribulose-1,5-bisphosphate carboxylase oxygenase*, PhD thesis, University of London.

Bainbridge, G., Madgwick, P. J., Parmar, S., Mitchell, R., Paul, M. J., Pitts, J., Keys, A. J. & Parry, M. A. J. (1995) Engineering Rubisco to change its catalytic properties, *J. Exp. Bot. 46*, 1269–1276.

Chène, P., Day, A. G. & Fersht, A. R. (1992) Mutation of asparagine 111 of Rubisco from *Rhodosprillum rubrum* alters the carboxylase/oxygenase specificity, *J. Mol. Biol. 225*, 891–896.

Curmi, P. M. G., Cascio, D., Sweet, R. M., Eisenberg, D. & Schreuder, H. (1992) Crystal structure of the unactivated form of Ribulose-1,5-bisphosphate carboxylase/oxygenase from tobacco refined at 2.0-Å resolution, *J. Biol. Chem. 267*, 16980–16989.

Gutteridge, S., Rhoades, D. F. & Herrmann, C. (1993) Site-specific mutations in a loop region of the C-terminal domain of the large subunit of ribulose bisphosphate carboxylase/oxygenase that influence substrate partitioning, *J. Biol. Chem. 268*, 7818–7824.

Harpel, M. & Hartman, F. C. (1992) Enhanced CO_2/O_2 specificity of a site-directed mutant of Ribulose-bisphosphate carboxylase/oxygenase, *J. Biol. Chem. 267*, 6475–6478.

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

Kane, H. J., Viil, J., Entsch, B., Paul, K., Morell, M. K. & Andrews, T. J. (1994) An improved method for measuring CO_2/O_2 specificity of Ribulose bisphosphate carboxylase-oxygenase, *Aust. J. Plant Physiol. 21*, 449−461.

Kettleborough, C. A., Parry, M. A. J., Burton, S., Gutteridge, S., Keys, A. J. & Phillips, A. L. (1987) The role of the N-terminus of the large subunit of ribulose-bisphosphate carboxylase investigated by construction and expression of chimaeric genes, *Eur. J. Biochem. 170*, 335−342.

Knight, S., Andersson, I. & Brändén, C.-I. (1990) Crystallographic analysis of Ribulose 1,5-bisphosphate carboxylase from spinach at 2.4 Å resolution, *J. Mol. Biol. 215*, 113−160.

Lorimer, G. H., Chen, Y.-R. & Hartman, F. C. (1993) A role for the ε-amino group of lysine-334 of ribulose-1,5-bisphosphate carboxylase in the addition of carbon dioxide to the 2,3-enediol(ate) of ribulose 1,5-bisphosphate, *Biochemistry 32*, 9018−9024.

Morell, M., Kane, H. J., Hudson, G. S. & Andrews, T. J. (1992) Effects of mutations at residue 309 of the large subunit of Ribulosebisphosphate carboxylase from *Synechococcus* PCC 6301, *Arch. Biochem. Biophys. 299*, 295−301.

Newman, J. & Gutteridge, S. (1990) The purification and preliminary X-ray diffraction studies of recombinant *Synechococcus* ribulose-1,5-bisphosphate carboxylase/oxygenase from *Escherichia coli*, *J. Biol. Chem. 265*, 15 154−15 159.

Newman, J. & Gutteridge, S. (1993) The X-ray Structure of *Synechococcus* Ribulose-bisphosphate carboxylase/oxygenase-activated quaternary complex at 2.2-Å resolution, *J. Biol. Chem. 268*, 25 876−25 886.

Parry, M. A. J., Madgwick, P. J., Parmar, S., Cornelius, M. J. & Keys, A. J. (1992) Mutations in loop six of the large subunit of ribulose-1,5-bisphosphate carboxylase affect substrate specificity, *Planta* (*Heidelb.*) *187*, 109−112.

Pierce, J., Tolbert, N. E. & Barker, R. (1980) Interaction of Rubisco with transition state analogues, *Biochemistry 19*, 934−942.

Pierce, J. (1986) Determinants of substrate specificity and the role of the metal in the reactions of ribulose-1,5-bisphosphate carboxylase, *Plant Physiol.* (*Bethesda*) *81*, 943−945.

Read, B. & Tabita, F. R. (1994) High substrate specificity factor ribulose bisphosphate carboxylase/oxygenase from eukaryotic marine algae and properties of recombinant Rubisco containing cyanoabterial Rubisco containing 'algal' residue modifications, *Arch. Biochem. Biophys. 312*, 210−218.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

Schneider, G., Lindqvist, Y. & Lundqvist, T. (1990) Crystallographic refinement and structure of ribulose-1,5-bisphosphate carboxylase from *Rhodospirillum rubrum* at 1.7 Å resolution, *J. Mol. Biol. 211*, 989−1008.

Schreuder, H. A., Knight, S., Curmi, P. M., Andersson, I., Cascio, D., Brändén, C.-I. & Eisenberg, D. (1993) Formation of the active site of ribulose-1,5-bisphosphate carboxylase/oxygenase by a dis-order transition from the unactivated to the activated form, *Proc. Natl Acad. Sci. USA 90*, 9968−9972.

Serianni, A. S., Nunez, H. A., Hayes, M. L. & Barker, R. (1982a) Chemical synthesis of monosaccharides enriched with carbon isotopes, *Methods Enzymol. 89*, 64−73.

Serianni, A. S., Pierce, J. & Barker, R. (1982b) Chemical synthesis of aldose phosphates enriched with carbon isotopes, *Methods Enzymol. 89*, 73−79.

Shibata, N., Yamamoto, H., Inoue, T., Uemura, K., Yokota, A. & Kai, Y. (1996) Crystallization and preliminary crystallographic studies of ribulose-1,5-bisphosphate carboxylase/oxygenase from a red alga, *Galderia partita*, with a high specificity factor, *J. Biochem.* (*Tokyo*) *120*, 1064−1066.

Thow, G., Zhu, G. & Spreitzer, R. J. (1994) Complementing substitutions within loop regions 2 and 3 of the alpha/beta-barrel active site influence the CO_2/O_2 specificity of chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase, *Biochemistry 33*, 5109−5114.

Uemura, K., Anwaruzzaman, Miyachi, S. & Yokota, A. (1997) Ribulose-1,5-bisphosphate carboxylase/oxygenase from the thermophillic red algae with a strong specificity for CO2 fixation, *Biochem. Biophys. Res. Commun. 233*, 568−571.

Wierenga, R. K., Borchert, T. V. & Noble, M. E. M. (1992) Crystallographic binding studies with triosephosphate isomerases: conformational changes induced by substrate and substrate-analogues, *FEBS Lett. 307*, 34−39.

Wildner, G., Schlitter, J. & Muller, M. (1996) Rubisco, an old challenge with new perspectives, *J. Biosci. 51*, 263−276.

Zhang, K. Y. J., Cascio, D. & Eisenberg, D. (1994) Crystal structure of the unactivated ribulose 1,5-bisphosphate carboxylase/oxygenase complexed with a transition state analog, 2-carboxy-D-arabinitol 1,5-bisphosphate, *Protein Sci. 3*, 64−69.

Zhu, G. & Spreitzer, R. J. (1994) Directed mutagenesis of chloroplast ribulosebisphosphate carboxylase/oxygenase, *J. Biol. Chem. 269*, 3952−3956.

Zhu, G. & Spreitzer, R. J. (1996) Directed mutagenesis of chloroplast ribulose 1,5-bisphosphate carboxylase/oxygenase, *J. Biol. Chem. 271*, 1894−1898.