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A site-specific mutation within the active site of ribulose-1,5-bisphosphate carboxylase of *Rhodospirillum rubrum*

S. Gutteridge[1], I. Sigal[2], B. Thomas[3], R. Arentzen, A. Cordova and G. Lorimer

Central Research and Development Department, E.I. du Pont de Nemours and Company, Experimental Station, Wilmington, DE 19898, USA

Present addresses: [1]Department of Biochemistry, Rothamsted Experimental Station, Harpenden, Herts AL5 2JQ, UK, [2]Merck, Sharp and Dohme Research Laboratories, West Point, PA 19486, and [3]Department of Biochemistry, State University of New York, Stony Brook, NY 11794, USA

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***In vitro* mutagenic techniques have generated an asp→glu substitution at residue 198 adjacent to the carbamate-divalent metal ion binding site of *Rhodospirillum rubrum* ribulose 1,5-bisphosphate carboxylase. A single C→A nucleotide change in the coding strand created the mutant and introduced a new *Eco*RI restriction site on the expression plasmid pRR2119. Although the carboxylase:oxygenase ratio remained the same, the mutant enzyme had slightly altered kinetic properties. The e.p.r. spectra of the quaternary complexes enzyme.activator carbamate.Mn^{2+}.2-carboxyarabinitol 1,5-bisphosphate and enzyme.activator carbamate.Mn^{2+}.4-carboxyarabinitol 1,5-bisphosphate for mutant and wild-type enzymes were different, indicating that the metal ion was in a slightly altered environment. These findings are consistent with the hypothesis that, besides the carbamate at lys 201, the carboxyl group of asp 198 contributes to the formation of the divalent metal ion binding site.**

Key words: active site/*in vitro* mutagenesis/site-specific mutation/ribulose bisphosphate carboxylase

Introduction

Ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) catalyzes the primary reactions of both photosynthetic and photorespiratory carbon metabolism (Lorimer, 1981a). Since photorespiration lowers the overall rate of photosynthesis, the enhancement of the carboxylase:oxygenase ratio is a potentially attractive means of increasing photosynthesis and hence of crop productivity (Somerville and Ogren, 1982). Variations in the partition coefficient (τ), the ratio of the

$$\tau = \frac{k_{cat}\,(\text{carboxylation})}{K_{m[CO_2]}\,(\text{carboxylation})} \Big/ \frac{k_{cat}\,(\text{oxygenation})}{K_{m[O_2]}\,(\text{oxygenation})}$$

specificity factors for carboxylation and oxygenation, have been reported for Rubiscos from taxonomically diverse organisms (Jordan and Ogren, 1981). However, the maximum and minimum τ values for 21 eukaryotic Rubiscos differ by less than a factor of 1.6. Nevertheless, even small increases in τ have the potential to enhance significantly photosynthesis in air.

The most common form of Rubisco is a hexadecamer consisting of eight large (~56 kd) subunits and eight small (~14 kd) subunits (Miziorko and Lorimer, 1983). The large subunits contain the sites for activation and catalysis. The function of the small subunits is unknown although they are required for catalysis (Andrews and Ballment, 1983). A simpler form of Rubisco, a catalytically active dimer of large subunits only, is found in the photosynthetic bacterium *Rhodospirillum rubrum* (Tabita and McFadden, 1974). The *R. rubrum* Rubisco shows many characteristics in common with the hexadecameric form. It catalyzes the oxygenation reaction (McFadden, 1974) and undergoes activation with CO_2 and Mg^{2+} by means of carbamate formation (Lorimer and Miziorko, 1980; Lorimer, 1981b; Donnelly *et al.* 1983). However, there is only ~25% overall homology between the amino acid sequences of the *R. rubrum* enzyme and the large subunit of the hexadecameric spinach enzyme (McIntosh *et al.*, 1980; Hartman *et al.*, 1982; Narang *et al.*, 1984). Striking homology is evident, however, in those regions identified by affinity labelling techniques as being within the domain of the catalytic site (Herndon *et al.*, 1982; Herndon and Hartman, 1984; Fraij and Hartman, 1983) and at the site for activator carbamate formation (Lorimer, 1981b; Donnelly *et al.*, 1983) (Figure 1). *R. rubrum* Rubisco thus represents a useful model system for probing the active site both spectroscopically and by the technique of site-specific mutagenesis.

The divalent metal ion binding site

During the activation of Rubisco by CO_2 and Mg^{2+} a carbamate is formed on the ϵ-amino group of lys 201 (Lorimer and Miziorko, 1980; Lorimer, 1981b). The most noteworthy feature of the sequence (Figure 1) adjacent to lys 201 is the presence of carboxyl residues at positions 198, 202, 203 and 204. This has prompted speculation (Lorimer, 1981b) that, together with the carbamate at lys 201, one or more of these carboxyl groups forms the divalent metal ion binding site (Figure 1). Strong evidence that the divalent metal ion binds to the carbamate has been obtained using paramagnetic metal ions such as Mn^{2+}, Co^{2+} and Ni^{2+} that substantially broaden the ^{13}C n.m.r. signal due to the carbamate (Pierce

(A)

Rr	Gly	Gly		Asp	Phe	Ile	Lys*	Asn	Asp	Glu
Spn	Gly	Gly	Leu	Asp	Phe	Thr	Lys*	Asp	Asp	Glu

(B)

	Gly	Gly	Asp	Phe	Ile	Lys*	Asn	Asp	Glu	
	GGC	GGC	GAC	TTC	ATC	AAG	AAC	GAC	GAG	
	GGC	GGC	GAA	TTC	ATC	AAG	AAC	GAC	GAG	
	Gly	Gly	Glu	Phe	Ile	Lys	Asn	Asp	Glu	

Fig. 1. The amino acid sequence surrounding the activator lysine of Rubisco. **(A)** The sequence of the activator region of the *R. rubrum* (Rr) enzyme (Donnelly *et al.*, 1983; Narang *et al.*, 1984) compared with the corresponding sequence of the spinach (Spn) enzyme (Lorimer, 1981b), aligned and anumerated with respect to spinach lys 201(*). This is only one of several sequences of the bacterial enzyme which are closely homologous to the higher plant enzyme. **(B)** The nucleotide sequence of the gene for *R. rubrum* Rubisco (Narang *et al.*, 1984) showing that the asp 198→glu 198 substitution can be achieved as a result of a single C→A nucleotide change on the coding strand. This single nucleotide change also creates a new *Eco*RI restriction site (GAATTC) which is underlined.

and Reddy, 1984).

The divalent metal ion influences the partitioning between carboxylation and oxygenation (Wildner and Henkel, 1979; Christeller, 1981; Jordan and Ogren, 1983) suggesting that, besides its involvement in activation, it is important in catalysis (Miziorko and Sealy, 1980; Miziorko *et al.*, 1982; Pierce *et al.*, 1980). The stoichiometry (Miziorko and Sealy, 1980; Miziorko *et al.*, 1982) (1:1:1:1) of the quaternary complex enzyme.activator carbamate.Mn^{2+}.2-carboxyarabinitol 1,5-bisphosphate, containing the tightly-bound, reaction-intermediate analog, 2-carboxyarabinitol 1,5-bisphosphate (2-CABP) (Pierce *et al.*, 1980; Schloss and Lorimer, 1982), also implies the same dual role. Recent e.p.r. (Miziorko and Sealy, 1984) and n.m.r. (Pierce and Reddy, 1984) evidence suggests that, in addition to coordination to the activator carbamate, the divalent metal ion also interacts with the carboxyl and hydroxyl groups at C-2 of 2-CABP. Thus, the carbamate-divalent metal ion complex at lys 201 constitutes part of the active site.

Site-specific mutagenesis offers an attractive means to probe this part of the active site with a view to establising structure-function relationships of individual amino acids. For example, the possible role of the carboxyl groups adjacent to lys 201 in the binding of the divalent metal ion.

Recently, the gene for Rubisco has been cloned from a genomic library of *R. rubrum* DNA (Somerville and Somerville, 1984) (Figure 2). The nucleotide sequence of the gene has been determined (Narang *et al.*, 1984). SDS-polyacrylamide gel electrophoresis of the purified recombinant enzyme revealed that it is ~3 kd larger than the native *R. rubrum* Rubisco (Lorimer, unpublished data). N-terminal amino acid sequence analysis showed that the recombinant enzyme is fused to a 25-residue segment of β-galactosidase (F. Hartman, personal communication). Nevertheless, the recombinant enzyme catalyzes both the carboxylation and the oxygenation of ribulose 1,5-bisphosphate. This constitutes the most compelling evidence that these two activities are associated with the same enzyme.

Results and Discussion

Rationale and strategy

In choosing the asp 198→glu 198 substitution we were influenced by a number of factors. Such a modest change would preserve the anionic nature of the site and is therefore unlikely to cause gross structural changes. We could reasonably expect that the mutant enzyme would be catalytically active, although perhaps with altered kinetic pro-

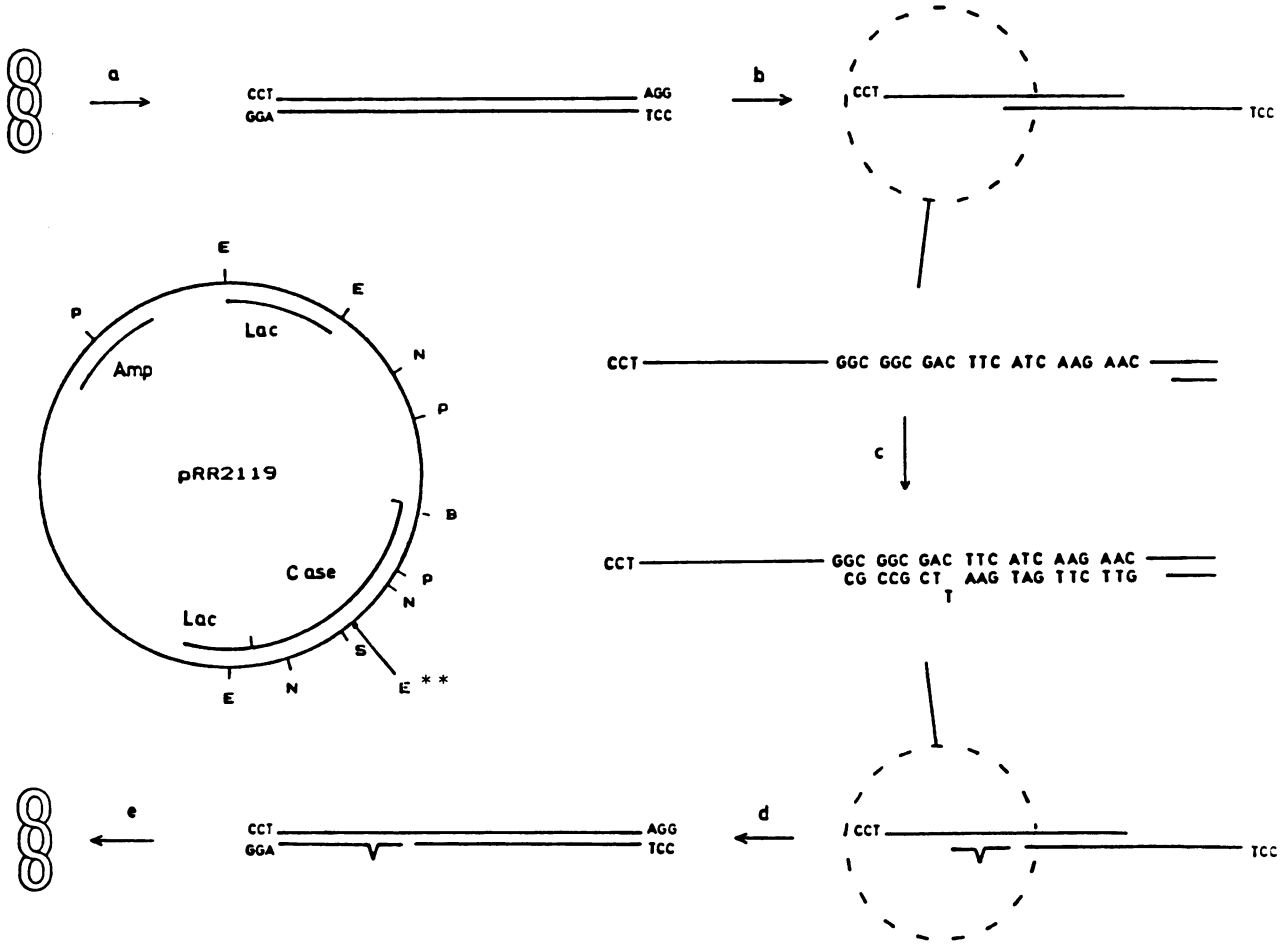


Fig. 2. The strategy adopted to introduce the single nucleotide mismatch and create the desired mutation. **(a)** The supercoiled plasmid pRR2119 was digested with the restriction enzyme *Stu*I (5 units/μg DNA) to generate linear, 'blunt-ended' double-stranded DNA. **(b)** The exonuclease activity of T4 DNA polymerase without dNTP was exploited to expose the region on the 5′→3′ strand of the linear DNA for hybridisation with the synthetic oligonucleotide. **(c)** The synthetic oligonucleotide primer composed of the 20 bases shown, including the single mismatch was synthesized (Caruthers *et al.*, 1982), purified (Fritz *et al.*, 1978) and phosphorylated (Maxam and Gilbert, 1980). Hybridisation was by heating at 55°C for 15 min and then cooling the solution to room temperature. **(d)** The Klenow fragment of DNA polymerase I with all 4 dNTP's generated the double-stranded DNA and ligation reactions **(e)** incorporated the mismatched oligonucleotide and regenerated the super-coiled plasmid. 10 ng plasmid DNA produced 2000 transformants/plate.The restriction map of pRR2119 shows the position of the *Stu*I (S) and new *Eco*RI sites, along with *Pst*I (P), *Bql*II (B) and *Nco*I (N) sites.

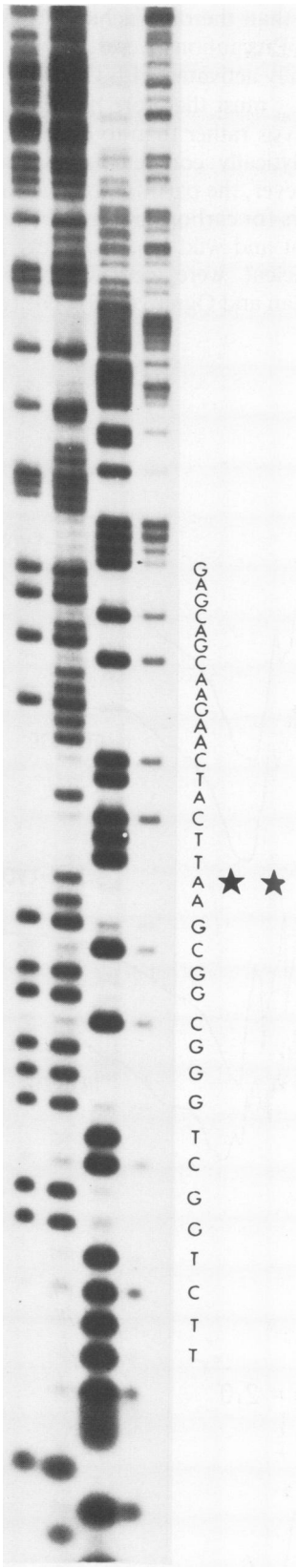


Fig. 3. Sequence analysis of the *Stu*I-*Pst*I restriction fragment of the mutant plasmid showing the C→A substitution responsible for the asp 198→glu 198 mutation and the new *Eco*RI restriction site GAATTC. Sequencing was performed by the method of Maxam and Gilbert (1980). From the left the four tracks are G, G+A, C+T, C.

perties. Inspection of the nucleotide sequence (Figure 1) showed that the asp 198→glu 198 substitution could be affected by a single C→A nucleotide change. More important, however, this mutation introduces a new *Eco*RI restriction site into a region of the gene which contains few convenient restriction sites and which encodes an enzymologically interesting region. The presence of a new *Eco*RI restriction site was also to provide a ready means of identifying mutants.

The strategy used to create the mutation is shown in Figure 2. It differs from other methods by using linear double-stranded DNA rather than single-stranded (Winter *et al.*, 1982) or nicked-duplex forms (Sigal *et al.*, 1982; Dalbadie-McFarland *et al.*, 1982). Here the plasmid pRR2119, amplified in *Escherichia coli* (strain HB101) was purified by CsCl-gradient ultracentrifugation. Thirty bases upstream from the intended mutation site is the sequence AGGCCT recognized by the restriction enzyme *Stu*I. Treatment of the plasmid with *Stu*I generated blunt-ended DNA (Figure 2). The 3′→5′ exonuclease activity of T4 DNA polymerse was used to expose the target site on the 5′→3′ strand. A 20-base synthetic oligodeoxyribonucleotide, complementary to this region except for a single mismatch, was annealed to the linear template. The 3′→5′ strand was repaired using T4 DNA polymerase in the presence of all four deoxyribonucleotide triphosphates and the circular super-coiled plasmid reformed by ligation with T4 DNA-ligase. *E. coli* (strain HB101) was transformed with the treated plasmid and grown in the presence of ampicillin as the selective agent. Mutant colonies were selected on the basis of their stronger hybridization to the ^{32}P-labelled synthetic oligonucleotide primer used above (Dalbadie-McFarland *et al.*, 1982). These colonies were then grown in the presence of ampicillin to produce plasmid for restriction analyses.

The plasmid from three of the colonies showed altered restriction patterns when digested with *Eco*RI, compared with the pattern normally seen for pRR2119. This was due to the plasmid acquiring a new *Eco*RI site as a result of these manipulations. The frequency of mutation was at least 1%. Further restriction analysis using combined digestion of the plasmid with *Eco*RI and *Pst*I or *Nco*I or *Bgl*II (data not shown) confirmed that the mutation was at the expected position. Sequence analysis of the isolated *Stu*I-*Pst*I fragment from the mutant plasmid by the Maxam-Gilbert method (Figure 3) confirmed that an adenine had become inserted in place of cytosine.

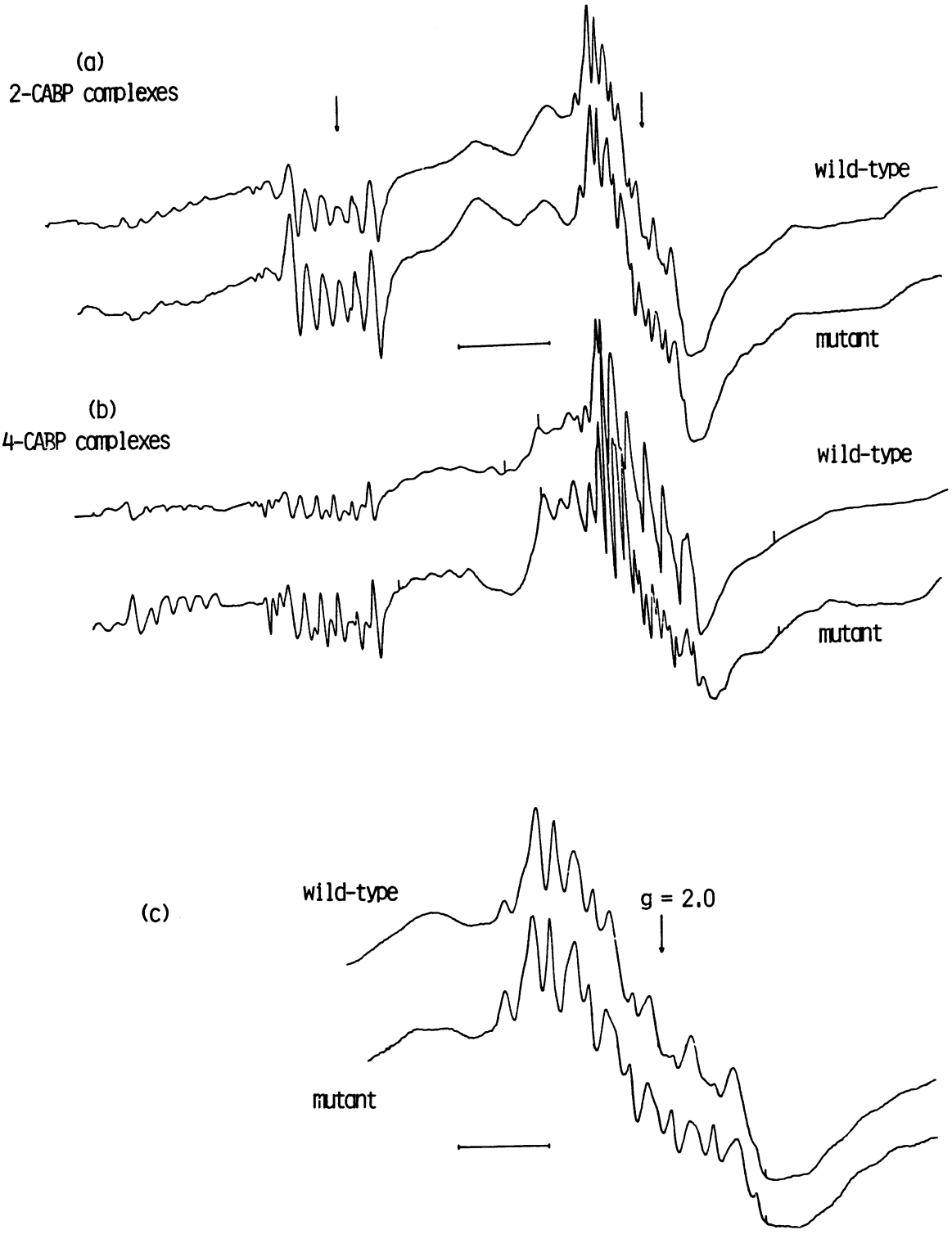
Table I. Kinetic properties of wild-type and mutant recombinant *R. rubrum* ribulose bisphosphate carboxylase-oxygenase[a]

	$k_{cat}(s^{-1})$	$K_{m[CO_2]}(\mu M)$[b]	$K_{m[RuBP]}(\mu M)$	τ[c]
Wild-type[d]	5.9±0.2	84±10	15±2	7.6
Mutant	4.8±0.2	142±16	13±2	7.6

[a]Enzyme was activated with 50 mM $NaHCO_3$, 20 mM $MgCl_2$, pH 8.2 for at least 5 min at 25°C. Assays (McFadden, 1974) were initiated by the addition of activated enzyme.
[b]Anaerobic assays.
[c]The partition coefficient was determined by a non-radiometric procedure to be described in greater detail elsewhere.
[d]The kinetic properties of authentic *R. rubrum* carboxylase are almost indistinguishable from the recombinant wild-type enzyme (see Jordan and Ogren, 1983; Christeller and Laing, 1978).

Properties of the mutant enzyme

A kinetic analysis of the mutant and wild-type enzymes was performed with *E. coli* cell-free extracts, using the tight binding (Pierce *et al.*, 1980; Schloss and Lorimer, 1982) reaction-intermediate analog 2-CABP, to determine the concentration of active sites. This was done to minimize the possibility that the small differences observed with the purified enzymes were not due to differences in the stability of the two enzymes. The results revealed a small difference in the kinetic properties of the two enzymes. The mutant enzyme catalyzed both carboxylation and oxygenation at rates which were some 20–30% lower than the rates achieved by the wild-type enzyme (Table I). Precautions were taken to ensure that both enzymes were fully activated with CO_2 and Mg^{2+}. The small differences in k_{cat} must therefore be attributed to subtle differences in catalysis rather than to differences in the concentration of catalytically competent (i.e., activated) enzyme molecules. However, the partition coefficient, the ratio of the specificity factors for carboxylation and oxygenation, was the same for mutant and wild-type enzymes. The values of the partition coefficient were very close to those reported previously (Jordan and Ogren, 1983). The two enzymes could



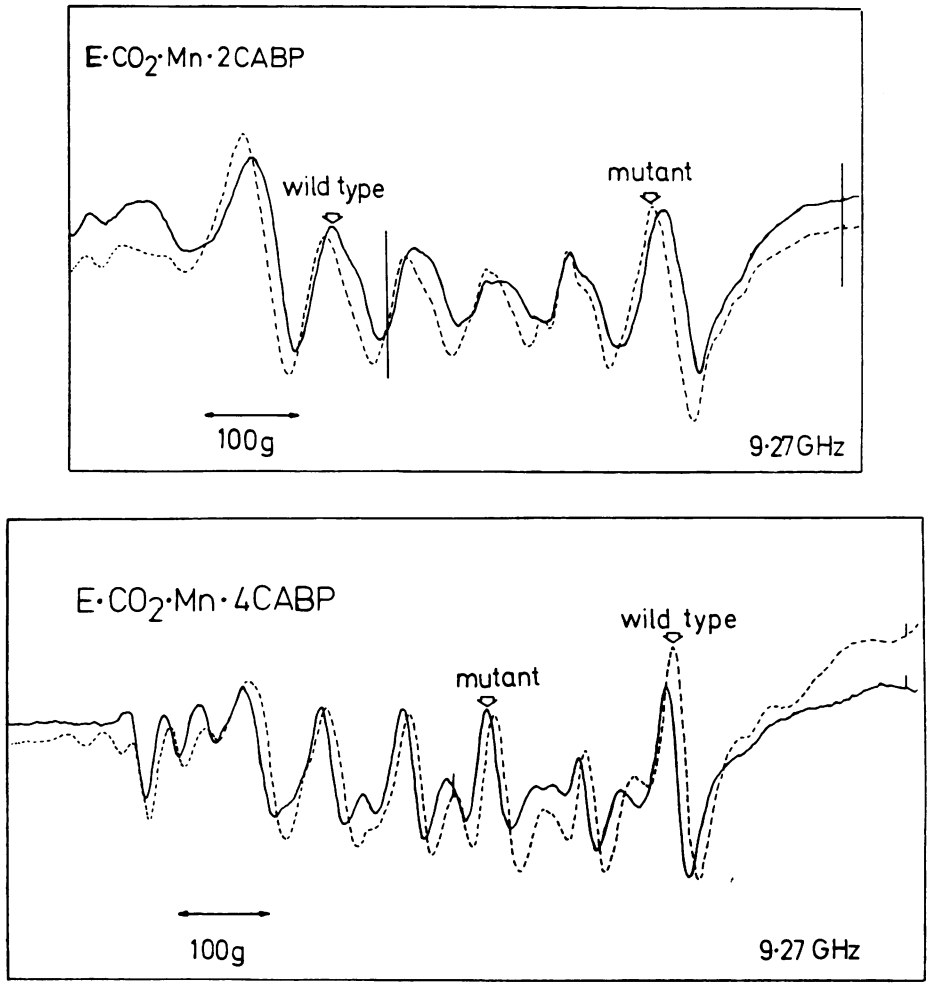


Fig. 4. E.p.r. spectra of the quaternary complexes (enzyme.activator carbamate.Mn^{2+}.CABP) formed with 2-CABP and 4-CABP of mutant and wild-type enzyme. Wide field spectra are shown for (**a**) the complexes containing 2-CABP and (**b**) the complexes containing 4-CABP. The g = 2 and g = 4 regions of the spectra of the 2-CABP complexes are shown in (**c**) and (**d**), respectively, to highlight the differences between the two enzyme species. The g = 4 regions of the spectra of the 4-CABP complexes are shown in (**e**). In (**d**) and (**e**) the spectra have been superimposed to show the downfield shift and increased resolution of the mutant enzyme. Microwave frequency was 9.27 GHz, modulation 6.3 g and power attenuation 10 dB.

not be distinguished upon the basis of their affinities for ribulose 1,5-bisphosphate. A small difference in the affinity for CO_2 was observed (Table I). In both cases the K_m values for the mutant and wild-type recombinant enzyme were similar to those previously reported for the native *R. rubrum* enzyme (Jordan and Ogren, 1983; Christeller and Laing, 1978).

The effect of the mutation on the nature of the structure of the divalent metal ion binding site was investigated by activating the enzymes with Mn^{2+} in place of Mg^{2+}. In the presence of the reaction-intermediate analogs 2-CABP and 4-CABP, the metal ion is bound essentially irreversibly at the active site (Pierce and Reddy, 1984; Miziorko and Sealy, 1980). Figure 4a and b shows the e.p.r. spectra of the quaternary complexes enzyme.activator carbamate.Mn^{2+}.2-CABP and 4-CABP, respectively formed with mutant and wild-type enzyme. Both generate almost identical Mn^{2+} spectra in terms of the positions of the various six-line features and typical of the spectrum expected for *R. rubrum* carboxylase (Miziorko and Sealy, 1984). However, closer inspection of the g = 2 and g = 4 regions (Figure 4c and d) indicate subtle, yet significant differences between the two species. The mutant carboxylase exhibits increased splitting and hence resolution of the superimposed features at both regions of the spectrum, including shifts to lower field by ~10 Gauss of the g = 4 derivative signal. Figure 4e is the same region of the spectrum of the quaternary complexes formed with 4-CABP. The spectral differences between the mutant and wild-type enzymes are more striking in terms of increased resolution and changes in signal position. The spectra do not permit identification of the metal ligands or definition of the geometry of the metal-binding site. However, it is clear that insertion of the methylene group accompanying the asp 198→glu 198 substitution has perturbed the divalent metal ion significantly. The precise manner by which this effect is achieved must await the completion of the crystallographic analysis (Schneider *et al.*, 1984).

Conclusion

The results described above indicate that oligonucleotide-directed site-specific mutagenesis can be applied successfully to introduce changes to the amino acid sequence of Rubisco at functionally important regions of the enzyme. The linear, double-stranded form of the expression plasmid pRR2119 can be used by exploiting the *Stu*I restriction site, generating mutant transformants. In the particular case described here the replacement of asp 198 by glu also introduced a new *Eco*RI restriction site into the Rubisco gene at a region devoid of useful sites. This also provided a rapid means for screening putative mutants.

In the absence of a crystallographic structure, the precise role of asp 198 is not known. It is present in all the dozen Rubiscos so far sequenced, both prokaryotic and eukaryotic, implying some degree of functional importance. Certainly it contributes to the high negative charge of this region of the protein, a fact that suggests that it may indeed by involved with the binding of the divalent metal ion. Accommodation of the extra methylene group of glutamate is sufficient to disturb the normal coordination of the divalent metal ion, as revealed by e.p.r. spectroscopy. This is accompanied by a modest decline in the catalytic activity of the enzyme, without altering the partitioning between carboxylation and oxygenation. Thus, this single mutation strengthens the hypothesis that these reactions occur at a common active site and that a reaction common to both carboxylation and oxygenation can be attenuated as a result of this single amino acid change. Whether or not changes in the partition coefficient can be brought about by substituting other amio acids by site-specific mutagenesis remains to be seen. There are grounds for cautious optimism (Wilkinson *et al.*, 1984).

Materials and methods

Restriction endonucleases, polymerases, ligases and DNA standards were purchased from Bethesda Research Labs., MD, USA and New England Biolabs, MA, USA. The restriction enzymes were used as recommended by the suppliers.

[γ-^{32}P]ATP was purchased from Amersham, UK. All other reagents were of the finest quality available.

Hybridisation and repair of the plasmid DNA

The plasmid was digested to a linear double-stranded form with restriction enzymes (see Figure 2a). The non-coding strand was shortened using T4 DNA polymerase (4 units/μg DNA) in the absence of deoxyribonucleotide triphosphates. Previous control experiments indicated that 30 min incubation at 37°C removed enough bases to expose the region for hybridisation. After denaturation of the enzyme and phenol extraction, the DNA was recovered by ethanol precipitation. The oligonucleotide was hybridised to the partial linear duplex DNA (60 ng oligonucleotide/μg DNA) by heating at 55°C for 15 min in 10 μl of 20 mM Tris-HCl; 50 mM NaCl, 10 mM $MgCl_2$, pH 7.8. The Klenow fragment of DNA polymerase I (5 units/μg template DNA) was added along with 1 mM each of the four deoxyribonucleotide triphosphates to synthesize double-stranded DNA. After 15 min at room temperature, ATP (1 mM final concentration) and 4 units of T4 DNA ligase were added to ligate the primer to the repaired DNA by incubating overnight at 4°C. The proteins were precipitated with an equal volume of phenol and the DNA in the aqueous phase desalted into 10 mM Tris-HCl, 1 mM EDTA, pH 7.6 by rapid gel filtration (P6-DG gel, Biorad, CA, USA) in a bench centrifuge. To repair the *Stu*I 'blunt-end' cut and ensure retrieval of super-coiled pRR2119, the plasmid was subjected to further ligation. $MgCl_2$ (6 mM) and β-mercaptoethanol (6 mM) were added to the plasmid solution along with 1 mM ATP and 8 units of T4 DNA ligase and incubation allowed to proceed for at least 24 h at 4°C. The recircularized plasmid was purified by phenol-extraction and ethanol precipitation and used to transform competent cells of *E. coli* strain HB101. Transformation was by methods described in Maniatis *et al.* (1982).

Location of colonies containing mutant plasmid

Colonies (270) of HB101 transformed with recircularized plasmid were prepared for *in situ* hybridization by the procedures described by Maniatis *et al.* (1982). The synthetic oligonucleotide probe was phosphorylated with [γ-^{32}P]ATP and T4-polynucleotide kinase (Maniatis *et al.*, 1982). Hybridization was performed by incubating the filter disc in 3 ml of 0.6 M NaCl, 0.09 M Na citrate, pH 7.0, containing 10% Denhardt's solution and 60 ng (4×10^6 c.p.m. ^{32}P) of the labelled probe. After 1 h at room temperature the disc was rinsed with 0.6 M NaCl, 0.09 M Na citrate, pH 7.0, dried on filter paper and autoradiographed. Further washings (5 min) at increasing temperatures were done with the same buffer. A final wash for 5 min within 0.5°C of the T_m (Dalbadie-McFarland *et al.*, 1982) for the synthetic probe (60°C) ensured clear identification of colonies containing mutant plasmid.

Sequence analysis of the mutagenised region

Ten micrograms of the mutant plasmid DNA, previously digested with 36 units of *Stu*I for 3 h at 37°C, were phosphorylated with [α-^{32}P]cordycepin-5'-triphosphate (20.0 μCi) (New England Nuclear) and 5 units of terminal deoxynucleotidyl transferase (P.L. Biochemicals) in 0.2 M potassium cacodylate, pH 7.2, 2 mM $CoCl_2$, 2 mM β-mercaptoethanol (total volume, 75 μl) for 1 h at 37°C. Two μl 0.1 mM unlabelled cordycepin-5'-triphosphate were added and the incubation continued for a further 20 min. The reaction was stopped by the addtion of 2 μl 0.5 M EDTA and 1.8 ml of 10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.1 mM EDTA in 50% ethanol. The solution was applied to a Whatman CF-11 column and the DNA eluted with water. The DNA was recovered by precipitation with ethanol and the precipitate redissolved in water, before being treated with *Pst*I (60 units, 2 h, 37°C). The 400-bp *Stu*I-*Pst*I fragment which included the new *Eco*RI site was isolated by gel electrophoresis on 5% polyacrylamide. The fragment was eluted from the gel with NACS buffer (Bethesda Research Labs.) and purified by passage through a column of NACS-52 (Bethesda Research Labs.). Ten micrograms of carrier RNA were added to the eluate and the mixture precipitated with ethanol. Sequencing was by the method of Maxam and Gilbert (1980).

Isolation of the recombinant enzyme

E. coli (strain HB101), transformed with either the wild-type or mutant plasmid, was cultured on a large scale (Pierce and Gutteridge, in preparation) to produce large quantities of the recombinant enzymes. The carboxylase was released from *E. coli* by treatment of cell pellets suspended in two volumes of 50 mM Tris-HCl, pH 8.1, containing 0.1 mM EDTA, 1 mM dithiothreitol and 1 mM p-toluene sulfonyl fluoride. Lysozyme and DNase (0.1 mg/ml) were also added to the solution, which was then incubated for 20 min at 35°C. Generally, between 10 and 15% of the total soluble protein was carboxylase at this stage. The large scale purification of the enzyme from the cell debris was by methods described elsewhere (Pierce and Reddy, 1984). The activity of the carboxylase was determined by incorporation of $^{14}CO_2$ into acid stable products (McFadden, 1974; Lorimer *et al.*, 1977).

Preparation of e.p.r. samples

[^{14}C]2-CABP and [^{14}C]4-CABP were prepared as previously described (Pierce *et al.*, 1980; Schloss and Lorimer, 1982). Two micromoles of enzyme promoter were incubated with a 2-fold molar excess of either [^{14}C]2-CABP or 4-CABP in the presence of 0.1 M Bicine-NaOH, 0.05 M $NaHCO_3$, 0.01 M $MnCl_2$, pH 8.2 for 10 h at 25°C. The solution was gel filtered on Sephadex-G25 *versus* 5 mM Bicine-NaOH, pH 8.2, 0.1% (v/v) glycerol. The protein fraction was pooled and concentrated by vacuum dialysis *versus* the same buffer to a final promoter concentration of 1–2 mM. The final CABP/promoter stoichiometry varied from 0.94 to 1.04. E.p.r. spectra were recorded on a Bruker instrument at 123 K.

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