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Ribulose 1,5-bisphosphate carboxylase

Effect on the catalytic properties of changing methionine-330 to leucine in the *Rhodospirillum rubrum* enzyme

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Oligonucleotide-directed mutagenesis of cloned *Rhodospirillum rubrum* ribulose bisphosphate carboxylase/oxygenase with a synthetic 13mer oligonucleotide primer was used to effect a change at Met-330 to Leu-330. The resultant enzyme was kinetically examined in some detail and the following changes were found. The $K_{m(CO_2)}$ increased from 0.16 to 2.35 mM, the $K_{m(\text{ribulose bisphosphate})}$ increased from 0.05 to 1.40 mM for the carboxylase reaction and by a similar amount for the oxygenase reaction. The $K_{i(O_2)}$ increased from 0.17 to 6.00 mM, but the ratio of carboxylase activity to oxygenase activity was scarcely affected by the change in amino acid. The binding of the transition state analogue 2-carboxyribitol 1,5-bisphosphate was reversible in the mutant and essentially irreversible in the wild type enzyme. Inhibition by fructose bisphosphate, competitive with ribulose bisphosphate, was slightly increased in the mutant enzyme. These data suggest that the change of the residue from methionine to leucine decreases the stability of the enediol reaction intermediate.

INTRODUCTION

The bifunctional photosynthetic enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) (RubisCO) is an important control enzyme in photosynthesis and photorespiration (Lorimer, 1981; Ogren, 1984). The carboxylation reaction is the first step in CO_2 fixation and the oxygenation reaction leads to CO_2 release, reducing net photosynthesis. Understanding the mechanism of the carboxylase/oxygenase reactions is critical in predicting whether attempts to alter the relative rates of carboxylation and oxygenation are feasible.

There is a firm theoretical understanding of the reaction mechanism of this enzyme (Calvin, 1954; Miziorko & Lorimer, 1983). However, because X-ray crystallographic studies have lacked sufficient resolution to determine the structure of RubisCO, no information is available on the spatial orientation of residues or substrates within the active sites or on which residues have a direct role in catalysis. Current knowledge of the active site is largely based on work by Hartman and coworkers (Hartman *et al.*, 1984) using a range of active site directed reagents. By studying both the higher plant spinach hexadecameric enzyme and the bacterial *Rhodospirillum rubrum* dimeric enzyme, Hartman's group have identified several regions of high homology. Within these regions, conserved lysine residues have been consistently labelled by these reagents, but more information on the precise roles of these amino acids is needed. To date His-44, Lys-166, Lys-329 and Met-330 have been identified as active site residues and Lys-191 as the CO_2 activator site, so called because it binds the CO_2 which activates the enzyme (Miziorko & Lorimer, 1983; Hearndon & Hartman, 1984; Hartman *et al.*, 1984; Fig. 1).

RubisCO DNA from *R. rubrum* has been cloned, sequenced and expressed in *Escherichia coli* (Somerville & Somerville, 1983; Nargang *et al.*, 1984). Two previous reports (Gutteridge *et al.*, 1984; Estelle *et al.*, 1985) using oligonucleotide-directed mutagenesis described changes in the CO_2 activator region of this RubisCO (Miziorko & Lorimer, 1983). We chose to work at the region around the catalytic site near Lys-329 because of previous results at this site. A single methionine had been chemically modified by the active site directed reagent 2-*N*-chloroamino-2-deoxypentitol 1,5-bisphosphate (Christeller & Hartman, 1982) resulting in changes in the kinetic parameters of RubisCO. At the same time, Fraij & Hartman (1982) labelled a specific methionine with the reagent 2-bromoacetylaminopentitol 1,5-bisphosphate and confirmed its position as Met-330 by peptide sequencing (Fraij & Hartman, 1983). Consequently, we chose to change Met-330 to leucine, the equivalent amino acid in higher plant enzymes (Miziorko & Lorimer, 1983), by using oligonucleotide-directed mutagenesis and we report the results in the present paper.

MATERIALS AND METHODS

Materials

Ribulose bisphosphate was either purchased from Sigma or synthesized (Laing & Christeller, 1976). In either case it was purified on a Dowex 1 X8 Cl^- column with a 0–0.2 M-HCl gradient. The eluted ribulose bisphosphate was freeze-dried, redissolved in water, the pH adjusted to 6.5 with Tris base and then again freeze-dried. The dried powder was stored at −70 °C. Fructose bisphosphate and phosphoglycolate were obtained from Sigma, and ATP was from Boehringer.

Abbreviations used: RubisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; CABP, CPBP, CRBP, the 1,5-bisphosphates of 2-carboxyarabinitol, 2-carboxypentitol and 2-carboxyribitol, respectively; kb, kilobases; bp, base pairs.

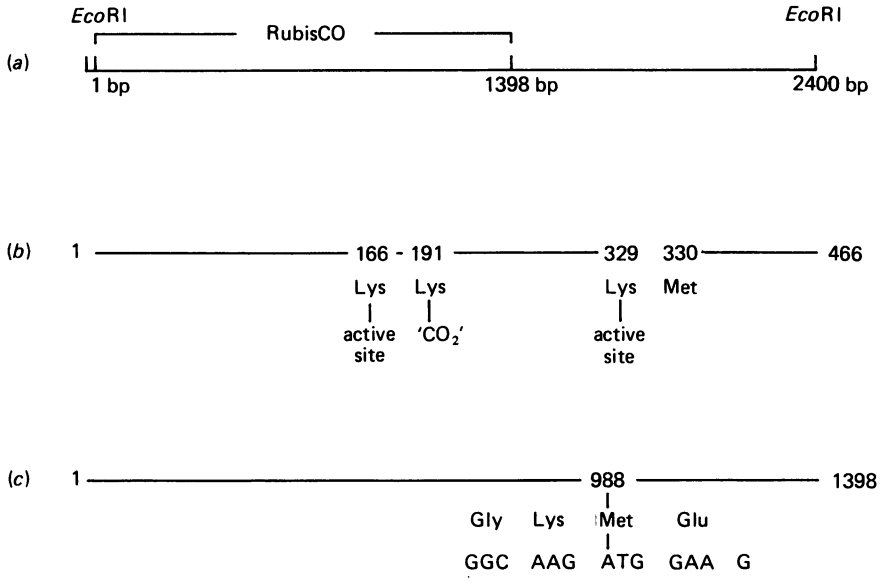

Fig. 1. Map of the *R. rubrum* RubisCO gene and enzyme

(*a*) Schematic presentation of the *Eco*RI fragment containing the *R. rubrum* RubisCO gene (Somerville & Somerville, 1983). (*b*) Identified lysine residues in *R. rubrum* RubisCO showing the position of Met-330. (*c*) Wild type DNA sequence of region used in mutagenesis. Nucleotide 988 was changed from A to C in the mutant.

$NaH^{14}CO_3$, $Na^{14}CN$ and [^{32}P]ATP came from Amersham. Phosphoglycolate phosphatase and glycolate oxidase were purified as described previously (Christeller & Laing, 1979). [^{14}C]Carboxyarabinitol 1,5-bisphosphate (CABP) was prepared as described by Pierce *et al.* (1980) except it was purified by the above method for ribulose bisphosphate. Unlabelled carboxypentitol bisphosphate (CPBP) was prepared as described for CABP, but it was not separated into its two isomers.

The primer 5′ CTTCCAGCTTGCC 3′ used for mutagenesis was synthesized on a polydimethylacrylamide-kieselguhr support by the phosphotriester route (Gait *et al.*, 1982), using a semi-manual synthetic apparatus (Omnifit, Cambridge, U.K.). The deprotected oligonucleotide was purified by elution from a DEAE-cellulose column (Whatman DE32) with a gradient of NaCl concentration in 0.1 M-sodium acetate/7 M-urea, pH 5.4 (Petersen & Reeves, 1969) and desalted by adsorption onto DE32 resin followed by elution with 2 M-triethylamine carbonate and lyophilization. Fractions from the columns were assayed and the purity of the final product was checked by electrophoresis of the oligonucleotides on polyacrylamide gels after terminal labelling with [^{32}P]ATP and T4 polynucleotide kinase (Maniatis *et al.*, 1982).

The *R. rubrum* RubisCO gene, obtained as a gift from C. Somerville, was cloned as a 2.4 kb *Eco*RI fragment in the plasmid pRR2119 (Somerville & Somerville, 1983). We recloned the *Eco*RI fragment into the vectors M13mp9 (Messing, 1984) and pEMBL8$^-$ (Dente *et al.*, 1983) using standard procedures (Maniatis *et al.*, 1982; Christeller *et al.*, 1985). *E. coli* strain JM101 was used for propagation of M13mp9 and strain JM83 for pEMBL8$^-$ with plasmid selection using ampicillin (Sigma; 50 μg/ml). 2XYT medium (Miller, 1972) was used throughout. Bacterial genotypes, especially for *lac*, and other conditions were as described in Christeller *et al.* (1985).

Methods

Oligonucleotide-directed mutagenesis was carried out as described by Zoller & Smith (1983) with the following modifications. The M13mp9–RubisCO ssDNA was incubated for only 2 h with the mutation primer and the M13 universal sequencing primer (for partial conversion of ssDNA to dsDNA) and the derived DNA was not purified on a sucrose gradient. Polyacrylamide gel electrophoresis was done as described by Maniatis *et al.* (1982).

For routine screening assays of RubisCO carboxylase activity, 2 ml of *E. coli* infected with M13 carrying the RubisCO gene insert were grown to late exponential phase. A 1 ml sample was harvested by centrifugation for 7 min in an Eppendorf centrifuge and resuspended in 100 μl of 100 mM-Tris (pH 8.0)/20 mM-$MgCl_2$/20 mM-$NaHCO_3$/10 mM-dithiothreitol (buffer A). Carboxylase activity was then determined by permeabilizing the cells with 20 μl of toluene (Tabita *et al.*, 1978), and assaying 10 μl in 0.25 ml of reaction mix containing 100 mM-Tris/HCl (pH 8.0)/20 mM-$MgCl_2$/5 mM-$NaH^{14}CO_3$ (1 mCi/mol) and 0.2 mM-ribulose bisphosphate for 6 min. Backgrounds were run without ribulose bisphosphate. Other details are described in Christeller *et al.* (1985).

Assay conditions to analyse the kinetic behaviour of the enzyme are described in the Figure and Table legends. The reaction vessels were 5 ml evacuated blood collection tubes pre-equilibrated with the relevant gas phase. Complete reaction mix (0.35–0.4 ml) was injected into the tube, the tube pre-equilibrated at 30 °C for 5 min and the assay started by injecting enzyme. The enzyme was pre-activated in 20 mM-$NaHCO_3$ and 20 mM-$MgCl_2$ at 30 °C for 10–20 min. Assays were stopped after 1 min by injecting 100 μl of 2 M-HCl. The carboxylase activity was measured by determining acid-stable radioactivity (labelled phosphoglycerate product) in a scintillation

counter. The oxygenase activity was determined either by converting the phosphoglycolate product to glyoxylate and measuring the phenylhydrazone derivative colorimetrically as described by Christeller & Laing (1978) or by using an oxygen electrode (Lorimer *et al.*, 1977).

Larger quantities of RubisCO were prepared by harvesting 4 × 500 ml cultures of late log phase M13- or pEMBL-infected *E. coli* by centrifugation, resuspending the cells in buffer A containing 0.5 mM-phenylmethanesulphonyl fluoride and 1 mM-EDTA, sonicating to break the cells and centrifuging to clear the solubilized RubisCO. Protein was precipitated by making the supernatant 20% in poly(ethylene glycol) and 20 mM-$MgCl_2$, and collected by centrifugation. The precipitate was redissolved in buffer A containing EDTA, and this solution was applied to a 30 cm × 2.5 cm DEAE-cellulose column equilibrated with buffer A containing EDTA. All operations were carried out at 4 °C. The RubisCO was eluted with a 0–0.2 M-NaCl gradient. This gave a 30-fold purification of the enzyme. Peak fractions were concentrated with 55% $(NH_4)_2SO_4$, centrifuged, redissolved in 20 mM-Tris/HCl buffer (pH 8.0) and desalted on a Sephadex G-25 column equilibrated in the same buffer. The enzyme was stored at −70 °C.

Data were fitted to kinetic equations by using nonlinear regression analysis as described in Laing & Christeller (1980).

RESULTS

Mutagenesis

Chemical modification of *R. rubrum* RubisCO methionine residue by Christeller & Hartman (1982) with a site-specific reagent resulted in a modified enzyme with a higher $K_{m(\text{ribulose bisphosphate})}$ and a reduced $V_{max.}$. Given the similarity in structure between this reagent and the reagent used by Fraij & Hartman (1982, 1983), it is likely that the chemically modified methionine is the same as that identified by Fraij & Hartman (1983) as Met-330. These results suggest that the Met-330 adjacent to active site Lys-329 (Hartman *et al.*, 1978) is important in catalysis, but that changes to this residue do not necessarily inactivate the enzyme. Lacking more specific information on structure–function relationships we decided to modify this Met-330 residue. The equivalent residue adjacent to active site Lys-329 in higher plants as determined from published sequences (Miziorko & Lorimer, 1983) is leucine. A methionine to leucine change can be accomplished by a single nucleotide change from ATG to CTG. This substitution also has the advantage of generating a new *Alu*I restriction enzyme site, allowing verification of the substitution.

We therefore did a computer sequence check comparison to verify that the sequence had no significant matching to other regions of DNA in the RubisCO gene or the M13mp9 DNA and then synthesized the 13mer oligonucleotide primer containing a one base mismatch. The template *R. rubrum* 2.4 kb *Eco*RI RubisCO DNA fragment was recloned from its original vector (Somerville & Somerville, 1983) into the *Eco*RI site of M13mp9. Phage with the fragment in the right orientation were identified by carboxylase activity (Christeller *et al.*, 1985). Of the recombinant plaques, 50% expressed activity enhanced by isopropylthiogalactoside (Christeller *et al.*, 1985). Although some clones were unstable and soon failed to express, stable lines were easily selected for subsequent work. *In vitro* mutagenesis followed the method of Zoller & Smith (1983). The primer was annealed to single-stranded M13 template DNA, extended and the partially double-stranded DNA was transformed into *E. coli* JM101. Putative mutants were initially detected by single plaque screening for carboxylase activity (Christeller *et al.*, 1985). Eight of 100 plaques showed reproducible low carboxylase activity (about 5–10% of wild type). Other plaques with intermediate levels of carboxylase activity segregated into either phages with no carboxylase activity, presumably through deletion of inserted DNA, or to phage expressing normal activity. Four independently derived stable low expression plaques were selected for further study.

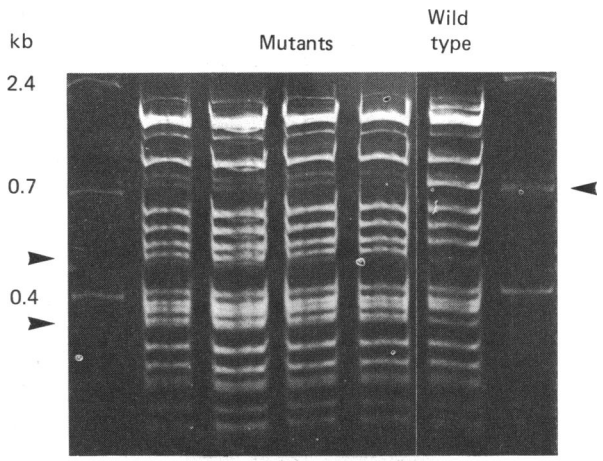

Fig. 2. *Alu*I digestion patterns

The double-stranded M13mp9 DNA from four mutants and from wild type was digested with *Alu*I restriction enzyme, and the digested fragments were analysed on a 3% polyacrylamide gel, the gel was stained with ethidium bromide and photographed under u.v. light (Maniatis *et al.*, 1982). An 805 bp fragment in wild type has been cut into 497 bp and 308 bp fragments in the mutants.

Confirmation of the nucleotide change

Hybridization. Annealing of ^{32}P-labelled primer to the mutant DNA and to cloned wild type DNA was determined over a range of temperatures (Zoller & Smith, 1983). Hybridization to wild type and mutant DNAs occurred at 40 °C but only the mutant DNAs continued to hybridize at 42 °C (results not shown). This result is consistent with complete homology between the 13mer and the mutant DNA and a one base difference between the 13mer and the wild type cloned *R. rubrum* DNA (Winter *et al.*, 1982; Zoller & Smith, 1983).

***Alu*I restriction enzyme digest pattern.** To verify that a new *Alu*I site had been generated, the complete *Alu*I digestion pattern was compared for DNA from the four mutants and wild type *R. rubrum* M13 clones (Fig. 2). We predicted that a 805 bp *Alu*I fragment should be cleaved into two fragments of 497 and 308 bp. As shown, one large fragment generated in wild type digested DNA was absent in the mutant DNA digestion patterns, while two smaller fragments of the expected size were generated. No other changes in the restriction pattern were apparent. No

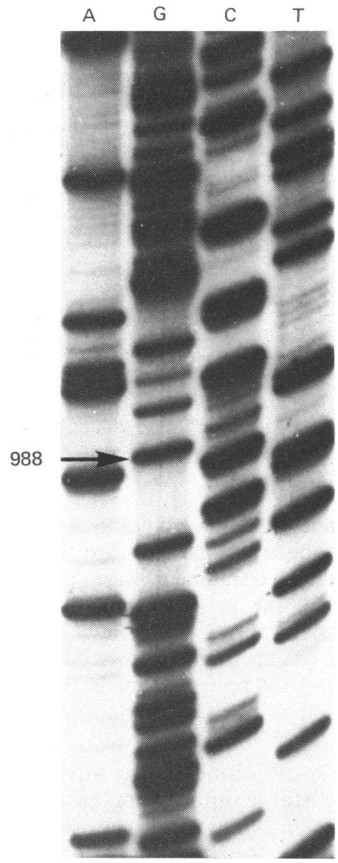

Fig. 3. Sequencing gel showing mutant sequence of complementary DNA strand (5′ CTTCCAGCTTGCC 3′)

The substituted base G is indicated at nucleotide 988.

other single base change in the primer could generate an *Alu*I site.

Nucleotide sequence. The mutant DNA sequence was determined by dideoxy sequencing (Sanger *et al.*, 1977) which confirmed cytosine at nucleotide 988 in the mutant DNA (Fig. 3).

Purification

Mutant and wild type enzymes behaved identically throughout purification, and hence could not be distinguished on the basis of net charge or M_r, as expected from a methionine to leucine change.

Initial studies

We then measured the $K_{m(CO_2)}$ and $K_{m(\text{ribulose bisphosphate})}$ on all four selected mutants. They all showed similar increases in these two K_m values (results not shown), verifying the reproducibility of the technique and its effect on the protein. In order to obtain sufficient expressed RubisCO protein to characterize fully, we recloned both wild type and mutant DNAs into the expression vector pEMBL8$^-$ (Dente *et al.*, 1983), confirmed their stability and thereby increased the level of expression per litre of culture about 20-fold (to approx. 20 mg/l of culture). Preliminary studies established no differences between wild type and mutant enzymes in the rate of activation or the response of activation to HCO_3^- and $MgCl_2$ concentration (results not shown). Hence

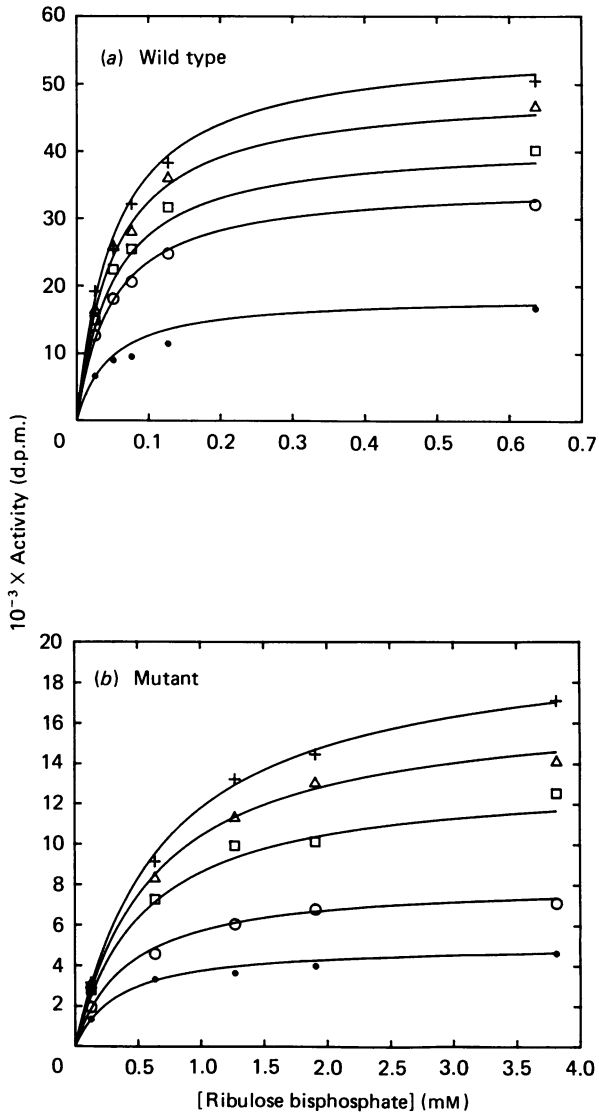

Fig. 4. Response of wild-type and mutant RubisCO to CO_2 and ribulose bisphosphate concentration

RubisCO reaction rates were determined under N_2 as described in the text. The reaction conditions were 100 mM-Tris/HCl (pH 8.0), 20 mM-$MgCl_2$, 4 μCi of $^{14}CO_2$/ml with CO_2 and ribulose bisphosphate as indicated below at 30 °C. RubisCO was preincubated for 10–20 min at 30 °C in 100 mM-Tris/HCl (pH 8.0), 20 mM-$MgCl_2$ and $NaHCO_3$ before initiating the reaction with 10 μl of this mixture. The concentrations of the enzyme in the reaction mixture were 3.2 μg of wild type/ml and 86 μg of mutant/ml. Assays were stopped after 60 s. The equilibrium between CO_2 and HCO_3^- in solution was calculated assuming a pK_1 of 6.33 (corrected for temperature and ionic strength). The amount of enzyme was calculated by incubating activated RubisCO with [^{14}C]CABP and separating the enzyme–CABP complex from the unbound CABP on a Sephadex G-75 column (see Fig. 5). The amount of mutant enzyme was corrected to infinite concentration of enzyme to correct for the lower affinity constant. (*a*) Wild-type RubisCO: ●, 62 μM-CO_2; ○, 183 μM; □, 269 μM; △, 474 μM; +, 890 μM. (*b*) Mutant RubisCO: ●, 269 μM-CO_2; ○, 474 μM; □, 890 μM; △, 1268 μM; +, 1672 μM. Note that the ribulose bisphosphate scales for the two graphs are different. The lines in Fig. 4 are calculated by using the constants described in Table 1.

Table 1. Derived parameters for wild-type and mutant RubisCOs

The values of V_C, K_r, K_{ir} and K_C were determined as described in the text and Fig. 4. The data were fitted to eqn. 1. The value of $K_{i(\text{fructose bisphosphate})}$ was calculated from an experiment where the inhibition by fructose bisphosphate was measured over a range of ribulose bisphosphate concentrations. A competitive inhibition model fitted well to the data. Similarly, the $K_{i(O_2)}$ was calculated from an experiment where the inhibition by O_2 was measured over a range of CO_2 concentrations. A competitive inhibition model fitted well to the data. The partition coefficient, $V_C K_O / V_O K_C$, was calculated from the slopes of the lines shown in Fig. 6. S.E.M. values are shown in parentheses.

Parameter (unit)	Wild type (WT)	Mutant (M)	Ratio (M/WT)
$V_{c(max.)}$ (μmol/min per mg)	6.1	1.23	0.2
K_r (μM)	55 (7)	1400 (370)	25
K_{ir} (μM)	43 (14)	230 (106)	5
K_c (μM CO_2)	159 (20)	2354 (471)	15
$K_{i(\text{fructose bisphosphate})}$ (mM)	3.0 (0.63)	1.9 (0.15)	0.63
$K_{i(O_2)}$ (μM)	167 (21)	6000 (1321)	36
$V_C K_O / V_O K_C$			
2 mM*	7.2 (0.07)	7.7 (0.09)	1.1
0.094 mM*	7.4 (0.3)	6.3 (0.06)	0.85

* Concentration of ribulose bisphosphate.

identical reactivation regimes were used in all further experiments.

Carboxylase activity

The response of the carboxylase activity of the mutant and the wild type enzymes to ribulose bisphosphate and HCO_3^- concentrations is shown in Fig. 4. It is immediately apparent that the $K_{m(\text{ribulose bisphosphate})}$ and $K_{m(CO_2)}$ are considerably increased in the mutant enzyme compared with wild type. The data in Fig. 4 were fitted to a velocity equation of the form:

$$v = V_{max.} . C . R / (K_c . K_{ir} + K_c . R + K_r . C + R . C) \quad (1)$$

where C is the CO_2 concentration, R is the ribulose bisophosphate concentration, K_c and K_r are the Michaelis constants for CO_2 and ribulose bisphosphate and K_{ir} is the inhibition constant for ribulose bisphosphate. $V_{max.}$ is the maximum velocity.

The calculated constants for the mutant and the wild type enzymes are tabulated in Table 1. Eqn. (1) is the equation for either a steady state ordered mechanism (with ribulose bisphosphate binding first) or a rapid equilibrium random mechanism (Segel, 1975). Laing & Christeller (1980) showed using initial velocity studies that soybean RubisCO has a random mechanism and this was confirmed by Roeske & O'Leary (1984) using isotope kinetic techniques for spinach enzyme. Subsequently, Roeske & O'Leary (1985) suggested that the enzyme from *R. rubrum* was essentially ordered in its mechanism with ribulose bisphosphate binding first. In the present paper, we will interpret the data on the basis of an ordered mechanism although the interpretation would be little affected if it were a random mechanism.

Because we had not purified the enzyme derived from the cloned gene to homogeneity, we needed a method to determine the amount of RubisCO protein present in an assay. To do this we measured the binding of the substrate analogue CABP to the enzyme, a method described by Hall *et al.* (1981). Wild type *R. rubrum* RubisCO binds CABP essentially irreversibly, as shown by the fact it does not dissociate during gel filtration (Fig. 5). Consequently, the binding of [^{14}C]CABP to wild type RubisCO is a valid measure of the amount of enzyme (Pierce *et al.*, 1980). However, when our mutant enzyme was similarly filtered, a considerable proportion of the [^{14}C]CABP was released and appeared as a second low-M_r peak. Upon running similar experiments with a smaller column and several concentrations of mutant RubisCO, the amount of bound CABP increased with increasing enzyme concentration (results not shown). We extrapolated the amount of bound CABP to infinite enzyme concentration and used this as an estimate of the amount of mutant RubisCO present. The binding of CABP to wild type RubisCO was independent of the concentration of RubisCO. Using the [^{14}C]CABP binding as a minimum measure of the amount of RubisCO present (Pierce *et al.*, 1980; Hall *et al.*, 1981) we calculated the $V_{max.}$ for the mutant and wild type enzymes (Table 1).

When the radioactive CABP–enzyme mix from the Sephadex G-75 column was incubated with a vast excess of unlabelled CPBP and passed through the G-75 column again, a proportion of the [^{14}C]CABP was released from the wild type enzyme (Fig. 5). Brown & Chollet (1982) have suggested that *R. rubrum* RubisCO binds CABP less tightly than higher plant large subunit/small subunit type RubisCOs. However, when the experiment was done using the mutant enzyme (with a lower ratio of unlabelled CPBP to [^{14}C]CABP–enzyme complex), virtually all of the radioactivity was released from the enzyme (Fig. 5). Similar results were reported by Estelle *et al.* (1985) with their mutant *R. rubrum* RubisCO.

Assuming a steady state ordered mechanism with ribulose bisphosphate binding first, the K_{ir} (Table 1) can be interpreted to represent the binding constant for ribulose bisphosphate in the absence of CO_2 (Segel, 1975), suggesting that the binding constants for the wild type and mutant enzymes were not as dissimilar as the K_r values would suggest. We tested this hypothesis by studying the inhibition of the enzyme by fructose bisphosphate, a dead end inhibitor of RubisCO, competitive with ribulose bisphosphate. The inhibition constant for such an inhibitor is often the binding

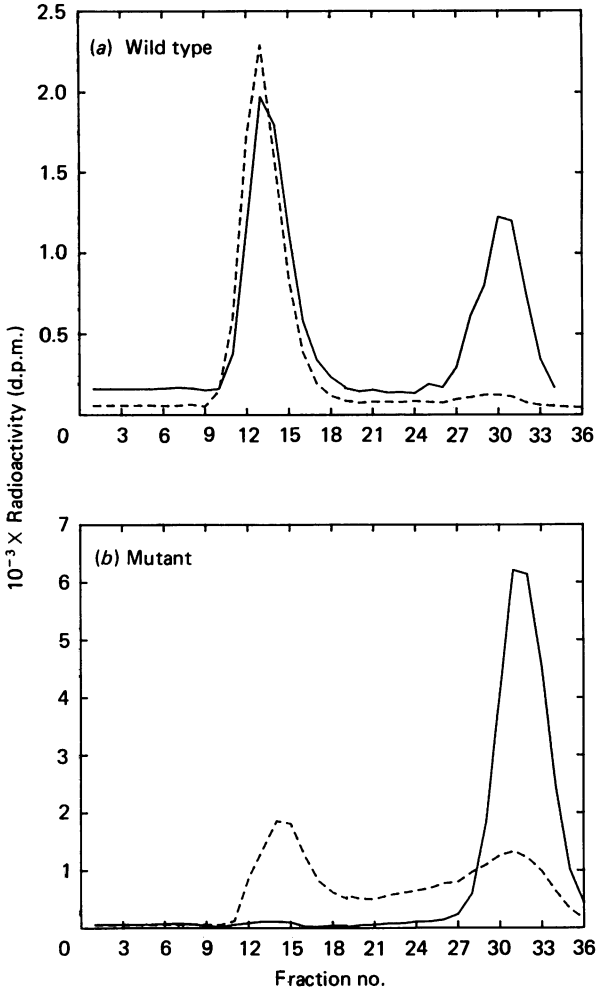

Fig. 5. CABP binding to RubisCO

RubisCO was preactivated by incubation in 100 mM-Tris/HCl (pH 8.0), 20 mM-$NaHCO_3$ and 20 mM-$MgCl_2$ for 15 min. Then [^{14}C]CABP (57.7 Ci/mol; final concentration 170 μM) was added and the mixture incubated for a further 10 min. The RubisCO–CABP complex was separated from the unbound CABP by gel filtration on a 1 cm × 30 cm Sephadex G-75 column equilibrated with 100 mM-Tris/HCl (pH 8.0), 20 mM-$MgCl_2$ and 50 mM-$NaHCO_3$ and the fractions were then collected and aliquots counted. To obtain CABP–enzyme complex, a sample of the fraction immediately preceding the maximum of the RubisCO–CABP peak was mixed with unlabelled CABP (12 mM final concentration, ratio of enzyme to CABP of 1:24000 for wild type RubisCO and 1:8000 for mutant RubisCO) and incubated for 30 min. A parallel sample was incubated without CABP. Both samples were passed through gel filtration columns as above and fractions were counted for radioactivity. (*a*) Wild-type enzyme, (*b*) mutant enzyme. Continuous lines show RubisCO incubated with excess unlabelled CABP and the broken lines show RubisCO incubated without excess CABP. The entire fraction was counted.

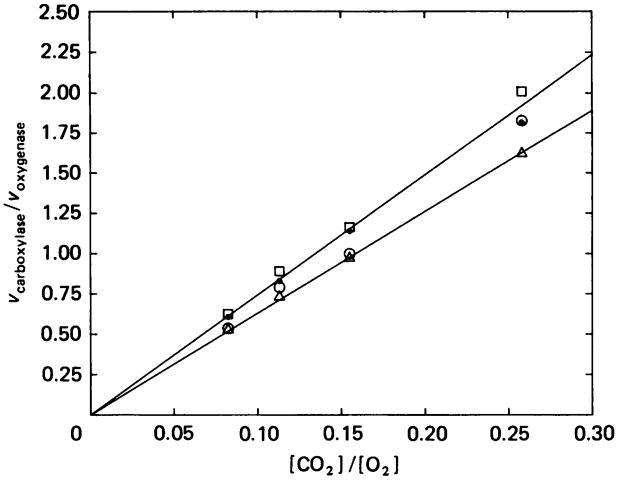

Fig. 6. Partition coefficient for RubisCO

The rates of carboxylation and oxygenation were measured simultaneously under 100% O_2 and at four CO_2 concentrations. The ratio of carboxylation to oxygenation is plotted versus the CO_2 concentration and the slope of this line when corrected for the oxygen concentration is the partition coefficient (Jordan & Ogren, 1983). Reaction conditions were 84 mM-Tris/HCl (pH 8.0), 22 mM-$MgCl_2$, 6.8 μCi of $^{14}CO_2$/ml at 30 °C. The enzyme was preincubated in 100 mM-Tris/HCl (pH 8.0), 20 mM-$MgCl_2$ and $NaHCO_3$ for 10–20 min before the reactions were initiated with enzyme. After 6 min, the reactions, were stopped, aliquots taken for determining acid-stable ^{14}C fixed and the remainder was neutralized and phosphoglycolate determined as described in the text. ●, 2.0 mM-ribulose bisphosphate, wild type enzyme; ○, 2.0 mM-ribulose bisphosphate, mutant type enzyme; □, 0.094 mM-ribulose bisphosphate, wild type enzyme; △, 0.094 mM-ribulose bisphosphate, mutant type enzyme. Regression of the ratio of carboxylase to oxygenase on CO_2 concentration had an intercept not significantly different from zero. Consequently, the regressions were forced through the origin and the slopes are tabulated in Table 1. The regression line for the data at 2 mM-ribulose bisphosphate (both enzymes) and at 0.094 mM-ribulose bisphosphate wild type enzyme were not significantly different and are represented by one line (upper).

constant for the inhibitor with the enzyme (Segel, 1975). We measured the inhibition by fructose bisphosphate as a function of ribulose bisphosphate and analysed the data. Non-linear regression showed that the inhibition pattern best fitted a competitive model. The inhibition constant for fructose bisphosphate was 1.9 mM for the mutant enzyme, compared with 3.0 mM for wild type (Table 1).

Oxygenase activity

We measured the $K_{m(O_2)}$ for the oxygenase reaction for wild type and mutant enzymes. Although a satisfactory $K_{m(O_2)}$ for the wild type enzyme could be calculated from the data, the response to O_2 from the mutant enzyme was essentially linear up to 100% oxygen, indicating that the $K_{m(O_2)}$ was considerably higher in the mutant than the wild type enzyme. Consequently, we measured the $K_{i(O_2)}$ in the carboxylase reaction as an estimate of the $K_{m(O_2)}$ (Laing *et al.*, 1974). The $K_{i(O_2)}$ was considerably raised in the mutant compared with the wild type enzyme (Table 1). The $K_{m(\text{ribulose bisphosphate})}$ measured at 100% oxygen in the oxygen electrode gave similar results to those obtained with the carboxylase reaction for the wild type and mutant RubisCOs (results not shown).

Specificity coefficient

The specificity coefficient (Jordan & Ogren, 1980, 1983) is the ratio of pseudo-first-order rate constants for the oxygenase and carboxylase reactions, (V_C/K_C, V_O/K_O), and is a measure of the relative rates of each reaction under limiting concentrations of CO_2 and O_2. The specificity coefficients for wild type and mutant enzymes were measured under 100% O_2 in the gas phase at high and low ribulose bisphosphate concentrations and a variety of CO_2 concentrations. A plot of the ratio of the carboxylase to oxygenase activity as a function of CO_2 concentration is shown in Fig. 6. The wild type and mutant enzymes had indistinguishable specificity coefficients (Table 1) under high ribulose bisphosphate but although the specificity coefficient of wild type enzyme was unchanged under low ribulose bisphosphate, that of the mutant enzyme was significantly lower.

DISCUSSION

Molecular biology

Our primer was a 13mer compared to the 20mers of Gutteridge *et al.* (1984) and Estelle *et al.* (1985). The 13mer was completely adequate for mutagenesis and the annealing under stringent conditions conformed closely to prediction (Winter *et al.*, 1983; Zoller & Smith, 1983).

Of the three expression vectors tested (M13, pEMBL and pUC), all three gave positive results for carboxylase, but the level of expression varied widely. The M13 system had the lowest level of activity, presumably reflecting demands on protein synthesis due to phage production. Expression, as expected, was dependent on addition of isopropylthiogalactoside. However, the activity expressed was sufficient and stable enough to screen directly for altered expression without recloning into another expression vector (Estelle *et al.*, 1985). Although the initial cloning of RubisCO DNA into M13 resulted in many plaques with variable activity, we were able to select stable lines easily and they have been used subsequently. The level of activity from the pUC vectors was always lower than that from pEMBL vectors, and we have therefore concentrated on the pEMBL vectors. Of 32 such clones with stable wild-type activity, one was routinely used because it consistently gave much higher levels of activity. We have subsequently discovered that this clone has a deletion on the vector, outside the RubisCO gene. In subsequent experiments investigating amino acid residue 330 we will be able to use the mutant containing a new *Alu*I site that we have generated and test for the creation of new mutants through the loss of that site. (The mutant is cloned into a normal-size pEMBL vector.)

Enzymology

The mutant enzyme differs from the wild type in one amino acid residue, the substitution of a leucine for the methionine at residue number 330 adjacent to a potentially catalytically active lysine (Hartman *et al.*, 1978). While the apparent binding constant for ribulose bisphosphate (represented by K_{ir} and $K_{i(\text{fructose bisphosphate})}$; see Segel, 1975) was slightly changed by the alteration of residue 330, the maximum velocity was reduced 5-fold in the mutant and the Michaelis–Menten parameters increased substantially for both the two alternative substrates CO_2 and O_2 and also for their common substrate ribulose bisphosphate. These data are consistent with the rate constants for substrate binding being less affected by the mutation than subsequent reaction steps. They are also consistent with the steady state concentration at substrate saturation of the reaction intermediate immediately prior to the first irreversible step also being less affected by the mutation. We conclude that the mutation has had the main effect of changing a rate constant(s) for an intermediate step in the reaction, a rate constant which largely determines the magnitude of all three Michaelis–Menten parameters.

CPBP consists of a mixture of the isomers 2-carboxyarabinitol bisphosphate and 2-carboxyribitol bisphosphate (CRBP). The binding of CABP to RubisCO has been interpreted as a two-stage process (Pierce *et al.*, 1980). The first reversible stage is followed by an essentially irreversible binding with an overall binding constant of less than 10^{-11} M. CRBP binds only in a reversible manner. The second, irreversible, binding has been suggested to be due to CABP having identical stereochemistry to the six-carbon intermediate formed by carboxylation of the enediol form of ribulose bisphosphate (Miziorko & Lorimer, 1983). Thus on the basis of the observed reversibility in the binding of CABP without an increase in the binding constant for the competitive inhibitor fructose bisphosphate and only a minor change in the value of K_{ir}, we would hypothesize that the initial first stage of binding of CABP is unaltered, but the irreversible binding of this reaction intermediate analogue is impaired. The currently accepted mechanism (Calvin, 1954; Miziorko & Lorimer, 1983) requires that, following binding, ribulose bisphosphate undergoes a slow reversible change to an enediol form which reacts with CO_2 to form a six-carbon intermediate. Carbon–carbon bond formation is essentially irreversible (G. H. Lorimer, personal communication) and rate-limiting. The six carbon intermediate then undergoes rapid hydrolysis to products. We therefore hypothesize that the effect of the mutation on the enzyme is to decrease the stability of the enediol–enzyme intermediate. Such an interpretation could be tested by the use of substrate isotope trapping techniques (Saver & Knowles, 1982; Sue & Knowles, 1982). Our interpretation would imply that the formation of the enediol intermediate would be more rate-limiting for the mutant than the wild type enzyme, more analogous to the situation in higher plant RubisCO (Roeske & O'Leary, 1984, 1985).

A major finding has been the lack of a marked effect on the partition coefficient. This is consistent with our hypothesis which does not require a direct effect on the rate constants involving O_2 and CO_2 directly. Given the substantial and matching decreases in the first order rate constants (V_C/K_C, V_O/K_O) it is likely that other areas of the active site are involved in CO_2 and O_2 binding.

The changes caused by the methionine to leucine substitution are marked, but any interpretation of the function of Leu-330 is limited by lack of structural information. The inherent difficulty in interpretation is highlighted by the fact that the conservative change of methionine to leucine has produced a mutant enzyme with kinetic parameters more divergent from the higher plant RubisCOs than even the wild type enzyme. The substitution of a leucine side chain for a methionine results in no change in charge or dipole, but small increases in steric hindrance and in hydrophobicity occur as determined by a comparison of side chain structures with relevant substituent constant scales (Williams, 1984)

for steric and hydrophobic interactions. Evaluation of the relative importance of electronic, steric and hydrophobic interactions has been done with esterases and acetyl transferase using a range of substrates (Hansch *et al.*, 1965). Activity correlations for substituted papains have also been made with Hammet constants for substituted papains (Carey *et al.*, 1984). Further modifications at this site might allow us to distinguish between the relative importance of the steric and hydrophobic factors.

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