Amino Acid Substitutions in the Small Subunit of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase That Influence Catalytic Activity of the Holoenzyme†

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ABSTRACT: Four unique amino acid substitutions were introduced by site-directed mutagenesis into the third conserved region of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) from *Anacystis nidulans* (*Synechococcus* sp., PCC6301), resulting in the formation of four mutant enzymes, I87V, R88K, G91V, and F92L. Wild-type and mutant proteins were purified after synthesis in *Escherichia coli*. These single amino acid substitutions do not appear to perturb intersubunit interactions or induce any gross conformational changes; purified mutant proteins are stable, for the most part like the wild-type holoenzyme, and exhibit nearly identical CD spectra. Three of the four mutants, however, are severely deficient in carboxylase activity, with $k_{cat} \leq 35\%$ of the wild-type enzyme. While the substrate specificity factors were the same for the mutant and wild-type enzymes, significant alterations in some kinetic parameters were observed, particularly in the Michaelis constants for CO_2, O_2, and RuBP. All four mutant proteins exhibited lower K_{CO_2} values, ranging from 37 to 88% of the wild-type enzyme. Two of the mutants, in addition, exhibited significantly lower K_{RuBP} values, and one mutant showed a substantial decrease in K_{O_2}. The effects of the single-site mutations in *rbcS* of this study strengthen the hypothesis that small subunits may not contribute directly to substrate specificity; however, individual residues of the small subunit substantially influence catalysis by large subunits.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)[1] is a bifunctional enzyme that is widely distributed among most photosynthetic and chemoautotrophic organisms. The enzyme catalyzes the rate-determining step and controls the flow of carbon of two competing metabolic pathways; it catalyzes both photosynthetic CO_2 fixation via the carboxylation of RuBP in the Calvin reductive pentose phosphate cycle, and it also catalyzes the oxygenation of RuBP in the photorespiratory pathway (Andrews & Lorimer, 1987; Tabita, 1988). Photorespiration, which leads to CO_2 release and lowers the overall rate of photosynthesis, is energy-wasteful. Considerable interest, therefore, lies in engineering a more efficient RubisCO by altering the relative rates of carboxylation and oxygenation, and there are indications that mutant enzymes with altered carboxylase:oxygenase ratios may be selected or constructed in the laboratory (Chen et al., 1988, 1990; Chen & Spreitzer, 1989; Smith et al., 1990).

The RubisCO holoenzyme of plants, algae, and most photosynthetic and chemolithoautotrophic bacteria is a hexadecamer composed of eight large (M_r ~52 000) subunits and eight small (M_r ~12 000) subunits (L_8S_8) (Andrews & Lorimer, 1987; Tabita, 1988). The large subunits have been well characterized. Among higher plants, green algae, and cyanobacteria, the amino acid sequence is highly conserved (≥75% identitiy) (Hudson et al., 1990); however, the large subunits from eucaryotic chromophytic and rhodophytic algae more closely resemble the protein of chemolithoautotrophic hydrogen bacteria and purple non-sulfur photosynthetic bacteria (Andersen & Caton, 1987; Hwang & Tabita, 1991; Valentin & Zetsche, 1989; Gibson et al., 1991). The binding sites for RuBP and activator CO_2, and the divalent metal cofactor are all located on the large subunit. The large subunit is also thought to determine the degree of partitioning between carboxylation and oxygenation (Andrews & Lorimer, 1985), although recent studies indicate that a nuclear-encoded protein may influence the CO_2/O_2 partition coefficient or specificity factor (Chen et al., 1990).

The amino acid sequence of the small subunit is less highly conserved, and relatively little is known about its function. Indeed, RubisCO enzymes from purple non-sulfur photosynthetic bacteria have been isolated that are composed of large subunits only (L_x), and the simplest RubisCO quaternary structure is that of the *Rhodospirillum rubrum* enzyme which is a homodimer of large subunits (Tabita & McFadden, 1974). It has thus been inferred that catalytic functions are restricted to the large subunit. The L_8S_8 RubisCO, however, requires the presence of both the large and small subunits for maximum catalysis (Andrews & Ballment, 1983), and it has recently been demonstrated that while the L_8 core of the *Anacystis nidulans* (*Synechococcus* sp. PCC6301) RubisCO exhibits carboxylase activity (Andrews, 1988; Lee & Tabita, 1990; Gutteridge, 1991; Smrcka et al., 1991), the k_{cat} is less than 1% that of the holoenzyme. Thus, while the small subunits may not be essential for catalysis, it is evident that they exert an important role in maximizing the activity of the enzyme.

To further investigate the role of the small subunit, site-directed mutagenesis studies have been initiated on the cyanobacterial (*Synechococcus* sp. PCC6301) *rbcS* gene. In

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[1] Abbreviations: RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; *rbcS*, small subunit gene; *rbcL*, large subunit gene; IPTG, isopropyl β-D-thiogalactopyranoside; TEM, 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 mM β-mercaptoethanol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TEMMB, 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 50 mM $NaHCO_3$; CD, circular dichroism.

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          5' CGT GAT TGC TAC ATC CGT GTC GCT GGC TTC GAC AAC ATC AAG CAG 3'
             GLY ASP CYS TYR ILE ARG VAL ALA GLY PHE ASP ASN ILE LYS GLN
                         *   *           *   *

IV87      5' GGT GAT TGC TAC GTC CGT GTC GCT G 3'
                         VAL

R88K         5' GAT TGC TAC ATC AAA GTC GCT GGC TTC 3'
                             LYS

R88Q         5' GAT TGC TAC ATC CAA GTC GCT GGC TTC 3'
                             GLN

G91V                   5' TC CGT GTC GCT GTC TTC GAC AAC AT 3'
                                   VAL

F92L                   5'    CGT GTC GCT GGC CTG GAC AAC ATC AAG 3'
                                       LEU
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FIGURE 1: Mutagenic oligonucleotides employed. Residues that have been changed are indicated by asterisks below the wild-type sequence (upper line). The underlined bases indicate nucleotides that have been changed from the wild-type sequence.

earlier mutagenesis studies with two cyanobacterial enzymes, residues of the small subunit were identified that participate in interactions between large and small subunits (Fitchen et al., 1990; Lee et al., 1991). In the present study, we have identified residues of the small subunit that exert large effects on the catalytic capacity of the holoenzyme yet do not affect the gross quaternary structure of the holoenzyme complex.

Experimental Procedures

Bacterial Strains, Phages, and Plasmids. *Escherichia coli* strain MV1190 [Δ(*lac-pro AB*),*thi*,*supE*, Δ(*srl-recA*)*306*::*Tn10*(*tet*^{r})(*F′ tra*D36,*pro*AB,*lac*I^{q}Z M15); strain CJ236 (*dut*-1,*ung*-1,*thi*-1,*rel*A-1;pCJ105(Cm^r)] (Kunkel et al., 1987), helper phage M13K07 (Viera & Messing, 1987), and the plasmids pBGL710 and pBGL520 (Lee & Tabita, 1990) were used in this study. *E. coli* strains MV1190 and CJ236 and the phagemid M13K07 were purchased from Bio-Rad (Richmond, CA). Plasmid pBGL710 contains the 2.2-kb *Pst*I fragment of pCS75 (Tabita & Small, 1985) encoding the *rbcL* and *rbcS* genes of *A. nidulans* (*Synechococcus* sp. PCC6301) in plasmid pTZ18R (Mead et al., 1986). Plasmid pBGL520 (Lee & Tabita, 1990) contains a 1.5-kb *Hin*dIII/*Pst*I fragment from pCS75 which encodes the *rbcL* gene in a pTZ18R vector.

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out according to the previously described method of Kunkel et al. (Kunkel, 1985; Kunkel et al., 1987) using the Muta-gene phagemid kit from Bio-Rad (Richmond, CA). The mutagenic oligonucleotide primers synthesized for this study are shown in Figure 1. Isoleucine at position 87 was replaced by valine; arginine at position 88 was replaced by lysine or glutamine; glycine at position 91 was replaced by valine; and phenylalanine was replaced by leucine at position 92. Single amino acid changes were confirmed by DNA sequencing as previously described (Lee et al., 1991).

Cell Culture and Preparation of Crude Extracts. Plasmid DNA for each mutant was used to transform competent *E. coli* MV1190 cells. Conditions for induction and expression of cloned *rbcL rbcS* genes have been described previously (Lee & Tabita, 1990). Briefly, cultures of MV1190 harboring the mutant RubisCO expression plasmid were grown in 1-L batch cultures using Luria–Bertani (Maniatis et al., 1982) medium containing 100 μg/mL ampicillin. Cells were grown at 37 °C with vigorous shaking. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM during exponential growth to induce the *lac* promoter, driving the expression of the mutant RubisCO. Cultures were allowed to grow for an additional 12–14 h prior to harvesting the cells. Substantial recombinant protein was expressed under these conditions.

Harvested cells were washed in TEM buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 mM β-mercaptoethanol) and resuspended in TEM with 1 mM phenylmethanesulfonyl fluoride at 1 g (wet weight) of cells/mL. Cells were lysed by passage through a French pressure cell at 1010 atm (1 atm = 1.013×10^5 Pa). Cell debris was removed by centrifugation, first at 12000*g* for 10 min and then at 100000*g* for 1 h to yield a crude extract. Total protein concentrations were determined by the standard Bio-Rad (Richmond, CA) dye binding assay using bovine serum albumin as a protein standard. The amount of RubisCO antigen present was established using rocket immunoelectrophoresis as previously described (Jouanneau & Tabita, 1986). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to a method previously described (Laemmli, 1970). Proteins were visualized with Coomassie Blue (0.1%) in methanol/water/acetic acid (5:5:1). The acrylamide concentration in the resolving gel was 5% under nondenaturing conditions and 15% under denaturing conditions.

Purification of Mutant RubisCO Proteins. Rocket immunoelectrophoresis analyses, using homogeneous wild-type recombinant holoenzyme as a standard, indicated that RubisCO comprised 10–15% of the total soluble protein in the crude extracts obtained from *E. coli* MV1190 cells harboring plasmids encoding the mutant enzymes. Wild-type and mutant enzymes were purified according to previously described methods (Jouanneau & Tabita, 1986; Lee & Tabita, 1990). Crude extracts were loaded onto green A–agarose columns equilibrated with TEMMB (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 50 mM $NaHCO_3$). The enzyme was eluted using a 0–1 M NaCl gradient in TEMMB. Fractions exhibiting activity were pooled and precipitated using ammonium sulfate to 70% saturation. The precipitate was resuspended and dialyzed against TEM buffer before being loaded onto a 0.2–0.8 M sucrose step gradient (Tabita & Small, 1985). Fractions of 1.5 mL were collected and assayed for RubisCO activity. The purity of the preparations and the integrity of the mutant enzymes were monitored using both nondenaturing and sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (SDS–PAGE). The amount of carboxylase purified was quantified using A_{280} readings and a published extinction coefficient of 12.6 for a 1% solution (Andrews & Ballment, 1983).

Measurement of CD Spectra. Circular dichroic (CD) spectra of wild-type and mutant proteins were measured on a Jasco 500 spectropolarimeter from 250 to 190 nm. All proteins were dialyzed against 25 mM potassium phosphate, pH 7.5, and immediately before being scanned were diluted to 4.93 μm in the same buffer. A spectrum of the buffer was subtracted from each protein spectrum.

Phenyl-Superose Chromatography. Phenyl-Superose chromatography was employed to analyze the hydrophobic characteristics of the G91V mutant enzyme compared to the wild-type enzyme. Samples, 250 μg, were diluted into a high-salt buffer solution, 1.7 M $(NH_4)_2SO_4$ in 25 mM KH_2PO_4, pH 7.8, and loaded onto a Pharmacia (Piscataway, NJ) phenyl-Superose column equilibrated with the same buffer. A gradient of 1.7–0 M $(NH_4)_2SO_4$ in 25 mM KH_2PO_4 (pH 7.8) was passed through the column to selectively desorb components according to their hydrophobicity. Fractions were collected with a Pharmacia FPLC apparatus at a flow rate of 0.5 mL/min.

Rubisco Assay. Rubisco activity was routinely measured using the RuBP-dependent incorporation of $^{14}CO_2$ into acid-stable 3-phosphoglyceric acid according to previously described methods (Whitman & Tabita, 1976). Specific activity is

expressed in terms of micromoles of CO_2 fixed per minute per milligram of antigen in crude extracts, or micromoles of CO_2 fixed per milligram of purified holoenzyme.

Kinetics. The CO_2/O_2 specificity factor or partition coefficient of each purified enzyme was determined by simultaneous assay of both carboxylase and oxygenase activities according to standard procedures (Spreitzer et al., 1982; Jordan & Ogren, 1981). Each sample was assayed in triplicate under both N_2 and O_2. The reaction buffer, containing 50 mM Bicine (pH 8.0) and 10 mM $MgCl_2$, was used in all specificity assays and for other kinetic measurements; the buffer was prepared using CO_2- and O_2-free distilled water that had been boiled for 10 min and then allowed to cool on ice under nitrogen. The reaction buffer was prepared and then bubbled with N_2 for an additional 30 min, or until the time the reaction was to begin. Serum vials containing 0.47 mL of the reaction buffer were capped with rubber stoppers and flushed with either N_2 or O_2 for 20 min prior to addition of enzyme. RubisCO was separately activated in the presence of excess Mg^{2+} and HCO_3^-; 30 μL of this enzyme solution and 10 μL of 0.1 M $NaH^{14}CO_3$ (3.7 Ci/mol) were then added and incubated for 10 min. The assay was then initiated with the addition of 10 μL of 13 mM [1-^{3}H]RuBP (11 Ci/mol). After 1 h at 25 °C, the reaction was terminated with 0.1 mL of 50 mM $ZnSO_4$ in 0.05 N HCl. At this time, 0.1 mL of 1.5 M sodium cacodylate was added (pH 6.3); 0.1 mL of a 0.2 M 3-phosphoglyceric acid, 12 mM RuBP, and 0.5 mM 2-phosphoglycolate solution was also added at this time to decrease the background in the glycolate-P analysis due to [^{3}H,^{14}C]PGA and [^{3}H]RuBP and to increase the recovery of [^{3}H]glycolate-P (Jordon & Ogren, 1981). Phosphoglycolate phosphatase, 0.3 unit in 40 μL, was then added, and samples were incubated at 30 °C for 30 min before the assay was terminated with 0.9 mL of 1 N formic acid. Carboxylase activity was determined by removing 0.75 mL of the reaction mixture, adding 0.1 mL of 1 N HCl, and drying the sample in an oven at 60 °C. After resuspension in 0.4 mL of 0.5 N HCL, the ^{14}C dpm from [^{14}C]phosphoglyceric acid was determined by counting with a ^{14}C,^{3}H dual-label dpm scintillation program. Oxygenase activity was determined by removing another 0.75 mL of the reaction mixture and loading it onto a Bio-Rad AG 1X8 formate column (6 × 70 mm). After elution with 3 mL of 1 N formic acid, the eluate was lyophilized, and ^{3}H dpm from [2-^{3}H]glycolate was determined using a ^{3}H single-label dpm scintillation program.

For all calculations, an O_2 concentration of 1.23 mM was employed for 100% O_2-flushed reactions (Spreitzer et al., 1982). CO_2 concentrations were calculated from the concentration of HCO_3^- using 6.12 as the pK' of CO_2/HCO_3^- at equilibrium.

For the determination of V_{CO_2}, K_{CO_2}, and K_{O_2}, enzymes were preincubated for 4 h at 4 °C in 10 mM $NaH^{14}CO_3$, 10 mM $MgCl_2$, and 50 mM Bicine, pH 8.0. Assays were conducted under both 100% N_2 (0% O_2) and 100% O_2. The reaction was initiated with the addition of 20 μL of activated enzyme (10 μg) to a 1.0-mL reaction mixture containing 0.4 mM RuBP, 10 mM $MgCl_2$, 50 mM Bicine, pH 8.0, and various concentrations of $NaH^{14}CO_3$. $NaH^{14}CO_3$ concentrations of 0.5, 1, 2, 3, 5, 8, and 15 mM were employed for assays of the mutant enzymes, while concentrations of 4, 6, 8, 10, 12, and 15 mM were employed for the wild-type enzyme. Reactions were terminated after 30 s with 0.5 mL of 3 M formic acid in methanol. After addition of 0.1 mL of 1 N HCl, samples were dried at 60 °C and resuspended in 400 μL of 0.5 N HCl, and the ^{14}C dpm from 3-[^{14}C]phosphoglyceric acid produced was conducted. V_{CO_2} and K_{CO_2} were calculated from the 100% N_2 assays using Scatchard plots. The K_{O_2} was derived from the ratio (R) of carboxylase activities under 100% N_2 versus 100% O_2 using the relationship of kinetic constants whereby $1/(R - 1) = K_{O_2}/[O_2] + K_{O_2}[CO_2]/K_{CO_2}[O_2]$ (Laing et al., 1975). The K_{O_2} was computed from the intercept of this plot. V_{O_2} was derived from the specificity factor, τ, V_{CO_2}, K_{CO_2}, and K_{O_2} using the equation $\tau = V_{CO_2}K_{O_2}/V_{O_2}K_{CO_2}$.

To determine the K_m for RuBP, purified enzymes were activated for 20 min at 25 °C in 0.475 mL of 5 mM $NaH^{14}CO_3$, 10 mM $MgCl_2$, and 50 mM Bicine, pH 8.0, under 100% N_2. RuBP, 25 μL, was added to initiate the reaction, bringing final concentrations to 17.5, 35, 52.5, 70, 87.5, 105, and 122.5 μM. After 1 min, reactions were terminated with the addition of 100 μL of 50 mM $ZnSO_4$ in 0.05 N HCl. Following the addition of 0.5 mL of 1 M formic acid and 0.1 mL of 1 N HCl, samples were dried, resuspended, and counted as before. Estimates of the K_m for RuBP were made from Scatchard plots.

Results

Carboxylase Activity in Crude Extracts of E. coli. In this study, four expression plasmids were constructed, each of which encodes an intact RubisCO large subunit gene together with a mutant small subunit gene harboring a single amino acid substitution. The RubisCO genes were expressed in *E. coli* under the control of the *lac* promoter. Crude extracts were prepared and assayed for their ability to catalyze RuBP-dependent fixation of $^{14}CO_2$ into acid-stable 3-phosphoglycerate product. Each of the mutant proteins exhibited differential levels of carboxylase activity, ranging from 9 to 53% of the activity of the wild-type enzyme.

Purification of the Mutant Proteins. To permit a closer examination of the properties of the mutant proteins, we attempted to purify each of the proteins using procedures previously established for wild-type recombinant RubisCO (Tabita & Small, 1985; Lee & Tabita, 1990). Unlike previously studied mutations in the small subunit, which influenced interactions with large subunits (Lee et al., 1991), the mutations investigated here resulted in the synthesis of RubisCO molecules which maintained their structure. This was initially suggested by the elution of enzyme activity from dye affinity columns and sucrose density gradients at the approximate position of the wild-type protein. The only exception was mutant R88Q, in which alteration of the charge at this position resulted in gross structural alterations such that normal large subunit–small subunit interactions were abolished, as evidenced by the recovery of separated large and small subunits from the gradient and the lack of holoenzyme activity in individual fractions (data not shown). This response is similar to the previously studied S16D, L21E, and Y54S mutant enzymes (Lee et al., 1991). The I87V, R88K, F92L, and G91V preparations were further compared using nondenaturing and SDS gel electrophoresis (Figure 2A). The identical electrophoretic mobilities of the wild-type and mutant carboxylases during nondenaturing gel electrophoresis of crude and purified preparations strongly suggested that the mutant proteins, like the wild-type enzyme, possessed the L_8S_8 hexadecameric structure. This was supported by SDS gel electrophoresis in which the wild-type and mutant RubisCO enzymes were shown to be indistinguishable (Figure 2B). It is apparent that each mutant protein exhibits the two characteristic bands, corresponding to the large (M_r = 53 000) and small subunit (M_r = 13 000) of the *Synechococcus* sp. PCC6301 RubisCO. Thus, these four single amino acid substitutions in the third conserved region of the small subunit do not prevent association

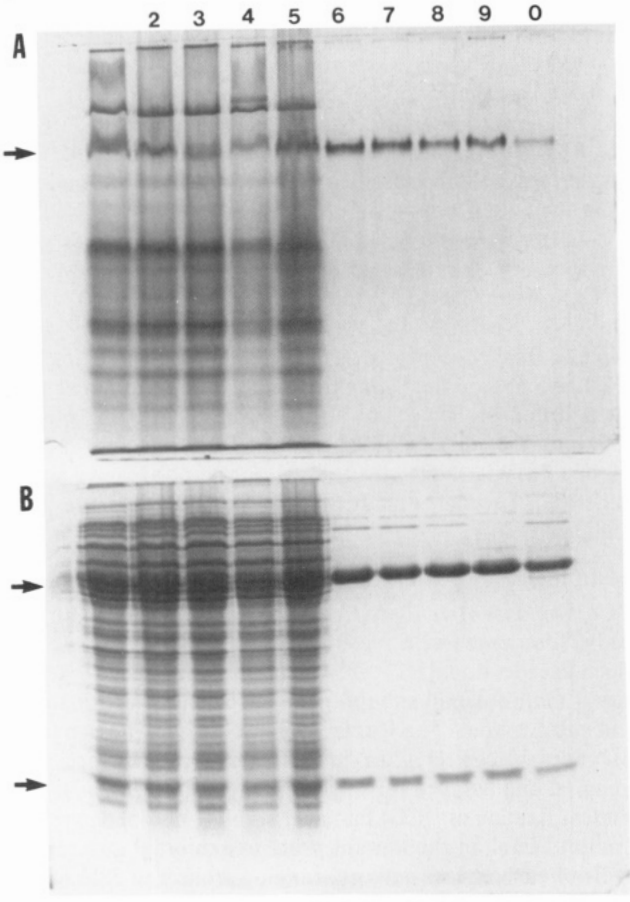

FIGURE 2: Polyacrylamide slab gel electrophoresis of wild-type and mutant RubisCO enzymes in the absence (A) and presence (B) of sodium dodecyl sulfate (0.1%). In both (A) and (B), *E. coli* crude extracts of wild-type and mutant proteins are shown in the lanes on the left, while the corresponding purified proteins are present in the lanes to the right. Lanes 1 and 6, wild-type RubisCO; lanes 2 and 7, R88K; lanes 3 and 8, F92L; lanes 4 and 9, I87V; lanes 5 and 10, G91V. The arrow in panel A points to the position of the L_8S_8 RubisCO. In panel B, the arrows point to the positions of the large and small subunits.

between large and small subunits.

The structural integrity of the isolated mutant proteins was further examined using circular dichroism (CD) (Figure 3). CD provides a measure of the relative amounts of α-helix, β-sheet, and random-coil structures present in a protein (Johnson, 1990). Gross structural changes resulting from a mutation in the wild-type sequence should produce alterations in the CD spectra of the mutants as compared to the wild type. The CD spectra from 190 to 250 nm of mutant G91V, and two of the other mutant proteins, R88K and I87V, differed very little from that of the wild-type enzyme, attesting to the conservation of the native quaternary structure. This was not the case, however, with the F92L mutant enzyme whose spectra differed from that of the wild-type protein (Figure 3). Differences in protein concentration cannot account for the variability in the CD spectra since measurements were made using identical protein concentrations.

It was noted that the G91V enzyme, unlike the wild type or any of the other three mutant proteins, precipitated in 0.1 M KH_2PO_4 buffer, pH 7.5. To further investigate this apparent difference in hydrophobicity, the mutant and wild-type enzymes were applied in a high-salt solution to a phenyl-Superose column (Figure 4). The wild-type enzyme adsorbed to the column and was eluted when the $(NH_4)_2SO_4$ concentration was reduced to 0% (Figure 4, top panel). The G91V mutant enzyme did not adsorb to the column (Figure 4, middle panel) when loaded in the same high ionic strength solution. A mixture of the wild-type and G91V proteins eluted at the expected positions from the column (Figure 4, bottom panel).

Table I: Specificity Factor and V_{max} Values of Purified Mutant RubisCO Enzymes[a]

strain	specificity factor	V_{CO_2}[b] (μmol min^{-1} mg^{-1})	V_{O_2}[c] (μmol min^{-1} mg^{-1})	V_{CO_2}/V_{O_2}
WT	40.8 ± 4.1	2.31 ± 0.04	0.18	12.8
I87V	39.5 ± 0.5	1.23 ± 0.13	0.12	10.3
R88K	38.5 ± 5.5	0.70 ± 0.09	0.12	5.8
G91V	41.0 ± 4.5	0.38 ± 0.00	0.03	12.7
F92L	44.6 ± 4.1	0.21 ± 0.06	0.03	7.0

[a] Enzymes were purified and specificity factors and component kinetic parameters were determined as described under Experimental Procedures. [b] V_{CO_2} values and standard errors were estimated by Scatchard plots. Lines were fitted by least-squares analysis. For each parameter, values represent the mean of at least three separate enzyme assays with sample ($n - 1$) standard deviations. All experiments included the wild-type enzyme as a control. [c] V_{O_2} values were derived according to the relationship $\tau = V_{CO_2}K_{O_2}/V_{O_2}K_{CO_2}$ where K_{O_2} and K_{CO_2} represent mean K_m values.

Table II: K_m Values of Purified Mutant RuBP Carboxylase/Oxygenase Enzymes

strain	K_{RuBP} (μM)	K_{CO_2} (μM)	K_{O_2} (μM)	K_{O_2}/K_{CO_2}
WT	21.6 ± 3.8	175 ± 21	565 ± 121	3.2
I87V	31.0 ± 4.9	123 ± 42	474 ± 23	3.9
R88K	51.2 ± 3.2	154 ± 2	1078 ± 97	7.0
G91V	9.0 ± 5.3	84 ± 4	294 ± 29	3.5
F92L	8.5 ± 3.2	66 ± 16	468 ± 36	7.1

Kinetic Properties of Mutant RubisCO Enzymes. The availability of purified wild-type and mutant enzymes enabled detailed comparisons to be made of the substrate specificity factor, a measure of the enzyme's ability to discriminate between CO_2 and O_2 (Jordan & Ogren, 1981), and other relevant kinetic constants (Tables I and II). There was little difference detected in the substrate specificity factor of the mutant enzymes compared to wild-type RubisCO (Table I). Wild-type RubisCO exhibited a specificity factor of 40.8, which closely approximated values previously reported for another cyanobacterial RubisCO (Jordan & Ogren 1981), but was somewhat less than the value reported by Gutteridge (1991) using a different method. The specificity factors of the mutant enzymes did not differ in a statistically significant manner from the wild-type enzyme. Although specificity factors for all the enzymes were similar, the maximal rates of carboxylation (V_{CO_2}) and oxygenation (V_{O_2}) for the mutant enzymes were, however, significantly different. The wild-type enzyme yielded a V_{CO_2} of 2.31 units/mg, a value which is also consistent with values previously obtained (Voordouw et al., 1987). All four of the mutant proteins showed a reduction in carboxylase activity. The I87V mutant enzyme showed a modest reduction, yielding a maximal rate of carboxylation of 1.23 units/mg, while the R88K, F92L, and G91V enzymes exhibited more dramatic decreases in activity, yielding V_{CO_2} values of 0.70, 0.21, and 0.38 units/mg, respectively. It is particularly apparent in the case of the F92L and G91V mutants that single amino acid substitutions in the small subunit drastically affect catalysis by the large subunits of the L_8S_8 holoenzyme complex.

All four of the mutant proteins also showed a reduction in oxygenase activity. The V_{O_2} of the I87V and the R88K mutants was 67% of the wild-type enzyme while the F92L and G91V enzymes exhibited V_{O_2} values that were approximately 17% that of the wild-type enzyme.

Significant differences were obtained for the Michaelis constants of the gaseous substrates and RuBP. The most

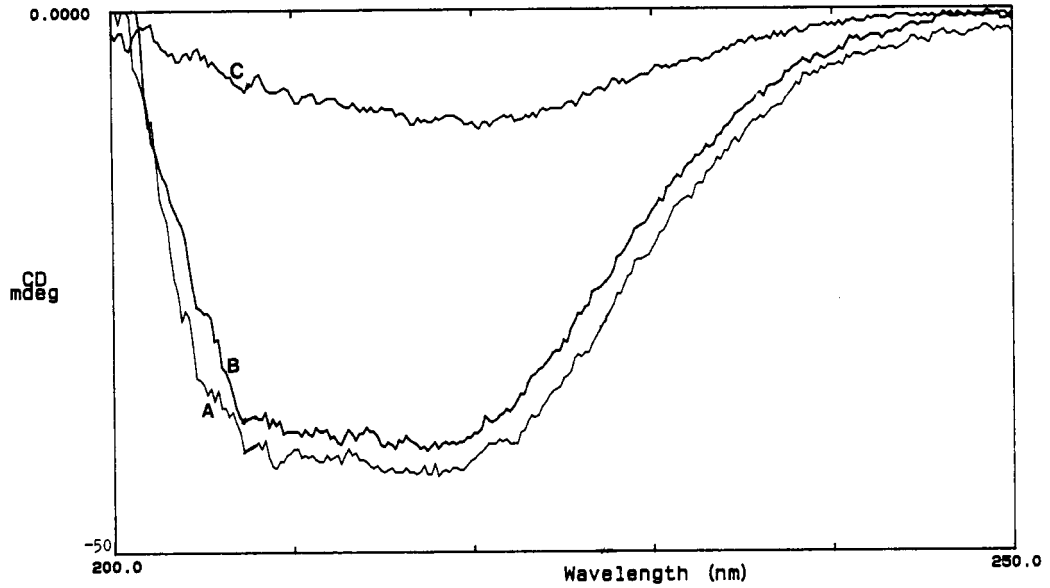

FIGURE 3: CD spectra of wild-type and mutant RubisCO proteins. Proteins were diluted in 25 mM potassium phosphate buffer, pH 7.5, to a molar concentration of 49.3 μM and scanned from 250 to 200 nm. (A) Wild-type RubisCO; (B) mutant G91V displaced (+1 mdeg); (C) mutant F92L.

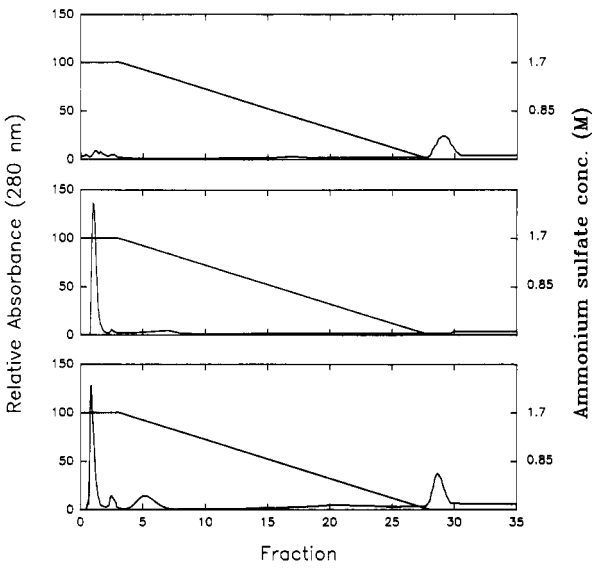

FIGURE 4: Hydrophobic column chromatography of purified holoenzymes. (Top) 250 μg of purified wild-type RubisCO diluted in a high-salt solution of 1.7 M $(NH_4)_2SO_2$ in 25 mM potassium phosphate, pH 7.8, was loaded onto a Pharmacia phenyl-Superose column and eluted with a linear gradient of 1.7–0 M $(NH_4)_2SO_4$; (middle) 250 μg of the purified G91V mutant enzyme was chromatographed under the same conditions; (bottom) a mixture of the wild-type RubisCO and G91V mutant enzyme chromatographed together.

notable differences were in the K_{CO_2} values. All four of the mutant enzymes showed a greater affinity for CO_2 than the wild-type RubisCO (Table II). The K_{CO_2} for the wild-type enzyme (175 μM) is consistent with previously reported measurements (Voordouw et al., 1987) and reflects the relatively poor affinity of cyanobacterial RubisCO for CO_2 compared to the plant enzyme (Badger, 1980; Andrews & Abel, 1981; Jordan & Ogren, 1981). The mutant enzymes possessed K_{CO_2} values that were 37–88% of the wild-type enzyme, with the F92L enzyme showing the highest affinity for CO_2 (K_{CO_2} of 66 μM). Less pronounced were differences in the Michaelis constants for the other gaseous substrate, O_2. The K_{O_2} for the wild-type *Synechococcus* enzyme was found to be 565 μM, a value slightly lower than obtained for the *Aphanizomenon flos-aquae* cyanobacterial enzyme (Jordan & Ogren, 1981) and for the enzyme from the two other cyanobacteria (Badger, 1980; Andrews, 1981). The K_{O_2} values obtained for the I87V and F92L mutant enzymes were comparable to that of the native enzyme (Table II). The R88K mutant enzyme, on the other hand, exhibited a lower affinity for O_2, having a K_{O_2} of 1078 μM, while the G91V mutant RubisCO exhibited a higher affinity for O_2 (K_{O_2} of 294 μM).

Differences were also observed in the K_{RuBP}. A K_{RuBP} of 21.6 μM was observed for the wild-type enzyme, equivalent to the K_{RuBP} reported by Badger (1980) for the enzyme from the cyanobacterium *Anabaena variablis*. While the I87V and the R88K enzymes, in particular, showed higher K_{RuBP} values, the K_{RuBP} for the G91V and F92L enzymes was significantly lower (Table II).

Discussion

To evaluate the role of the small subunit of RubisCO, four unique amino acid substitutions were made in the third highly conserved region of the small subunit of the *Anacystis nidulans* (*Synechococcus* sp. PCC6301) RubisCO. The residues we chose to substitute, Ile-87, Arg-88, Gly-91, and Phe-92, are invariant or conservatively substituted in all known sequences of the RubisCO small subunits, implying some degree of functional importance (Hwang & Tabita, 1991). Information extrapolated from X-ray crystallography studies of the spinach enzyme (Knight et al., 1990) suggests that Ile-87 and Gly-91 are within a β-sheet buried in the interior of the small subunit and have a solvent-accessible surface area of less than 5%. Arg-88 and Phe-92 are also within a β-sheet; these strictly conserved residues participate in interactions at the interface between the small and large subunits.

The mutant enzymes exhibit properties consistent with and similar to the wild-type hexadecameric protein and show similar abilities to bind to dye–ligand columns, perhaps reflecting a common hydrophobic fold. The CD spectra of the purified mutant proteins, with the exception of the F92L mutant protein, are identical to that of the native RubisCO. These observations indicate that substitutions we have made in Ile-87, Arg-88, and Gly-91 of the small subunit, yielding mutant proteins I87V, R88K, and G91V, respectively, may not be required to maintain the gross three-dimensional structure of the holoenzyme. However, a more drastic change

in position 88, resulting in the alteration of arginine to glutamine, yielded a protein that was drastically deficient in its capacity to maintain its structure through even gentle enzyme purification protocols.

The altered CD profile of the F92L mutant enzyme suggests that the loss of the aromatic phenylalanine at this position eliminates or interferes with specific interactions that occur in the native quaternary structure. Because of its potential for forming hydrophobic bonds and its ability to pack tightly against other flat molecules, phenylalanine is a suitable residue for participating in intersubunit interactions. A leucine substitution at this position may be less suitable for this function and may lead to imperfect alignment of the large subunit dimer (the basic building unit of the holoenzyme) (Knight et al., 1990). The carboxylase and oxygenase activities of the F92L protein are also severely impaired by the leucine substitution. When purified the carboxylase activity of the F92L mutant is only 10% and the oxygenase activity is about 17% that of the wild-type enzyme. The reduced activities may be explained by the differential affinity the mutant has for substrate molecules. The F92L mutant enzyme's K_{CO_2} and K_{RuBP} are both nearly 3-fold lower than the native enzyme. Preliminary studies with the competitive inhibitor 6-phosphogluconate support these findings, the K_i for the activated mutant enzyme being significantly lower than that of the wild-type RubisCO. The lower Michaelis constants for RuBP and the gaseous substrate CO_2 may reflect difficulties in the capacity of the enzyme to release product, which in turn might lead to a decrease in maximal reaction rates for carboxylation. Obviously, further studies are required to explain the relationship between the Michaelis values and the reduced activity of this mutant enzyme.

Extrapolating from X-ray structural studies of the highly homologous spinach RubisCO indicates that Arg-88 is also an intersubunit contact residue (Knight et al., 1990). The carboxylase activity of the R88K mutant protein in both the crude and purified states was between 34 and 38% that of the wild-type enzyme while the oxygenase activity of the purified enzyme was about 70% of the wild-type enzyme. This reduction in carboxylase activity may be a reflection of the enzyme's decreased affinity for RuBP. The R88K mutant possesses an affinity for CO_2 that appears to be slightly greater than the native RubisCO. There is, however, more than a 2-fold increase in K_{RuBP} and a 3-fold increase in K_{O_2}. Intersubunit contacts demand a precise fit of hydrophobic side chains, charge pairing, and side chain as well as backbone hydrogen bonds. Arginine's guanidino group, a group which is sometimes important for the binding of phosphate ligands, may be important in terms of subunit packing. The presence of arginine's guanidino group coupled with the fact that arginine is not a hydrophobic residue, whereas lysine is, may be suffient to cause very subtle changes in the conformation of the holoenzyme when lysine replaces arginine. These subtle changes in turn may manifest themselves in the Michaelis constants for RuBP and O_2, thereby altering the carboxylase and oxygenase activities.

Alteration of arginine to glutamine at position 88 causes a drastic change in the capacity of the protein to maintain a stable quaternary structure, as seen by the instability of the protein on sucrose density gradients. These results are entirely consistent with Arg-88 playing an important role in intersubunit contacts.

The accommodation of two extra methylene groups when valine replaces glycine in the core of the small subunit impinges upon both the solubility and the catalytic activity of the enzyme. Unlike the wild-type enzyme, the G91V mutant enzyme precipitates in a 0.1 M phosphate buffer and fails to adsorb to a phenyl-Superose column. These observations suggest that the amino acid substitution has caused major changes in the hydrophobic characteristics of the holoenzyme. Glycine may facilitate the precise packing of adjacent groups or impart some degree of flexibility at this position that valine with its two bulky methylene groups cannot provide. The ineptness of valine at this position is highlighted by the dramatic effects of the amino acid substitution on the enzyme's catalytic potential. The carboxylase activity of the purified G91V enzyme was only 16% and the oxygenase activity only 17% that of the wild-type enzyme. Significant differences were also noted in the Michaelis constants of the mutant protein compared to the wild type. The K_{RuBP} and the K_{CO_2} of the G91V mutant are both less than half of the wild-type enzyme. The K_{O_2} of the G91V enzyme is also nearly 50% lower than the wild-type enzyme. Again, no alterations were evidenced in the substrate specificity factor as changes in the K_{O_2}/K_{CO_2} ratio are balanced by changes in the V_{CO_2}/V_{O_2} ratio.

The effects of substituting a valine for isoleucine at position 87 were less pronounced than some of the other mutations. The carboxylase activity of the purified I87V protein was 53% and the oxygenase activity 70% that of the wild-type. Both the K_{CO_2} and K_{O_2} values of the I87V enzyme appear to be slightly lower than that of the wild-type enzyme, while the K_{RuBP} appears to be slightly greater. That such a seemingly conservative amino acid substitution in the core of the small subunit can influence the kinetic properties of the enzyme emphasizes the importance of the small subunit in regulating the catalytic potential of the holoenzyme.

The function of the small subunit of RubisCO has been in question for a number of years. It is well established that the active site as well as substrate binding sites of the enzyme are located on the large subunit. Recent studies by Schneider et al. (1990a,b) suggest that the small subunit can modulate substrate binding by inducing conformational changes at the active site. The present work further supports this idea. The characteristics of the four mutant proteins demonstrate that residues in the third highly conserved region of the small subunit have an important role in determining the catalytic properties of the *Synechococcus* RubisCO. Though the amino acid substitutions do not alter the partitioning between carboxylation and oxygenation, they have a substantial effect on the maximal reaction rates and the Michaelis constants for the substrates. The most pronounced effects exhibited by each of the four mutants were a reduction in both the carboxylase and oxygenase activities and a variable decrease in K_{CO_2}. Variations in the Michaelis constants for RuBP and O_2 were also observed for the mutant enzymes.

Voordouw et al. (1987) have also performed site-directed mutagenesis to examine the contribution of small subunits to RubisCO activity. In these studies, tryptophan residues at positions 55 and 58 of the small subunit of the *A. nidulans* RubisCO were replaced with phenylalanine. While the specificity factor and the K_{CO_2} for the W55F and W58F mutant proteins remained unchanged, the V_{CO_2} for the two mutant enzymes was significantly lower than the wild type. Unique to the present investigation is the finding that mutations in the small subunit have the capacity to alter the Michaelis constants and catalytic activity of the hexadecameric holoenzyme. Of course, one would like to know whether subtle conformational changes in the small subunit could alter the active-site geometry to facilitate substrate binding in such a manner that the stability of one of the transition states in the

overall reaction pathway might be altered to decrease or increase catalytic efficiency. Indeed, it will be crucial to determine if the conformational change imparted by the small subunit occurs prior, or subsequent, to the binding of the substrate.

Results from the present investigation support the hypothesis that the small subunit may not contribute directly to the specificity of the gaseous substrates (Andrews & Lorimer, 1985); however, it should be noted that only a limited number of mutants or hybrid large subunit/small subunit molecules have been studied at this time. This study also supports the idea that small subunits are functionally involved in governing carboxylase and oxygenase activities and play a role in modulating key kinetic constants. The recent development of a photosynthetic bacterial indicator strain in which the *Synechococcus* RubisCO genes may be expressed in a RubisCO deletion mutant of *Rhodobacter sphaeroides*, resulting in restoration of CO_2-dependent growth (Falcone & Tabita, 1991), suggests that second-site revertants of the mutant proteins studied in this investigation may be isolated which retain the high affinity for CO_2 but regain the activity of the wild-type enzyme.

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