Serine-376 Contributes to the Binding of Substrate by Ribulose-bisphosphate Carboxylase/Oxygenase from *Anacystis nidulans*†

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ABSTRACT: Site-directed mutagenesis was used to change Ser376 in the active site of ribulose-1,5-bisphosphate carboxylase/oxygenase from the cyanobacterium *Anacystis nidulans* to Cys, Thr, or Ala. When expressed in *Escherichia coli* and purified, the mutant enzymes exhibited carboxylase activities that were reduced by 99% or more with respect to the activity of the wild-type enzyme. The K_m values for ribulose bisphosphate at pH 8.0, 30 °C, were elevated from 46 μM for wild-type enzyme to 287, 978, and 81 μM for mutants in which Cys, Thr, or Ala, respectively, replaced Ser376. The Cys and Thr variants were almost devoid of oxygenase activity whereas the Ala variant had 16% as much oxygenase as wild-type enzyme, suggesting that this mutation had greatly elevated the oxygenase:carboxylase ratio.

The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)[1] plays a critical role in autotrophic carbon reduction by trapping an estimated 10^{11} tons of atmospheric CO_2 globally each year in the commitment step of the Calvin–Benson cycle (Miziorko & Lorimer, 1983). This figure becomes even more striking when one considers that the alternate catalytic activity in this bifunctional enzyme, namely, its oxygenase activity, inexplicably opposes its own carboxylase activity and is responsible for the net loss of CO_2 through the process of photorespiration (Andrews & Lorimer, 1978). The extremely significant agronomic consequences of the carboxylation and oxygenation reactions catalyzed by RuBisCO have directed much attention to the study of this enzyme, especially as a target for improvement by genetic manipulation.

RuBisCO has been studied in diverse organisms including higher plants and photosynthetic bacteria, and generally exists as a hexadecamer of eight ca. 55-kDa large (L) subunits and eight ca. 14-kDa small (S) subunits (McFadden, 1980). One exception is RuBisCO from the bacterium *Rhodospirillum rubrum* which exists simply as the L_2 dimer (Tabita & McFadden, 1974).

Recent crystallographic data (Chapman et al., 1988; Andersson et al., 1989, Schneider et al., 1990; Knight et al., 1990) have greatly enhanced the understanding of the RuBisCO active-site domains, two of which are shared between two adjoining L subunits. Such X-ray crystallographic studies have aided the rational design of mutagenesis studies and have indeed confirmed much previously obtained affinity labeling and directed mutagenesis data.

Recent results from our laboratory have suggested an active-site serine residue in RuBisCO from spinach by vanadate photomodification. In the photoinactivation, which was considerably reduced in the presence of RuBP or a competitive inhibitor, the side chain of one or more Ser residues was oxidized to the aldehyde. The latter could be reduced back to Ser with concomitant reactivation of the enzyme (Mogel & McFadden, 1989). Representations obtained by crystallography are consistent with this finding and place Ser379 in the active site of the spinach enzyme and the corresponding Ser (368) in the same domain of the *R. rubrum* enzyme.

In the spinach crystal structure, Ser379 is positioned with its serine hydroxyl in proximity to the 3-OH (or 4-OH) and one 5-phosphate O of the bisubstrate analogue CABP (Andersson et al., 1989; Knight et al., 1990); in the representation of the *R. rubrum* enzyme with bound RuBP, Ser368 is in proximity to oxygens on both 1- and 5-phosphates of this substrate (Lundquist & Schneider, 1991). Such observations suggest that this serine is involved in substrate binding. Nevertheless, its proximity to C-3 of CABP in the spinach structure has lead to speculation that Ser379 may be the essential base that promotes the enolization of RuBP by deprotonation of C-3 (Andersson et al., 1989; Knaff, 1988) in spite of the very high pK_a of the Ser OH.

Certainly the importance of this serine residue is supported by sequence alignments which show it to be invariant in RuBisCO from diverse organisms, including that of the cyanobacterium *Anacystis nidulans* (Shinozaki et al., 1983; Hudson et al., 1990). In the present study, we have utilized site-directed mutagenesis to replace Ser376 with Cys, Thr, and Ala in the L subunit of RuBisCO from *A. nidulans* in order to evaluate its contribution to the activity of the enzyme.

Experimental Procedures

Materials. RuBP sodium salt, Tris-HCl, MOPS, β-mercaptoethanol, and α-tolylsulfonyl fluoride were purchased from Sigma Chemical Co. DABCO was obtained from Aldrich. CABP was a gift from S. N. Mogel (Mogel & McFadden, 1990).

The plasmid pCS75 (Tabita & Small, 1985), a pUC9 derivative, served as the source of the *A. nidulans* RbcL/RbcS gene tandem. Phage M13mp18 (Messing, 1983) was used for mutagenesis experiments. *Escherichia coli* JM105, *the rpsL, endA, shcB15, hspR4,* Δ(*lac-proAB*) [F′ *traD36, proAB, lacI*^{q} *Z*ΔM15] (Yanisch-Perron et al., 1985), was used as a host for M13 vectors and for expression of the RbcL and RbcS genes. The plasmid pCS88, a pUC19 derivative (McFadden & Small, 1988), expresses the wild-type RbcL/RbcS gene tandem from *A. nidulans*.

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[1] Abbreviations: CABP, 2-carboxyarabinitol 1,5-bisphosphate; DABCO, diazabicyclooctane; L, large subunit of RuBisCO; MOPS, 3-(*N*-morpholino)propanesulfonic acid; RuBisCO, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose 1,5-bisphosphate; S, small subunit of RuBisCO; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Synthesis of Oligonucleotides. Oligonucleotides were synthesized on a Pharmacia gene assembler by the phosphoramidite method (Sinha et al., 1984) and purified by polyacrylamide gel electrophoresis. Mutagenic deoxyoligonucleotides 24 bases in length were constructed in order to facilitate the Ser376 → Thr and Ser376 → Ala substitutions while a deoxyoligonucleotide 25 bases in length was constructed to accomplish the Ser376 → Cys substitution. All three cases were predicted to result in a single base mismatch.

Oligonucleotide-Directed Mutagenesis and Plasmid Reconstruction. Details of this procedure have been previously reported (Haining & McFadden, 1990). Briefly, the 619 bp *Eco*RI/*Sph*I restriction fragment from pCS75 which encodes a carboxyl-terminal portion of the RuBisCO L subunit was subcloned into M13mp18. Site-directed mutagenesis was performed according to the Olson and Burke cotransformation method (Zoller & Smith, 1983), after which putative mutants were identified by hybridization with ^{32}P-labeled mutagenic oligonucleotides (Zoller & Smith, 1984). The desired mutations were confirmed by dideoxy sequencing (Sanger et al., 1977) using the Sequenase system (U.S. Biochemical).

To restore the entire RbcL gene, each mutant *Eco*RI/*Sph*I fragment was excised from the M13 replicative form and then ligated into the similarly cut pCS75ΔSS, a PCS75 derivative which lacks the *Eco*RI fragment encoding the entire RbcS gene. To reconstruct the RbcL/RbcS gene tandem, the RbcS gene within the *Eco*RI fragment was subsequently ligated into the *Eco*RI site of the resulting pCS75ΔSS analogue to produce the mutant pCS75 analogue. Finally, the *Pst*I fragment encompassing the entire RbcL/RbcS gene tandem was excised and ligated into the multiple cloning site of pUC19 to generate pCS88 (or analogues). The resultant mutant pCS88 plasmids—pGL S376C, pGL S376T, and pGL S376A (Ser → Cys, Ser → Thr, and Ser → Ala at position 376 of the RuBisCO L subunit, respectively)—were sequenced through a 390-base region flanking the altered codon to confirm the desired mutations.

Plasmid Expression. Starter cell cultures of *E. coli* JM105 harboring pCS88 or the mutant plasmids were used to inoculate TYE media (1% tryptone, 0.5% yeast extract, and 1% NaCl) containing 200 μg/mL penicillin G. After 6 h of growth at 37 °C with shaking, 1 mM isopropyl thiogalactoside was added, and growth was allowed to continue for an additional 9 h.

Purification of RuBisCO. Following growth, RuBisCO was purified from cells by sucrose density gradient centrifugation essentially as described by Haining and McFadden (1990). Cells expressing mutant and wild-type carboxylases were harvested by centrifugation, washed with TEMMB buffer (50 mM Tris-HCl, 0.1 mM EDTA, 50 mM $NaHCO_3$, 10 mM $MgCl_2$, and 1 mM β-mercaptoethanol, pH 7.5 at 25 °C), and repelleted by centrifugation. After resuspension in TEMMB buffer supplemented with 1 mM α-tolylsulfonyl fluoride, cells were broken by sonic disruption. A cell-free extract was then prepared by centrifuging the broken cells for 30 min at 17500*g*. Following the precipitation of proteins within the cell-free extract with 40% $(NH_4)_2SO_4$ and subsequent centrifugation for 10 min at 10000*g*, the resulting pellets were resuspended in TEMMB buffer and dialyzed against the same buffer. Dialysates were loaded onto 0.2–0.8 M linear sucrose gradients made with TEMMB buffer and then subjected to 2.5 h of centrifugation in a Beckman VTi50 rotor at 50000 rpm. Fractions containing the highest carboxylase activities were retained for subsequent studies and are referred to as "first gradient purified" preparations. For convenience, most manipulations involved enzyme preparations at this level of purification.

Further purification was accomplished by dialyzing pooled fractions of first gradient purified enzyme preparations against TEMMB buffer and then concentrating the dialysates. These samples were subjected to 2.5 h of centrifugation in a Beckman VTi50 rotor at 50000 rpm through stepped sucrose gradients constructed with 0.2, 0.4, 0.6, and 0.8 M sucrose in TEMMB buffer. Resulting fractions enriched in carboxylase activity are referred to as "second gradient purified" preparations.

Carboxylase Assay. Carboxylase activity was measured after 10-min activation essentially by the method of McFadden et al. (1975) which is based upon the incorporation of $^{14}CO_2$ supplied as $H^{14}CO_3^-$, into the acid-stable product 3-phospho-D-glycerate. Reaction times were 2 min unless otherwise noted. Protein concentrations were determined (Bradford, 1976) using the Bio-Rad dye reagent with bovine serum albumin as a standard. For studies involving CABP, 100 μM CABP was added 2 min prior to the initiation of the reaction by RuBP.

Chemiluminescent Oxygenase Assay. Oxygenase activity was determined using a modification of the highly sensitive procedure developed by Mogel and McFadden (1990). This assay measures oxygenase-dependent chemiluminescence of Mn^{2+}-activated RuBisCO. First gradient purified enzyme preparations were first dialyzed into MMBB buffer (50 mM MOPS, 1 mM $MnCl_2$, 1 mM $NaHCO_3$, and 1 mM β-mercaptoethanol, pH 7.5); 0.2 mg protein was then added to MMBB buffer to yield a final volume of 1.5 mL. After addition of DABCO to a final concentration of 0.5 mM, the enzyme solution was equilibrated in a water bath to 25 °C for 10 min and then transferred to a 3-mL fluorescence cuvette. Next, in a Beckman LS-9000 liquid scintillation counter, background counts (always found to be linear with respect to time) were recorded manually every 5 s for a duration of 2 min. Next, oxygenase reactions were initiated by the addition of RuBP (0.1 mL) to 1 mM, counts were again recorded as described above, and background count rates were then subtracted to obtain initial rates. For studies involving CABP, 50 μM CABP was added prior to the 10-min equilibration at 25 °C.

Polyacrylamide Gel Electrophoresis and Western Blotting. Polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (1970) in the presence or absence of 0.1% sodium dodecyl sulfate. Ultrapure grade acrylamide and molecular weight standards were obtained from Bio-Rad Laboratories. Western blotting was performed (Torres-Ruiz & McFadden, 1988) using alkaline phosphate conjugated secondary antibodies for visualization of immunoreactive bands.

Results

Structure of Mutant and Wild-Type Proteins. Analysis of first gradient purified mutant and wild-type RuBisCOs by nondenaturing polyacrylamide gel electrophoresis and Western blotting reveals that the electrophoretic mobilities of mutant proteins are nearly identical to that of the wild-type protein (Figure 1A). Analysis of the same preparations by denaturing polyacrylamide gel electrophoresis and subsequent Western blotting (Figure 1B) indicates the presence of assembled enzyme containing both large and small subunits. Taken together, these results suggest that the mutant proteins exist as fully assembled oligomers.

Carboxylase Activities of Mutant and Wild-Type Proteins. All gradient-purified mutant enzyme preparations displayed significantly decreased but detectable RuBP-dependent CO_2

Table I: Carboxylase Activities of Mutant and Wild-Type Proteins[a]

plasmid expressed	Preparation A, first sucrose gradient: sp act.[b]	% wild-type	act. (+CABP)	second sucrose gradient: sp act.[b]	% wild-type
pCS88	1420 ± 11	100	0.3	2200	100
pGL S376C	10 ± 2	0.7	0	27	1
pGL S376T	3 ± 0.1	0.2	0	5	0.2
pGL S376A	3 ± 0.1	0.2	0	5	0.2

plasmid expressed	Preparation B, second sucrose gradient: sp act.[b]	% wild-type	act. (+CABP)	K_m(RuBP) (μM)	sp act./K_m(RuBP)
pCS88	3388 ± 132	100	2	46 ± 11	74
pGL S376C	25 ± 2	0.8	0	287 ± 87	0.087
pGL S376T	12 ± 1	0.3	0	978 ± 92	0.012
pGL S376A	11 ± 1	0.3	0	81 ± 6	0.14

[a] Proteins were expressed from the plasmids pCS88 (wild-type), pGL S376C, pGL S376T, and pGL S376A (Cys, Thr, and Ala mutants, respectively), purified by one or two sucrose gradients as indicated, and then assayed for carboxylase activity. Where indicated, assays were conducted in the presence of 100 μM CABP. Specific activities are expressed as nanomoles of CO_2 fixed per minute per milligram of protein at pH 8.0, 30 °C. [b] Assayed at 13 mM RuBP.

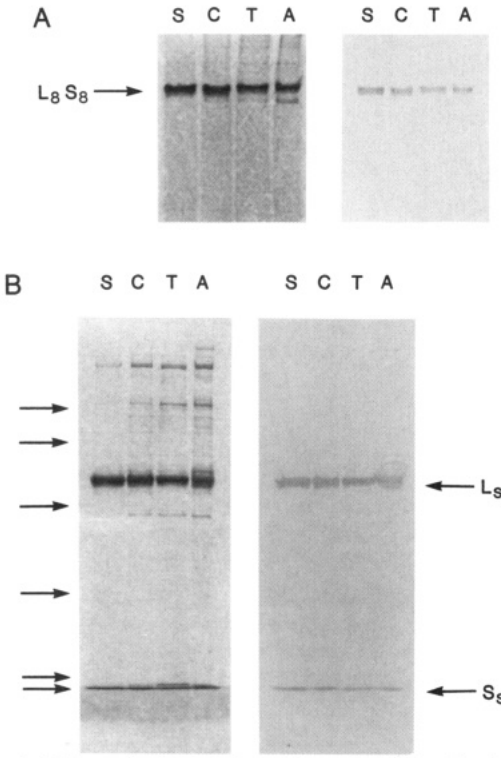

FIGURE 1: Polyacrylamide gel electrophoresis (left) and subsequent Western blot analysis (right) of first gradient purified enzyme preparations (preparation A, Table I) in the absence (A) or presence (B) of sodium dodecyl sulfate. S indicates the wild-type enzyme with Ser at position 376. C, T, and A indicate the Cys, Thr, and Ala mutants, respectively. In panel A, 5 μg of protein/lane was loaded in duplicate on a 7% nondenaturing gel which was cut in half after electrophoresis and either stained with Coomassie blue or immunoblotted using antibodies raised against RuBisCO holoenzyme from *A. nidulans* as a probe. In panel B, 6 μg of protein samples identical to those in panel A was loaded in each lane in duplicate on a 10% denaturing gel. Following electrophoresis, the gel was cut in half and either stained or immunoblotted using two antibodies raised against RuBisCO holoenzyme from *A. nidulans* and the RuBisCO S subunit also from *A. nidulans* as probes. Positions of molecular mass markers are indicated by arrows in the left margin and correspond (from top to bottom) to phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

fixation (Table I). CO_2 fixation due to RuBisCO-independent processes could be ruled out since all CO_2 fixation observed was inhibited by the addition of the RuBisCO-specific bi-substrate analogue CABP.

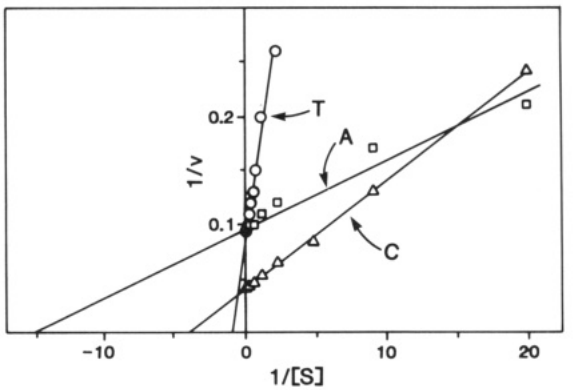

FIGURE 2: Lineweaver–Burk double-reciprocal plots at 30 °C, pH 8.0, for mutant carboxylases. Second gradient purified enzymes (preparation B, Table I) were expressed from the plasmids pGL S376C (C, △), pGL S376T (T, ○), and pGL S376A (A, □). Best-fit straight lines were derived from 11 data points and resulted in correlation coefficients of 1.00, 0.99, and 1.00 for C, T, and A, respectively. For best graphical presentation, no data points are shown for 1/[S] values of 0.038, 0.051, and 0.077 and an 1/[S] value of 77 for A (the Ala variant). Lineweaver–Burk analysis for the wild-type enzyme expressed from the plasmid pCS88 (data not shown) produced a line with a $1/v$ intercept = 3.04×10^{-4} and a 1/[S] intercept = −23.8 (correlation coefficient = 0.99). The K_m and V_{max} values presented in Table I were computed with the k cat 1.3 program (BioMetallics, Inc.) which fits the Michaelis–Menten equation directly to v vs [S] data using nonlinear regression after approximation of K_m and V_m values from the Lineweaver–Burk plots.

Of the three position 376 variants constructed, the Cys variant, when assayed with 13 mM RuBP, exhibited the most activity. For example, in preparation A, this mutant retained 0.7% and 1% of the wild-type carboxylase activity after the first and second sucrose gradients. The Thr and Ala mutants both retained 0.2% of the wild-type specific activity at both levels of purification when assayed with 13 mM concentration of RuBP. Closely similar results were obtained with preparation B (Table I). Double-reciprocal plots are linear for mutant proteins (Figure 2) and wild-type enzyme but reveal divergent slopes and intercepts. The derived value for K_m(RuBP) for the Ala mutant was almost twice that for wild-type enzyme but those for the Cys and Thr mutants were considerably more elevated (Table I). The ratio of the specific activity to K_m(RuBP) for carboxylase catalysis was greatly reduced for all mutant enzymes (Table I), suggesting disporportionate effects of substitution on k_{cat} and K_m(RuBP).

Oxygenase Activities of Mutant and Wild-Type Proteins. Using a highly sensitive chemiluminescent assay, oxygenase

Table II: Initial Rates of Oxygenase-Dependent Chemiluminescence of Mutant and Wild-Type Proteins[a]

plasmid expressed	chemiluminescence (oxygenase act.) initial rate	% wild-type	initial rate (+CABP)	carboxylase act. % wild-type
pCS88	36380 ± 5159	100	0	100
pGL S376C	68 ± 8	0.2	0	0.7
pGL S376T	7 ± 3	0.02	0	0.2
pGL S376A	5790 ± 1038	16	0	0.2

[a] Initial rates of chemiluminescence expressed in cpm were derived from Figure 3. CABP concentrations were 50 μM where indicated. Carboxylase activities of comparably purified mutant enzyme preparations expressed as a percentage of wild-type activity were reported for preparation A in Table I, and are shown here for comparison.

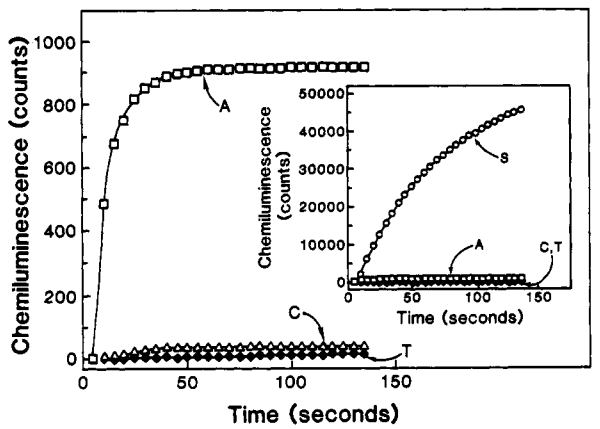

FIGURE 3: Oxygenase-dependent chemiluminescence of Mn^{2+}-activated mutant and wild-type enzyme preparations (0.125 mg of protein/mL). RuBisCO was purified through the first sucrose gradient from cells which had expressed the plasmids pCS88 (wild-type) (S, ○), pGL S376C (C, △), pGL S376T (T, ♦), and pGL S376A (A, □). The inset shows the chemiluminescence of the wild-type enzyme in relation to that of the mutant enzymes. Rates are shown for 0.2 mg of enzyme in all cases.

activities of mutant enzymes purified through the first sucrose gradient were measurable, although greatly reduced relative to that determined for the wild-type enzyme (Table II). As was observed for carboxylation, the addition of CABP severely inhibited the rates of chemiluminescence for all the enzyme preparations tested. Unlike the relatively constant rates of chemiluminescence exhibited with the wild-type and Thr mutant, the Ala and Cys mutants both produced chemiluminescence only for a short duration following the initiation of the reaction with RuBP. This chemiluminescence then abruptly and completely ceased within the first minute of the reaction (Figure 3).

The initial rates of chemiluminescence for the Cys and Thr mutants were 0.2% and 0.02%, respectively, of that for the wild-type enzyme, displaying roughly the same reduction in oxygenase activity as was observed for carboxylase activity. Strikingly, however, the initial rate of chemiluminescence observed with the Ala mutant was 16% of that for the wild-type, in marked contrast to the almost null carboxylase activity in the same mutant (Table II). For purposes of comparison, the rates of RuBP-dependent O_2 consumption measured by an O_2 electrode were 10 and 11% for the Ala mutant vs wild-type enzyme in the presence of Mg^{2+} and Mn^{2+}, respectively (data not shown).

Attempts to measure the CO_2/O_2 specificity factor (Jordan & Ogren, 1981) for the Ala mutant were unsuccessful because the maximal carboxylase velocity (and K_m for CO_2) could not be reliably measured (F. R. Tabita, personal communication). This reflects the extremely low specific activity for the carboxylase of this mutant (Table I).

Discussion

On the basis of crystallography, RuBisCO is a parallel β–α-barrel enzyme in which the carboxy-terminal domain of a given L subunit contributes predominantly to the active-site domain but with contributions from the N-terminal domain of an adjoining L subunit (Schneider et al., 1990; Knight et al., 1990; Farber & Petsko, 1990). There are two active sites shared by each L_2 dimer. In each carboxy-terminal domain, there are eight β-sheets at the interior of the barrel connected by loops to eight helical strands of opposite direction. In the L_8S_8 enzyme from spinach, the binding site for the 1-phosphate of CABP is contributed primarily by loops 7 and 8. In contrast, the binding site for the 5-phosphate of CABP is contributed almost exclusively by loops 5 and 6 (Knight et al., 1990). The present research on the closely similar L_8S_8 enzyme from *A. nidulans* implicates Ser376 in RuBP binding. In the spinach enzyme, the counterpart Ser379 is found in β-sheet 7 of the carboxy-terminal domain.

In the present work, the replacement of Ser376 with Cys, Thr, or Ala results in mutant L_8S_8 enzymes with carboxylase activities reduced by 99% or greater, supporting the idea that Ser376 is crucial for RuBisCO catalysis. However, since methyl groups cannot contribute to any functional chemistry, the fact that the Ala mutant retained 0.3% of the wild-type activity indicates that Ser376 is not absolutely essential.

In all substitutions of Ser376, the K_m for RuBP is elevated, suggesting that Ser376 may play an important role in binding RuBP. This interpretation is consistent with its placement close to the 3-OH and one 5-phosphate O of CABP in active-site representations of the L_8S_8 enzyme based on X-ray crystallography (Knight et al., 1990). Since sulfhydryls form considerably weaker hydrogen bonds than hydroxyls, a substrate binding function may account for the finding that the seemingly conservative replacement of Cys for Ser results in a 99% reduction in activity and a 5-fold increase in the K_m for RuBP. Moreover, the still lower catalytic activity and further elevated K_m for RuBP exhibited through the substitution of Ser376 by Thr, another potential hydrogen bond participant, suggest that steric constraints are extremely high. The possibility that Ser376 may function as the base that deprotonates C-3 of RuBP to promote enolization—previously indicated as plausible by X-ray crystallography—seems unlikely on the basis of the present study. In addition, evidence has accumulated implicating Lys175 in the spinach enzyme (or Lys66 in *R. rubrum*) in the deprotonation of C-3 of RuBP (Hartman & Lorimer, 1988).

The use of a novel chemiluminescent oxygenase assay in this study allowed the measurement of mutant oxygenase activities that would fail detection by a standard oxygen electrode. It is not clear why the chemiluminescence produced by the Ala and Cys mutants ceased so abruptly, however, appearing as if it was inhibited completely after a few catalytic cycles. The chemiluminescence of Mn^{2+}-activated RuBisCO from spinach has been attributed to the emission of singlet O_2 (Mogel & McFadden, 1990) which can have highly deleterious effects on protein structure and function. Therefore, one explanation for the observed behavior is that the Ala and Cys mutants are for unknown reasons more susceptible to singlet O_2 than are the Thr mutant and wild-type enzymes.

Characterization of the Ser376 → Ala mutant has demonstrated that alterations in RuBisCO structure can disproportionately affect the carboxylation and oxygenation pro-

cesses. The Ala substitution resulted in a mutant enzyme which was almost devoid of carboxylase activity yet retained 16% of the oxygenase activity. These findings suggest that the CO_2/O_2 specificity factor may have been altered in favor of the oxygenase activity. A previous study has also identified a mutation in the L subunit of RuBisCO from the alga *Chlamydomonas reinhardtii* that reduced the CO_2/O_2 specificity factor by 37% (Chen & Spreitzer, 1989). Unlike the active-site Ser → Ala mutation reported here, this mutation was characterized as a Val → Ala substitution localized to residue 331 (in *C. reinhardtii*) which lies near the catalytically essential Lys334. Recently, two suppressor mutations in the *C. reinhardtii* L subunit gene for the Ala331 variant have been made. In these, Thr342 or Gly344 have been replaced by Ile or Ser with a consequent increase in the CO_2/O_2 specificity factor for the enzyme (Chen et al., 1991). Ala331 is in loop 6, and residues 342 and 344 are in α-helix 6 in the spinach enzyme (Knight et al., 1990). The present data establish that a marked alteration in the ratio of carboxylase to oxygenase can be achieved by directed mutagenesis of the L-subunit gene for RuBisCO, in the region specifying β-sheet 7 in the L subunit. Although in the wrong direction to be of potential physiological importance, our finding is of considerable theoretical interest and may augur well for the production of mutant enzymes with elevated carboxylase:oxygenase ratios.

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