Leucine 332 influences the CO₂/O₂ specificity factor of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Anacystis nidulans*

GARRETT J. LEE, KELLY A. McDONALD, AND BRUCE A. McFADDEN

Department of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164-4660

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Abstract

The role of Leu 332 in ribulose-1,5-bisphosphate carboxylase/oxygenase from the cyanobacterium *Anacystis nidulans* was investigated by site-directed mutagenesis. Substitutions of this residue with Met, Ile, Val, Thr, or Ala decreased the CO₂/O₂ specificity factor by as much as 67% and 96% for the Ile mutant in the presence of Mg²⁺ and Mn²⁺, respectively. For the Met, Ile, and Ala mutants in the presence of Mg²⁺, no loss of oxygenase activity was observed despite the loss of greater than 65% of the carboxylase activity relative to the wild-type enzyme. In the presence of Mn²⁺, carboxylase activities for mutant enzymes were reduced to approximately the same degree as was observed in the presence of Mg²⁺, although oxygenase activities were also reduced to similar extents as carboxylase activities. Only minor changes in $K_m(\text{RuBP})$ were observed for all mutants in the presence of Mg²⁺ relative to the wild-type enzyme, indicating that Leu 332 does not function in RuBP binding. These results suggest that in the presence of Mg²⁺, Leu 332 contributes to the stabilization of the transition state for the carboxylase reaction, and demonstrate that it is possible to affect only one of the activities of this bifunctional enzyme.

Keywords: chemiluminescence; CO₂ fixation; CO₂/O₂ specificity factor; rubisco; site-directed mutagenesis

Rubisco plays a pivotal role in photosynthetic growth by catalyzing the initial step in CO₂ fixation. The enzyme's inherent inability to discriminate between CO₂ and O₂ markedly decreases net carbon assimilation by virtue of both the competition by O₂ for the common substrate RuBP and the subsequent release of CO₂ through photorespiration initiated by the oxygenation of RuBP (Andrews et al., 1973).

The carboxylation to oxygenation efficiency of rubisco is believed to dictate photosynthetic productivity and is influenced by the maximal velocities for carboxylation and oxygenation, as well as the Michaelis constants for

CO₂ and O₂. The CO₂/O₂ specificity factor, τ , expressed as $(V_c/V_o)(K_o/K_c)$ kinetically describes the carboxylation efficiency of rubisco as the ratio of apparent second-order rate constants for the two opposing reactions (Jordan & Ogren, 1981).

CO₂/O₂ specificity factors measured for enzymes from a variety of photosynthetic organisms indicate that selective pressures have increased τ values in the enzymes from more evolutionarily advanced species (Jordan & Ogren, 1981), although an enzyme with an unusually high τ value has been identified in the marine diatom *Cyclindrotheca* sp. strain N1 (Read & Tabita, 1991). Likewise, protein engineering has greatly enhanced the prospects for investigating the structure/function relationships that determine CO₂/O₂ partitioning and may ultimately lead to the construction of enzymes with increased efficiency that are agriculturally beneficial.

In addition to the variability observed for carboxylation/oxygenation efficiencies in nature, variation in the oligomeric structure of rubisco holoenzymes has also been observed. Generally the enzyme exists in higher plants, algae, and cyanobacteria as a hexadecamer consisting of

Reprint requests to: Bruce A. McFadden, Department of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164-4660.

Abbreviations: CABP, 2-carboxy-D-arabinitol-1,5-bisphosphate; HEPPS, N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid); K_c , Michaelis constant for CO₂; K_o , Michaelis constant for O₂; L, large subunit of rubisco; PAGE, polyacrylamide gel electrophoresis; RuBP, D-ribulose-1,5-bisphosphate; rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; S, small subunit of rubisco; τ , CO₂/O₂ specificity factor; V_c , maximal velocity for carboxylation; V_o , maximal velocity for oxygenation.

eight 55-kDa L subunits and eight 15-kDa S subunits (McFadden, 1980). A notable exception is the L_2 enzyme from *Rhodospirillum rubrum*, which exists as a dimer of L subunits (Tabita & McFadden, 1974).

Regardless of the differences in structure, however, all forms of the enzyme require activation steps to achieve catalytic competence. The initial step in activation involves the carbamylation of an active-site Lys residue (Lys 201 in higher plant enzymes) with a molecule of CO_2 that is distinct from substrate CO_2 (Lorimer, 1981). Carbamylation is then followed by coordination of a divalent metal to the carbamate and a number of active-site residues. Mg^{2+} has been shown to support the highest carboxylation to oxygenation efficiency, although other divalent metals such as Mn^{2+} can support both activities (Weissbach et al., 1956; Christeller, 1981). A unique aspect of Mn^{2+} -activated rubisco is the production of chemiluminescence resulting from the oxygenase reaction (Mogel & McFadden, 1990). This property has been previously exploited to monitor the oxygenase activities of mutant and wild-type enzymes (Lee & McFadden, 1992).

Crystallographic analysis of rubisco from higher plant species such as tobacco (Curmi et al., 1992), spinach (Andersson et al., 1989; Knight et al., 1990), and the bacterium *R. rubrum* (Lundqvist & Schneider, 1989, 1991) have demonstrated that the carboxyterminal domain of L subunits consists of an eight-membered β/α barrel in which the most catalytically critical residues are positioned on loops connecting the β -strands to the α -helices. In the tobacco crystal structures, comparison of the unactivated enzyme (Curmi et al., 1992) (Fig. 1A; Kinemages 1, 2) with the activated quaternary complex with bound bisubstrate analog CABP (D.S. Eisenberg, unpubl.) (Fig. 1B; Kinemages 1, 2) reveals that loop 6, which contains residues Lys 334 and Leu 335, is particularly mobile. Relative to the Ser OH at position 379, the C_γ of Leu 335 moves approximately 11 Å to partially cover the active site in the CABP form, suggesting that this mobility serves as a mechanism to allow access of RuBP to the active site prior to reaction.

Sequence comparisons of rubisco from diverse organisms such as higher plants, green and nongreen algae, cyanobacteria, and photosynthetic bacteria show Lys 334 to be completely conserved and Leu 335 to be conserved in all enzymes except in that from *R. rubrum* where Leu has been replaced by Met (Knight et al., 1990; Hwang & Tabita, 1991). In the *R. rubrum* enzyme both the Met and Lys residues have been previously studied by directed mutagenesis. The analogous Lys 329 was found to be catalytically essential (Soper et al., 1988), whereas the substitution of the analogous Met 330 by Leu (the only substitution evaluated) decreased the carboxylase activity by 80%, but had no effect on the CO_2/O_2 specificity factor (Terzaghi et al., 1986).

We report here that replacement of the corresponding Leu 332 with a variety of residues in L_8S_8 rubisco from

Fig. 1. X-ray crystal structure of the rubisco active site from tobacco in the unactivated form (A) and the activated form with CABP (bold lines) (B). Glu 60 and Asn 123 are contributed by the neighboring L subunit. The corresponding residue numbers for the *A. nidulans* enzyme are three positions less than those for the tobacco enzyme.

the cyanobacterium *Anacystis nidulans*, an enzyme structurally similar to higher plant enzymes, markedly affects CO_2/O_2 partitioning.

Results

Purification of wild-type and mutant enzymes

Throughout the purification process, mutant enzymes behaved similarly to the wild-type enzyme and were isolated in highly purified states (Fig. 2). In addition, the identical behavior of the mutant enzymes relative to the wild-type enzyme during nondenaturing and denaturing PAGE indicates that the native mutant enzymes are fully assembled holoenzymes (Fig. 2).

CO_2/O_2 specificity factors

When activated and studied in the presence of either Mg^{2+} or Mn^{2+} , all mutant enzymes containing substitu-

Fig. 2. Polyacrylamide gel electrophoresis of purified wild-type and mutant enzymes in the absence (A) or presence (B) of SDS. At the top, L indicates the wild-type enzyme with Leu at position 332. M, I, V, T, and A indicate the Met, Ile, Val, Thr, and Ala mutants, respectively. Three micrograms of protein were loaded per lane and stained with Coomassie blue after electrophoresis. Positions of the holoenzymes and individual subunits are indicated in the right margin. Positions of the molecular mass markers are shown 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).

tions at Leu 332 had substantially lowered τ values compared to the wild-type enzyme (Fig. 3) using a new method to measure τ (Lee & McFadden, 1993). For example, in the presence of Mg^{2+} , the specificity factor of the Ile variant was reduced by 67%, whereas in the presence of Mn^{2+} , the specificity factor was reduced by 96%. With regard to the wild-type enzyme, use of Mn^{2+} instead of Mg^{2+} decreased the τ value by 61%. In contrast to the reduction observed for the wild-type enzyme, all but the Met mutant showed much larger declines in τ values as a result of studies in the presence of Mn^{2+} .

Kinetic parameters

Use of Mg^{2+} in both activation and catalysis resulted in decreased V_c/V_o ratios for all mutants relative to that of the wild-type enzyme (Table 1). These decreased ratios result from larger decreases in V_c relative to V_o . With re-

Fig. 3. CO_2/O_2 specificity factor determination. The slope of each line is equivalent to the specificity factor, τ , and was determined in the presence of Mg^{2+} (A) or Mn^{2+} (B) at 30 °C, pH 8.0, for the wild-type and mutant proteins, as indicated. In all cases, lines were generated by linear regression with the origin included. The symbol representation and reaction times are as follows: ■, wild type, 10 min; ●, L332 Met, 20 min; ○, L332 Ile, 40 min; ▲, L332 Val, 40 min; △, L332 Thr, 40 min; and □, L332 Ala, 40 min.

spect to carboxylase activity, the most deficient enzyme, the Ile 332 mutant, still retained 14% of the wild-type activity. With respect to the oxygenase activity, notably, the Met, Ile, and Ala substitutions had no effect on V_o values, despite the fact that the corresponding V_c values were appreciably decreased by 65% or greater. However, use of Mn^{2+} instead of Mg^{2+} had little effect on the V_c/V_o ratios of the mutants relative to that of the wild-type enzyme because of similar decreases in V_o and V_c (Table 1).

In the presence of Mg^{2+} the apparent affinities for CO_2 were highly variable from mutant to mutant, indicating no particular trends (Table 2). Compared to the wild-type K_c value, the K_c values for the Met and Ala mutants remained relatively unchanged, decreased for the Ile mutant, and increased for the Val and Thr mutants. Changing the metal ion from Mg^{2+} to Mn^{2+} reduced the wild-type K_c value from 221 μM to 15 μM (Table 2). Likewise, the K_c values for the mutant enzymes also greatly decreased in the presence of Mn^{2+} but not pro-

Table 1. Maximal velocities for carboxylation and oxygenation of RuBP by wild-type and mutant enzymes

Enzyme	V_c , app ($\mu\text{mol}/\text{min}/\text{mg}$)	V_o ($\mu\text{mol}/\text{min}/\text{mg}$)	V_c/V_o
Mg²⁺			
Wild type	4.23 $\pm$ 0.19 (100%)	0.15 $\pm$ 0.02 (100%)	28.2
L332 Met	1.47 $\pm$ 0.10 (35%)	0.16 $\pm$ 0.02 (107%)	9.2
L332 Ile	0.61 $\pm$ 0.01 (14%)	0.15 $\pm$ 0.01 (100%)	4.1
L332 Val	1.65 $\pm$ 0.15 (39%)	0.10 $\pm$ 0.02 (67%)	16.5
L332 Thr	2.27 $\pm$ 0.14 (54%)	0.10 $\pm$ 0.01 (67%)	22.7
L332 Ala	1.25 $\pm$ 0.04 (30%)	0.15 $\pm$ 0.03 (100%)	8.3
Mn²⁺			
Wild type	1.46 $\pm$ 0.02 (100%)	0.54 $\pm$ 0.01 (100%)	2.7
L332 Met	0.67 $\pm$ 0.05 (46%)	0.24 $\pm$ 0.02 (44%)	2.8
L332 Ile	0.33 $\pm$ 0.02 (23%)	0.14 $\pm$ 0.01 (26%)	2.4
L332 Val	0.39 $\pm$ 0.04 (27%)	0.17 $\pm$ 0.01 (31%)	2.3
L332 Thr	0.88 $\pm$ 0.03 (60%)	0.15 $\pm$ 0.01 (28%)	5.9
L332 Ala	0.66 $\pm$ 0.07 (45%)	0.29 $\pm$ 0.03 (54%)	2.3

portionally to the 14-fold decrease observed for the wild-type enzyme. The K_c values in the presence of Mn²⁺ relative to wild type were decreased for the Met mutant, unchanged for the Ile mutant, and increased for the Val, Thr, and Ala mutations. K_o values could not be reliably measured using a previously described method (Chen et al., 1988).

$K_{m(\text{RuBP})}$ values measured in the presence of Mg²⁺ (Table 2) were decreased from 51 μM to 33–35 μM for the Ile, Val, and Ala mutants. The value for the Met mutant enzyme was essentially unaltered. Only the Thr mutant enzyme exhibited an elevated $K_{m(\text{RuBP})}$ of 131 μM .

Oxygenase-dependent chemiluminescence

Because of its simplicity, previous studies from our laboratory have utilized a Mn²⁺, RuBP-dependent chemiluminescent assay to monitor rubisco oxygenase activity

Table 2. Michaelis constants for wild-type and mutant enzymes

Enzyme	Mg ²⁺		Mn ²⁺
	K_c , app (μM)	$K_{m(\text{RuBP})}$, app (μM)	K_c , app (μM)
Wild type	221 $\pm$ 19	51 $\pm$ 3	15 $\pm$ 3
L332 Met	182 $\pm$ 16	50 $\pm$ 3	7 $\pm$ 2
L332 Ile	98 $\pm$ 2	35 $\pm$ 5	13 $\pm$ 2
L332 Val	380 $\pm$ 14	33 $\pm$ 4	37 $\pm$ 8
L332 Thr	457 $\pm$ 4	131 $\pm$ 8	54 $\pm$ 4
L332 Ala	234 $\pm$ 20	33 $\pm$ 6	76 $\pm$ 8

(Mogel & McFadden, 1990; Lee & McFadden, 1992). The use of the assay was extended to the present research, where it was found that Mn²⁺-activated wild-type and mutant *A. nidulans* enzymes produced highly divergent rates of chemiluminescence when measured with nearly saturating O₂ (Table 3). The Met mutant exhibited a rate 77% higher than the wild-type rate, whereas the Ile mutant retained 16% of the wild-type rate. Still further reductions were observed for the Val, Thr, and Ala mutants, which retained less than 1% of the wild-type chemiluminescence activity. These relative chemiluminescence rates, however, did not correlate with the oxygen consumption measured by an O₂ electrode under similar conditions (Table 3).

The lack of correlation between the rates of oxygenase-dependent chemiluminescence and oxygen consumption for the Mn²⁺-activated wild-type and mutant enzymes suggests that the two processes are not tightly coupled. These results question the validity of using the chemiluminescent assay for the general purpose of comparing mutant to wild-type oxygenase activities. However, previous comparison of the Ser 376 Ala mutant with the wild-type enzyme demonstrated a reasonable correlation between chemiluminescence and oxygen consumption; the mutant produced 16% of the wild-type chemiluminescence and 11% of the wild-type oxygen consumption (Lee & McFadden, 1992). Because chemiluminescence has been attributed to the interaction of two molecules of singlet oxygen produced from two molecules of bound triplet oxygen during the course of the oxygenase reaction (Mogel & McFadden, 1989), certain mutations can presumably alter the rate of singlet oxygen production, its release, and/or the subsequent interaction of two molecules of singlet oxygen. The positioning of Leu 332 over the active site would be consistent with all three possibilities.

Discussion

In this study, Leu 332 of the L subunit has been substituted with the hydrophobic amino acids Met, Ile, Val, and Ala, in addition to Thr, a potential hydrogen bond do-

Table 3. Comparison of oxygenase-dependent chemiluminescence and maximal velocities for oxygenation for Mn²⁺-activated enzymes

Enzyme	Chemiluminescence (cps)	V_o ($\mu\text{mol}/\text{min}/\text{mg}$) ^a
Wild type	7,048 $\pm$ 463 (100%)	0.54 $\pm$ 0.01 (100%)
L332 Met	12,593 $\pm$ 876 (177%)	0.24 $\pm$ 0.02 (44%)
L332 Ile	1,127 $\pm$ 49 (16%)	0.14 $\pm$ 0.01 (26%)
L332 Val	58 $\pm$ 12 (0.8%)	0.17 $\pm$ 0.01 (28%)
L332 Thr	20 $\pm$ 5 (0.3%)	0.15 $\pm$ 0.01 (28%)
L332 Ala	34 $\pm$ 4 (0.5%)	0.29 $\pm$ 0.03 (54%)

^a Reported in Table 1.

nor. Regardless of whether Mg^{2+} or Mn^{2+} is used for activation and subsequent rate measurements, it is evident that Leu 332 plays an important role in maintaining the relatively high CO_2/O_2 specificity factor of 43 for rubisco from *A. nidulans* and presumably in other enzymes where this residue is conserved. Since the *R. rubrum* rubisco with a low τ value of 15 (Jordan & Ogren, 1981) is the sole known exception in which Met replaces Leu, the same substitution has been carried out in the present research and indeed results in τ value of 20. In contrast, the converse replacement of Met 330 by Leu in the *R. rubrum* enzyme failed to alter the specificity factor (Terzaghi et al., 1986). This lack of change is therefore probably largely related to the fundamental structural differences between the dimeric *R. rubrum* enzyme and enzymes similar to the *A. nidulans* enzyme, which possess L_8S_8 oligomerization (Tabita, 1988).

To our knowledge, with Mg^{2+} as the activating ion, the Leu 332 Ile mutation reported here results in the largest alteration in the CO_2/O_2 specificity factor reported for mutations localized to the C-terminal domain of the large subunit. In addition to the present results, other studies have revealed different mutations within loop 6 that reduce τ values in the enzymes from *A. nidulans* (Parry et al., 1992) and the alga *Chlamydomonas reinhardtii* (Chen & Spreitzer, 1989). We have previously described the mutation Ser 376 Ala in *A. nidulans* rubisco (Lee & McFadden, 1992), which localizes to β -strand 7, approximately two residues removed from loop 7 in the L subunit. This mutant exhibits 10% of the wild-type oxygenase activity but only 0.1% of the wild-type carboxylase activity, suggesting drastic alterations in the carboxylation to oxygenation efficiency. Inspection of the CABP form of the tobacco crystal structure (Fig. 1B; Kinemage 1) indicates that the equivalent Leu 335 is proximal to the equivalent Ser 379. However, the analogous substitution in the *R. rubrum* enzyme, Ser 368 Ala, has been recently reported to result in a 60% increase in CO_2/O_2 specificity despite a substantial decrease in carboxylase activity (Harpel & Hartman, 1992). These contrasting results further demonstrate appreciable differences between the enzymes from *A. nidulans* and *R. rubrum*. Nonetheless, collectively the results provide compelling evidence that in general, the regions in or near loops 6 and 7 heavily influence CO_2/O_2 specificity.

In the present investigation, marked differences were observed for τ when the wild-type and mutant enzymes were studied in the presence of Mn^{2+} as opposed to Mg^{2+} . For the wild-type enzyme, the twofold decrease in τ as a consequence of Mn^{2+} usage follows the previously observed trend for reduced efficiency in the presence of Mn^{2+} rather than Mg^{2+} for rubisco from a variety of species (Jordan & Ogren, 1983). However, the failure of the mutants to follow strictly a similar twofold reduction underscores the unique physical relationship between a particular side chain and a given metal ion. Because, ac-

cording to the model in Figure 1B and Kinemage 1, the side chains at position 332 (335 in tobacco) would be somewhat distant from the metal ion, it is plausible that the interaction is mediated through transition states for carboxylation versus oxygenation, incoming CO_2 or O_2 , or through neighboring residues. The latter case may be plausible, since the model shown in Figure 1B and Kinemage 1 indicates that the C δ 2 of Asn 123 (Asn 120 in *A. nidulans*), one of the potential metal-coordinating residues, is 4.5 Å from the C β of Leu 335. Of particular relevance is the recent observation that substitution of the analogous Asn 111 with Gly in the *R. rubrum* enzyme resulted in a greater than 10-fold decrease in τ (Chene et al., 1992). This and the present results suggest that the indicated Leu and Asn residues may mutually determine CO_2/O_2 specificity.

With respect to the wild-type *A. nidulans* enzyme, the observation that V_c decreased in the presence of Mn^{2+} is consistent with previously reported findings (Wildner & Henkel, 1979; Tabita, 1988). Furthermore, the previously reported enhancements in V_o in the presence of Mn^{2+} is also consistent with the findings of the present study. The majority of in vitro studies reported have focused upon rubisco studied with Mg^{2+} . In the presence of equimolar Mg^{2+} and Mn^{2+} , in vitro studies indicate that the enzyme would preferentially bind Mn^{2+} , since soybean rubisco has been shown to have a 15-fold higher affinity for Mn^{2+} than Mg^{2+} (Wildner & Henkel, 1979). Certainly the presence of manganese ion in the O_2 -evolving complex of photosystem II (Hansson & Wydrzynski, 1989) suggests that Mn^{2+} could indeed be available to rubisco. In the future, measurement of the ratio of Mg^{2+} to Mn^{2+} forms of rubisco in vivo may enable a better estimate of the ratio of CO_2 assimilation to respiratory capacity of host autotrophic organisms.

The decrease in V_c without a change in V_o for the Leu 332 mutants in the presence of Mg^{2+} demonstrate for the first time that one V_{max} value of this dual-function enzyme can be decreased by directed mutagenesis without affecting the other value. For example, the Met, Ile, and Ala variants have wild-type levels of oxygenase activity despite substantial decreases in carboxylase activity. The low V_c/V_o ratios relative to the wild-type enzyme for all the mutants in the presence of Mg^{2+} must largely contribute to the overall decreased CO_2/O_2 specificity factors, although the K_c/K_o ratios also contribute to the τ values. However, the relatively unaltered V_c/V_o ratios observed in the presence of Mn^{2+} implies that with Mn^{2+} , the affinities for O_2 must be sufficiently high to result also in decreased CO_2/O_2 specificity factors.

In the present series of substitutions for Leu 332, selective suppression of the carboxylase activity versus the oxygenase activity in the presence of Mg^{2+} suggests that Leu 332 may function in catalytic step(s) unique to the carboxylase reaction. One possibility would be the stabilization of the transition state for carboxylation. Based on

results of the substitution of the analogous Met 330 by Leu in the *R. rubrum* enzyme, the Met 330 Leu mutation was instead hypothesized to destabilize the enediol intermediate that arises from deprotonation of C-3 on RuBP (Terzaghi et al., 1986). Formation of this intermediate is common to both the carboxylation and oxygenation reactions and precedes the attack by either gaseous substrate (Saver & Knowles, 1982). In addition, this earlier study found that substitution of Met 330 with Leu resulted in an enzyme with 27-fold decreased affinity for RuBP, indicating that Met 330 may also play a role in RuBP binding. By comparison, the present results demonstrate that Leu 332 in the *A. nidulans* enzyme does not contribute to RuBP binding. Taken together, these differences suggest that Met 330 contributes to earlier steps in the reaction catalyzed by the *R. rubrum* carboxylase while Leu 332 of the *A. nidulans* carboxylase contributes to later steps, perhaps during or after the commitment toward either the carboxylation or oxygenation pathway has been made.

The results of the present research add to the growing body of data that demonstrate the importance of loop 6 in determining CO₂/O₂ specificity. Substitution of Val by Ala at position 331 of the L subunit of the *C. reinhardtii* enzyme (328 in *A. nidulans*) decreased the CO₂/O₂ specificity factor by 37% (Chen & Spreitzer, 1989). Multiple amino acid substitutions have been identified that complement the effect of the original mutation. These suppressor mutations have been identified as Thr 342 Ala and Gly 344 Ser (Chen & Spreitzer, 1989; Chen et al., 1991), both of which also localize to loop 6. In the *A. nidulans* enzyme, substitution of Ala 340 by Glu reduced the specificity factor by 17%, although substitution of Val 331 by Ala, analogous to the previously identified mutation in the *C. reinhardtii* enzyme, failed to alter τ (Parry et al., 1992). An interesting result from this same study was the finding that replacement of residues 338–341 with a cassette emulating higher plant sequences increased τ by 6%. In light of these and the present results, further investigations of the effects of loop 6 substitutions may in fact result in an enzyme with significantly higher efficiency.

Materials and methods

Materials

HEPPS was purchased from United States Biochemical, NaH¹⁴CO₃ from New England Nuclear, bovine erythrocyte carbonic anhydrase from Sigma, ascorbic acid and K₃PO₄ from Baker, and (NH₄)₆Mo₇O₂₄·4H₂O from Baker. RuBP was either purchased from Sigma or synthesized as described (Horecker et al., 1958) and purified by Dowex 1-X8-Cl[−] chromatography. CABP was synthesized by S.N. Mogel as described (Pierce et al., 1980).

Methods

Plasmids and strains

The pUC19 derivative pCS88 (McFadden & Small, 1988) expresses the 2.1-kb Pst I fragment containing the *rbcL/rbcS* gene tandem from *A. nidulans*. Phagemid pRH90 (Haining & McFadden, in prep.), a derivative of the pAlter phagemid (Promega) containing the Pst I fragment from pCS88, was used for site-directed mutagenesis. *Escherichia coli* strains BMH71-18 mut S and JM109 were used for mutagenic manipulations. *Escherichia coli* strain JM105 was used for expression of the final plasmid constructs.

Site-directed mutagenesis

Site-directed mutants were constructed as described (Haining & McFadden, in prep.) using the Promega Altered Sites Mutagenesis System. Appropriate oligonucleotides 25 bases in length were synthesized to enable the Leu 332 → Ile, Thr, and Ala mutations, and oligonucleotides 23 bases in length were synthesized to enable the Leu 332 → Met and Val mutations. The sequences of these mutagenic oligonucleotides were searched against the sequence of the *rbcL/rbcS* gene tandem revealing that they should hybridize specifically with the target sequence. The mutations were confirmed by dideoxy sequencing (Sanger et al., 1977) using the Sequenase system (United States Biochemical). The mutagenic Pst I fragments were subcloned into pUC18 to produce the plasmids pGL L332M, pGL L332I, pGL L332V, pGL L332T, and pGL L332A, which express the Leu → Met, Ile, Val, Thr, and Ala mutations at position 332 of the L subunit, respectively. Mutations were again confirmed by sequencing 150 bases flanking the target codon.

Expression and purification of rubisco

Rubisco was purified from *E. coli* strain JM105 expressing the wild-type (pCS88) and mutant plasmids by centrifugation through a single linear sucrose gradient as previously described (Lee & McFadden, 1992) except that isolation buffer (50 mM HEPPS, 1 mM NaHCO₃, 1 mM MgCl₂, 0.1 mM EDTA, 1 mM β -mercaptoethanol, pH 8.0, at 30 °C) was substituted for TEMMB buffer (50 mM Tris-HCl, 50 mM NaHCO₃, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM β -mercaptoethanol, pH 7.5, at 25 °C). Protein concentrations were measured using the Bio-Rad protein assay with bovine serum albumin as a standard. Enzymes were stored in 15% glycerol at −70 °C with no loss of activity.

Kinetic analyses

All analyses were performed with at least three replicates on enzymes from at least two independent preparations.

CO₂/O₂ specificity factor determination. CO₂/O₂ specificity factors (τ) were determined according to the

relationship $\tau = (v_c/v_o)([O_2]/[CO_2])$ (Laing et al., 1974). In this method (Lee & McFadden, 1993) initial velocity ratios, v_c/v_o , were determined at varying $[CO_2]/[O_2]$ ratios by the relationship $v_c/v_o = (\text{moles } CO_2 \text{ fixed})/(\text{moles RuBP consumed} - \text{moles } CO_2 \text{ fixed})$. CO_2 fixation was determined in one aliquot by $^{14}CO_2$ incorporation into acid-stable 3-phosphoglycerate and the RuBP remaining determined in another aliquot by analyzing inorganic phosphate specifically hydrolyzed from position 1 of RuBP under alkaline conditions. The slope of v_c/v_o vs. $[CO_2]/[O_2]$ is the specificity factor. Rubisco was activated on ice for 30 min, then at 30 °C for 5 min prior to the initiation of reactions in 41 mM HEPPS, pH 8.0, 10 mM $MgCl_2$ or $MnCl_2$, and 10 mM $NaH^{14}CO_3$ (0.4 mCi/mmol). The final reaction conditions were as follows: 50 mM HEPPS, pH 8.0, 30 °C, 10 mM $MgCl_2$ or $MnCl_2$, 0.4 mM RuBP, 560 μM dissolved O_2 , and 12.7, 30.0, 48.0, or 66.0 μM CO_2 . Reaction times are indicated in Figure 3.

V_c and K_c determination. V_c and K_c values were determined under the same conditions described for specificity factor measurements except that reaction vials were flushed with 100% N_2 and the CO_2 concentrations at equilibrium were 12.7, 25.4, 44.4, 63.4, 82.4, 101, 152, and 208 μM . Reactions were terminated after 30 s with the addition of 100 mL of glacial acetic acid. Kinetic parameters were calculated with the k-cat 1.3 program (Biometallics).

$K_m(RuBP)$ determination. Values were determined using the assay previously described (McFadden et al., 1975) in the presence of 0.005, 0.02, 0.06, 0.1, 0.5, 1.0, 4.0, and 10.0 mM RuBP at pH 8.0 and 30 °C. Reaction times were 30 s.

Determination of O_2 consumption. Enzymes were activated for 30 min on ice, then for 5 min at 30 °C in 50 mM HEPPS, 20 mM $MgCl_2$ or $MnCl_2$, and 8 mM $NaHCO_3$, pH 8.0. Activated enzymes were introduced into the reaction chamber of a Clark type O_2 electrode (Hansatech) containing HEPPS reaction buffer supplemented with either $MgCl_2$ or $MnCl_2$ and previously bubbled to saturation with 100% O_2 . After sealing the reaction chamber, reactions were initiated with the addition of RuBP. Final reaction conditions were 50 mM HEPPS, 0.4 mM RuBP, 20 mM $MgCl_2$ or $MnCl_2$, 1.5 mM $NaHCO_3$, 880 μM O_2 , pH 8.0 at 30 °C. Any rubisco-independent O_2 consumption was monitored after termination of reactions with 50 μM CABP. Previous results indicated CABP abolished all wild-type and mutant activity.

Oxygenase-dependent chemiluminescent assay. Chemiluminescence was measured using minor modifications of previously described methods (Mogel & McFadden, 1990; Lee & McFadden, 1992). Enzyme (0.1 mg) was activated on ice for 30 min, then for 5 min at 30 °C in the

presence of 38 mM HEPPS, 20 mM $MnCl_2$, and 10 mM $NaHCO_3$, pH 8.0. During enzyme activation, HEPPS reaction buffer containing $MnCl_2$ was combined with RuBP in 7-mL polyethylene scintillation vials (Kimble) and stoppered with septum caps. Vials were degassed with house vacuum for 10 min, then flushed with 100% O_2 for 30 min. Reactions were initiated by the addition of activated enzymes. Counts were monitored every 5 s in a Beckman LS-9000 liquid scintillation counter. Final reaction conditions were 50 mM HEPPS, 0.4 mM RuBP, 20 mM $MnCl_2$, 1.25 mM $NaHCO_3$, 950 μM O_2 , pH 8.0, at 30 °C.

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