Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5768–5772, August 1990
Botany

Residues in three conserved regions of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase are required for quaternary structure

JOHN H. FITCHEN*, STEFAN KNIGHT†, INGER ANDERSSON†, CARL-IVAR BRANDEN†, AND LEE MCINTOSH*‡

*Michigan State University–Department of Energy Plant Research Laboratory, and ‡Department of Biochemistry, Michigan State University, East Lansing, MI 48824; and †Department of Molecular Biology, Swedish University of Agricultural Sciences, Uppsala Biomedical Center, P.O. Box 590, S-75124 Uppsala, Sweden

Communicated by N. Edward Tolbert, December 26, 1989 (received for review April 27, 1989)

ABSTRACT **To explore the role of individual residues in the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39), small subunits with single amino acid substitutions in three regions of relative sequence conservation were produced by directed mutagenesis of the *rbcS* gene from *Anabaena* 7120. These altered small subunits were cosynthesized with large subunits (from an expressed *Anabaena rbcL* gene) in *Escherichia coli*. Mutants were analyzed for effects on quaternary structure and catalytic activity. Changing Glu-13S (numbering used is that of the spinach enzyme) to Val, Trp-67S to Arg, Pro-73S to His, or Tyr-98S to Asn prevented accumulation of stable holoenzyme. Interpretation of these results using a model for the three-dimensional structure of the spinach enzyme based on x-ray crystallographic data suggests that our small subunit mutants containing substitutions at positions 13S and 67S probably do not assemble because of mispairing or nonpairing of charged residues on the interfacing surfaces of the large and small subunits. The failure of small subunits substituted at positions 73S or 98S to assemble correctly may result from disruption of intersubunit or intrasubunit hydrophobic pockets, respectively.**

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39) catalyzes the first committed step of both the reductive and oxidative photosynthetic carbon cycles (for review, see ref. 1). The holoenzyme in cyanobacteria and plants is composed of eight large subunits (LSUs) and eight small subunits (SSUs). LSUs form the catalytic sites of the enzyme and provide all catalytically essential residues (2). The contribution of the SSUs to the function of the holoenzyme is unresolved.

The importance of the SSUs to the catalytic activity of the enzyme is demonstrated by a 200-fold drop in the V_{max} when the SSUs are removed (2). Activity can be restored by reconstitution of the resulting LSU octamer with isolated SSUs (2), but the structural requirements for this restoration and the mechanism by which activity is effected are not understood.

Several systems have been described that allow the use of site-directed mutagenesis to study the role of individual residues in the SSU. These systems are based on the observation that a normal holoenzyme is assembled when cyanobacterial *rbcL* and *rbcS* genes, encoding the LSUs and SSUs, respectively, are expressed in *Escherichia coli* (3–6). Some of the mutant enzymes produced have shown reduced activity (7, 8); however, the causes of the reductions in activity have not been determined and specific functions have not been assigned to individual residues in the SSU.

We have used directed mutagenesis of *rbcS* from the cyanobacterium *Anabaena* 7120 and coexpression with *Anabaena rbcL* in *E. coli* to investigate the function of individual residues in three conserved regions of the SSU. In this paper we present data from four single-amino acid substitutions, representing the three regions of broadest sequence conservation, that were analyzed for their effect on catalytic function and holoenzyme formation. To better understand the function of these residues in the native holoenzyme, these amino acids have been examined in the context of a model for the three-dimensional structure of spinach RuBisCO (9). For each of the four mutations, we discuss the structural implications of the substitution made and propose structural explanations for the results observed.

MATERIALS AND METHODS

Construction of Plasmids. DNA manipulations were according to standard protocols (10).

pL56, a plasmid to direct the expression of *Anabaena* RuBisCO LSUs in *E. coli*, was constructed by excising a *Pvu* II–*Sca* I fragment including the *lac* promoter and *Anabaena rbcL* coding sequence from pANX105 (6) and subcloning it, with the aid of *Sal* I linkers, into the *Sal* I site of the cosmid vector pWH4 (11).

p205-3, a "phagemid" plasmid for production of single-stranded template DNA for mutagenesis, was made by inserting a 0.5-kilobase *Sca* I–*Dra* I fragment containing the *Anabaena* 7120 *rbcS* protein coding region into pGC1 (12), which had been cleaved with *Bam*HI and treated with S1 nuclease to create appropriate ends for ligation. A plasmid with the orientation placing the 5′ end of *rbcS* adjacent to the *Xba* I site of the vector was designated p205-3.

pSewt, pSe13, pSe67, pSe73, and pSe98, plasmids to direct the expression of authentic or substituted *Anabaena* RuBisCO SSUs in *E. coli*, were made by subcloning *Xba* I–*Eco*RI fragments from p205-3 and its mutant derivatives into the *Xba* I and *Eco*RI sites of pUC19 (13). The *rbcS* coding region is not in the reading frame of the *lacZ* fragment of the vector and translation of *rbcS* mRNA must begin at Met-1S.

Mutagenesis. Single-stranded DNA templates of p205-3 were generated by superinfection with M13K07 as described (14). Oligonucleotides for each conserved region were synthesized as mixtures of oligonucleotides without and with one, two, or more differences from the wild-type sequence. Mutagenesis was performed as described (15). Mutants were identified by sequencing the appropriate region of randomly selected progeny plasmids from the mutagenesis protocol using the dideoxynucleotide chain-termination method (16). The entire *rbcS* cassette was sequenced in putative single mutants to exclude the possibility of additional spurious mutations.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SSU, small subunit of RuBisCO; *rbcS*, gene encoding SSU; LSU, large subunit of RuBisCO; *rbcL*, gene encoding LSU.

Growth of Cells. Cells were grown in LB medium (10). Antibiotics were added when appropriate at the following concentrations: ampicillin at 50 mg/liter; kanamycin at 30 mg/liter. Cultures (300 ml) for analysis of mutant or wild-type proteins were started with a 1-ml inoculum and grown for 24 hr at 37°C with vigorous aeration.

Preparation of Bacterial Extracts and Assay of Carboxylase Activity. Cells were harvested by centrifugation, resuspended in assay buffer (50 mM Tricine, pH 8.0/10 mM $MgCl_2$/10 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride), and disrupted by ultrasonic treatment (6). The cell extract was centrifuged at 100,000 × *g* for 1 hr. Unwashed pelleted material was resuspended in SDS/PAGE sample buffer (17) and boiled for 2 min. Saturated ammonium sulfate solution was added to a portion of the 100,000 × *g* supernatant fraction to a final concentration of 40% (wt/vol) ammonium sulfate. Aggregated proteins were collected by centrifugation at 10,000 × *g* for 15 min, resuspended in assay buffer, and dialyzed against assay buffer. The carboxylase activity of this preparation was determined as ribulose 1,5-bisphosphate-dependent incorporation of radioactivity from $^{14}CO_2$ into acid-stable forms (6). Protein was quantified by a modified Lowry procedure (18).

Assay for Holoenzyme Formation. A 2.5-mg sample of the crude 100,000 × *g* supernatant fraction was loaded on a 5.2-ml sucrose gradient (0.2–0.8 M sucrose in 50 mM Tris·HCl, pH 8.0/0.1 mM EDTA/1 mM 2-mercaptoethanol/50 mM $NaHCO_3$/10 mM $MgCl_2$) (19) and centrifuged at 50,000 rpm in a Beckman VTi 65 rotor (239,000 × *g*) for 70 min at 4°C. Three 300-μl fractions centered around the position of 18S particles [as estimated by sedimentation of purified spinach RuBisCO, a gift from S. C. Somerville (Michigan State University) in equivalent gradients] were pooled and proteins in an 80-μl sample were resolved by SDS/PAGE (ref. 20 as modified in ref. 17) and electrophoretically transferred to a nitrocellulose membrane (17). SSU was detected using SSU-specific polyclonal antibodies and protein-A-linked alkaline phosphatase according to standard immunodetection protocols (21).

Structural Interpretation of Mutants. The model of the spinach $(LSU)_8(SSU)_8$ RuBisCO holoenzyme was derived from an isomorphous electron density map with phase angles refined by fourfold averaging. The structures of both the LSUs and the SSUs have been briefly described (9, 22). The model was examined on a graphics display using FRODO (23) and the steric effects of mutations were examined by substituting appropriate side chains. Accessibility calculations were made using the program of Lee and Richards (24). Secondary structures in wild-type and substituted SSUs were predicted with the algorithm of Chou and Fasman (25).

RESULTS AND DISCUSSION

Synthesis and Analysis of Authentic and Substituted Proteins. *Experimental system.* Transformation of *E. coli* with the *rbcL* and *rbcS* expression plasmids pL56 and pSewt, respectively, results in the synthesis of functional heteromultimeric RuBisCO (Fig. 1). This result is similar to that observed with RuBisCO genes from other cyanobacteria expressed in *E. coli* (3–5) and with both *Anabaena* 7120 genes expressed as an operon on a single plasmid (6). The RuBisCO produced from such systems is apparently identical to the native enzyme, although posttranslational processing and modification of LSU, known to occur in higher plants (26), has not been investigated.

Mutagenesis. Oligonucleotide mixtures were used to generate a collection of mutations in each of three relatively conserved regions of the small subunit. The mutations presented in this paper result in the following substitutions: Glu-13S → Val, Trp-67S → Arg, Pro-73S → His, and Tyr-98S → Asn (Fig. 1).

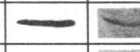
Substitution	Activity	18S	Pellet
None (wt)	51 ± 17		
Glu 13S → Val	0.0		
Trp 67S → Arg	0.0		
Pro 73S → His	0.0		
Tyr 98S → Asn	0.0		

FIG. 1. Loss of carboxylase activity and absence of holoenzyme resulting from substitutions in the SSU. Residue numbering is that of spinach. Activity is expressed as nmol of CO_2 fixed per min per mg of protein and was assayed on a crude RuBisCO preparation from dually transformed (expressing LSU and native or substituted SSU) *E. coli*. The last two columns are sections of Western blots of SDS/polyacrylamide gels after immunodetection of SSU. The samples for the lanes in the 18S column were taken from sucrose gradients used to separate holoenzyme from unassembled subunits and included the region where assembled unsubstituted holoenzyme migrates. The samples for the pellet column were from unwashed 10,000 × *g* pellets of the ultrasonically disrupted cells and were prepared by partial solubilization in boiling 3% (wt/vol) SDS sample buffer (20).

Analysis of enzyme activity. Cultures of *E. coli* containing pL56 and either pSewt or one of the mutant *rbcS* plasmids were grown and extracts were prepared for analysis. Relatively crude preparations were used for analysis to avoid the possible loss of an aberrant but still active or assembled enzyme. A very low level of activity (less than 18 pmol of CO_2 fixed per min per mg of total protein) was detected in control preparations from cells with pL56 but lacking an SSU-expressing plasmid. This activity is most likely the residual activity from LSU octamers (2) and sets the lower limit of detectable activity by mutant enzymes at about 0.2% of wild-type activity. Oxygenase activity was not measured since partitioning between carboxylation and oxygenation appears to be a property determined solely by the LSUs (27). All four of the mutants were found to lack detectable carboxylase activity (Fig. 1).

Analysis of quaternary structure. To explore a possible reason for the lack of activity in these mutants, extracts were analyzed by sedimentation through sucrose density gradients to separate assembled holoenzyme from unassembled subunits. After centrifugation, no SSU was detectable for any of the mutants at the position in the gradient where 18S holoenzyme is normally found (Fig. 1). The sensitivity of our detection was such that levels of SSU greater than 2% of that in the nonsubstituted case would have been detected (data not shown). Low levels of SSU were sometimes detected at the top of the gradients (data not shown) and were reliably detected in crude pellets (Fig. 1) indicating mutant SSU was being synthesized as expected.

Correlation of Mutants with the Three-Dimensional Structure. To gain insight into the structural roles of the substituted residues and to try to understand how the substitutions made would interfere with formulation or stability of the correct quaternary structure, we examined residues 13S, 67S, 73S, and 98S and residues in their vicinities in a model for the three-dimensional structure of spinach RuBisCO. The amino acid sequences of the SSUs from *Anabaena* and spinach are 41% identical overall but are more similar in the regions of our substitutions (Fig. 2).

Glu-13S → Val. Glu-13S is in the interface region between the LSUs and the SSUs and is accessible to solvent in the free SSU but is buried in the $(LSU)_8(SSU)_8$ complex (Table 1). It is conserved in SSUs of known sequence (1, 7, 31) except

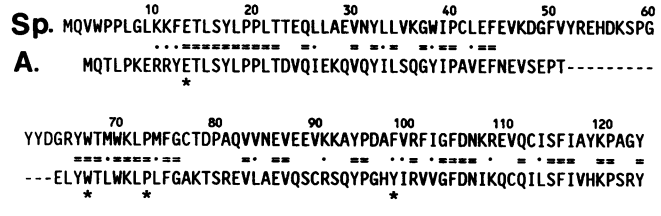


FIG. 2. Comparison of spinach (Sp.) (ref. 28, corrected in refs. 29 and 9) and *Anabaena* 7120 (A.) (30) RuBisCO SSU sequences. The positioning of the gap in the *Anabaena* sequence is based upon the three-dimensional structure of the spinach protein in which residues 52S–63S form a loop. Similar (·) and identical (=) residues in the two sequences are indicated. Residues that were mutated are marked with an asterisk.

those of the hydrogen bacterium *Alcaligenes eutrophus* (31) and the eukaryotic alga *Olisthodiscus luteus* (32), which are quite different in this region of the SSU. Glu-13S is close to Lys-164L (Fig. 3*C*) and there is an intersubunit salt bridge between them. The surrounding region is highly charged (Fig. 3*C*). There are two nearby internal salt bridges in LSU, Glu-234L–Arg-421L and Asp-198L–Arg-167L. These four LSU residues are conserved in all LSUs including those of the *Rhodospirillum rubrum* enzyme (33), in which there are no SSUs. In *R. rubrum* the residue corresponding to residue 164L is an arginine.

Removal of the negative charge on Glu-13S in the mutant by replacement with valine could decrease the attraction between LSU and SSU, and, if they were to assemble correctly, it would result in an unpaired positive charge of Lys-164L buried in the interface between them. In addition to decreased stability of the assembled enzyme, this unpaired charge might interfere with proper alignment of the other charges in this region. Assembly and activity are probably both quite sensitive to small disruptions in this region since it is at the junction of the C and N domains of the LSU (22).

Trp-67S → Arg. The conserved Trp-67S is in a quite polar environment near the interface between SSU and the two LSUs, subunits B and D (see Fig. 3*A* for designation of LSU). In this region, Glu-13S interacts with subunit B as discussed for the previous mutant, and Glu-43S forms a salt bridge to Arg-187L of subunit D (Fig. 3*D*), which is conserved except in *R. rubrum* (33). This location of Glu-43S on the surface of SSU, which is in contact with LSU in the holoenzyme, is reflected in the residue being less accessible in the holoenzyme than in free SSU (Table 1). Arg-100S is situated between Trp-67S and Glu-43S with the plane of the guanidinium group roughly parallel to the indole ring.

In the mutant, positive charge on the introduced arginine would repel Arg-100S, possibly forcing it closer to Glu-43S. This might result in a new intrasubunit salt bridge between the two residues. At the very least, the negative charge in the immediate environment of Glu-43S on the interfacing surface of SSU would be diminished and the intersubunit attraction leading to a salt bridge between Glu-43S and Arg-187L in the wild-type enzyme would be reduced. Alternatively, the presence of two positively charged arginines close together could cause one of them to form a salt bridge with Glu-13S, preventing this residue from bridging to Lys-164L of LSU subunit B. In either case, assembly would be negatively affected. Additionally, the substitution by an arginine side chain for the bulky tryptophan, which is almost completely buried (Table 1), would leave an internal cavity in the SSU that could destabilize local structure.

Pro-73S → His. The conserved residue Pro-73S is situated in a hydrophobic patch on the surface of SSU, which also includes conserved residues Leu-72S, Phe-75S, Phe-104S, and Met-69S (leucine in *Anabaena*) (Fig. 3*E*). This patch forms an area of hydrophobic interaction with LSU subunit C involving Leu-74L. This leucine residue is conserved in all LSUs except for LSUs from *R. rubrum* (33). Consistent with their forming an intersubunit hydrophobic pocket, the accessibility of all of these residues to solvent decreases upon association of SSU and LSU (Table 1).

Replacement of the proline side group by a polar histidine side chain would alter this pocket and make a more polar environment unsuitable for interaction with Leu-74L from the LSU. It is also possible that the proline residue is important for determining the local conformation of this region of the polypeptide chain. A search through a data base of refined structures (23) showed that the majority of protein segments with similar conformation had a proline residue either at, or adjacent to, this position.

Tyr-98S → Asn. Residue 98S is a phenylalanine in spinach and is in the middle of an internal hydrophobic core in SSU and does not interact directly with any LSU residue (Fig. 3*F*). In addition to residue 98S this core is comprised of Phe-12S (tyrosine in *Anabaena*), Pro-120S, Tyr-123S, and the β- and γ-carbons of Lys-119S.

Phe-12S and the nearby residue Phe-50S contribute to interactions with LSU subunit B. The main chain atoms of Phe-12S and the β- and γ-carbons of Phe-50S are near (within 5 Å) the main chain atoms of residues 232L–234L (Fig. 3*F*). Accordingly, the accessibility of residues 12S and 50S decreases upon association of subunits (Table 1). Introduction of an asparagine at position 98S would increase the polarity of the region and could alter the core such that the subunit interactions involving residues 12S and 50S are adversely affected.

In SSU from *Silene pratensis*, residues 98S and 50S are histidines (34) and, therefore, have polar side chains in this core. Compared to other organisms, however, the polypeptide chain of SSU from this organism is shorter, and residue 123S is absent. The effect of this evolutionary change, as modeled by removal of Tyr-123S in the spinach structure, would be to open up the core and make it accessible to solvent. It is, therefore, logical that, in concert with the shorter chain, some of the hydrophobic residues in this region would have evolved into more polar residues in this organism.

Table 1. Solvent accessibility of selected residues in model for three-dimensional structure of spinach RuBisCO

Residue	% accessible: Monomer in isolation	% accessible: Holoenzyme	% decrease due to intersubunit contact
Phe-12S	16.1	4.6	11.5
Glu-13S	62.3	19.1	43.2
Glu-43S	14.8	3.3	11.5
Phe-50S	33.9	21.1	12.8
Trp-67S	11.2	4.6	6.6
Met-69S	40.2	10.8	29.4
Leu-72S	69.4	24.4	45.0
Pro-73S	20.3	4.2	16.1
Phe-75S	80.3	54.1	26.2
Phe-98S	1.8	1.8	0.0
Phe-104S	23.9	4.1	19.8
Lys-119S	33.6	33.6	0.0
Pro-120S	23.8	23.8	0.0
Tyr-123S	49.8	49.8	0.0
Leu-74L	58.3	8.9	49.4

Accessible surface area was calculated for individual residues in the model for the three-dimensional structure of spinach RuBisCO and in the context of the appropriate SSU or LSU monomer alone, using the program of Lee and Richards (24), and is expressed as a percent of the accessible surface area of a tripeptide containing the amino acid found at that position.

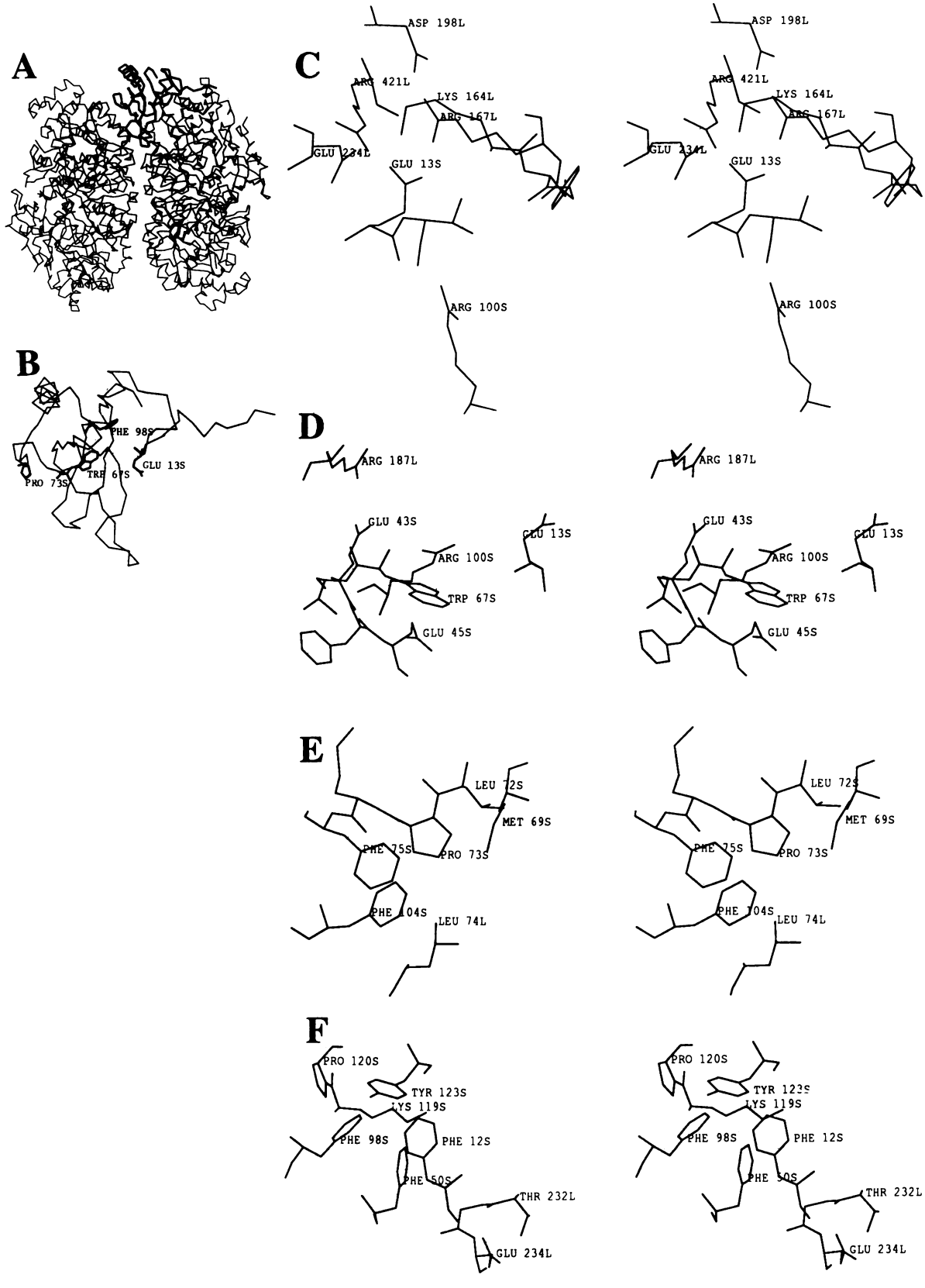


FIG. 3. Drawings from the model for three-dimensional structure of spinach RuBisCO. (*A*) SSU (bold lines) shown in relation to four LSUs. The intact holoenzyme is cube-shaped with the main body of the cube formed by four LSU dimers (two dimers are shown here). The SSUs are wedged between the ends of the dimers forming clusters of four each on the top and the bottom of the cube. The LSU dimer shown on the left is formed by LSU subunits C and D and the dimer shown on the right is formed by LSU subunits A and B. (*B*) SSU in roughly the same orientation as in *A* showing the location of residues 13S, 67S, 73S, and 98S. All nonhydrogen atoms are shown for these residues. Serial α-carbons are shown and connected for all other residues. (*C–F*) Stereo drawings of residues in the vicinities of residues 13S (*C*), 67S (*D*), 73S (*E*), and 98S (*F*).

Conformation and Assembly. The preceding discussions have as a premise the near-normal folding of the mutant SSUs. In the experimental system used, it could not be determined whether the substituted proteins differed in overall structure or in stability or, if there was a difference, whether it was a cause or a consequence of the observed failure to assemble into holoenzyme. To address the possibility that one or more of the substitutions might cause misfolding of the protein resulting in a structure grossly different from the wild-type SSU, the algorithm of Chou and Fasman (25) was used to predict secondary structures in the wild-type protein and in each of the substituted proteins. The predictions for the substituted proteins were not significantly different from the predictions for the wild-type protein, and the structures predicted corresponded well to the structures observed in the spinach protein (results not shown). This analysis suggests that the observed lack of assembly was due to disruption of specific interactions rather than gross conformational changes.

Assembly of cyanobacterial RuBisCO in *E. coli* requires the *E. coli groEL* and *groES* gene products (35). GroEL protein shows extensive sequence similarity to the RuBisCO binding protein, which is found in plant chloroplasts and which is probably involved in assembly of RuBisCO in green plants (36). Although it is possible that the substitutions described in this work prevent holoenzyme assembly by interfering with a necessary (but undemonstrated) interaction of the *E. coli* GroEL or GroES with the SSU, we consider this interpretation to be unlikely in light of the ability of isolated SSU to assemble *in vitro* with preassembled LSU octamers.

Recent experiments have identified a region of higher plant SSU that is important for assembly in higher plant RuBisCO (37). This region that includes residues 49S–54S of the spinach protein is missing in the SSU from cyanobacteria.

Other Mutations. Other previously reported SSU mutants have all been shown to have catalytic activity, and, by implication, to form a stable holoenzyme. A Trp-67S → Phe substitution in *Anacystis nidulans rbcS* (ref. 7, referred to as Trp-54) was shown to reduce V_{max} 2.5-fold without altering either the K_m or the partition coefficient (relative substrate specificity for CO_2 and O_2) of the enzyme. This result is in contrast to our result for an arginine substitution at position 67S but fits well with our explanation that it is the additional positive charge introduced by the arginine substitution, rather than the removal of the tryptophan, *per se*, which prevented assembly. Altered interaction with the LSU, despite ability to assemble on a gross level, could lead to the reduction in V_{max} seen in the phenylalanine-substituted enzyme. Trp-70S was also changed to phenylalanine (ref. 7, referred to as Trp-57) with results very similar to those found for Trp-67S → Phe. Mutations resulting in pairs of substitutions in amino acids 10S, 11S, 14S, 16S, 19S, and 20S were shown to lead to reduced activity in crude preparations (8) but the amount of holoenzyme was not quantified. It is possible then that these mutations result in an enzyme of reduced activity that, as with Trp-70S → Phe and Trp-67S → Phe, could be due to deleteriously altered LSU–SSU interactions.

In the work presented here, we have identified specific residues widely separated in the primary sequence that play structurally important roles in the SSU and its interaction with the LSUs. Disruption of the intrasubunit and intersubunit interactions of these and neighboring residues by amino acid substitutions can completely prevent formation of stable holoenzyme. To further understand assembly as well as stability of the holoenzyme, similar analyses of a larger number of mutations should be done.

This work was supported in part by grants from the McKnight Foundation, the Department of Agriculture Competitive Research Grants Program (83-CRCR-1-1290), and the Department of Energy (AC02-76ER01338).

1. Miziorko, H. M. & Lorimer, G. H. (1983) *Annu. Rev. Biochem.* **52,** 507–535.
2. Andrews, T. J. (1988) *J. Biol. Chem.* **263,** 12213–12219.
3. Gatenby, A. A., van der Vies, S. M. & Bradley, D. (1985) *Nature (London)* **314,** 617–620.
4. Christeller, J. T., Terzaghi, B. E., Hill, D. F. & Laing, W. A. (1985) *Plant Mol. Biol.* **5,** 257–263.
5. Tabita, F. R. & Small, C. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6100–6103.
6. Gurevitz, M., Somerville, C. R. & McIntosh, L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6546–6550.
7. Voordouw, G., de Vries, P. A., van den Berg, W. A. M. & de Clerck, E. P. J. (1987) *Eur. J. Biochem.* **163,** 591–598.
8. McFadden, B. A. & Small, C. L. (1988) *Photosynthesis Res.* **18,** 245–260.
9. Knight, S., Andersson, I. & Branden, C. I. (1989) *Science* **244,** 702–705.
10. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
11. Herrero, A., Elhai, J., Hohn, B. & Wolk, C. P. (1984) *J. Bacteriol.* **160,** 781–784.
12. Myers, R. M., Lerman, L. S. & Maniatis, T. (1985) *Science* **229,** 242–247.
13. Yanisch-Perron, C., Vieira, J. & Messing, J. (1985) *Gene* **33,** 103–119.
14. Vieira, J. & Messing, J. (1987) *Methods Enzymol.* **153,** 3–11.
15. Nakamaye, K. L. & Eckstein, F. (1986) *Nucleic Acids Res.* **14,** 9679–9698.
16. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
17. Elthon, T. E. & McIntosh, L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8399–8403.
18. Larson, E., Howlett, B. & Jagendorf, A. (1986) *Anal. Biochem.* **155,** 243–248.
19. Berhow, M. A., Saluja, A. & McFadden, B. A. (1982) *Plant Sci. Lett.* **27,** 51–57.
20. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
21. Harlow, E. & Lane, D. (1988) *Antibodies* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
22. Andersson, I., Knight, S., Schneider, G., Lindqvist, Y., Lundqvist, T., Branden, C. I. & Lorimer, G. H. (1989) *Nature (London)* **337,** 229–234.
23. Jones, T. A. & Thirup, S. (1986) *EMBO J.* **5,** 819–822.
24. Lee, B. & Richards, F. M. (1971) *J. Mol. Biol.* **55,** 379–400.
25. Chou, P. Y. & Fasman, G. D. (1978) *Adv. Enzymol.* **47,** 45–148.
26. Mulligan, R. M., Houtz, R. L. & Tolbert, N. E. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1513–1517.
27. Andrews, T. J. & Lorimer, G. H. (1985) *J. Biol. Chem.* **260,** 4632–4636.
28. Martin, P. G. (1979) *Aust. J. Plant Physiol.* **6,** 401–408.
29. Takruri, I. A. H., Boulter, D. & Ellis, R. J. (1981) *Phytochemistry* **20,** 413–415.
30. Nierzwicki-Bauer, S. A., Curtis, S. E. & Haselkorn, R. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5961–5965.
31. Andersen, K. & Caton, J. (1987) *J. Bacteriol.* **169,** 4547–4558.
32. Boczar, B. A., Delany, T. P. & Cattolico, R. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4996–4999.
33. Nargang, F., McIntosh, L. & Somerville, C. (1984) *Mol. Gen. Genet.* **193,** 220–224.
34. Smeekens, S., van Oosten, J., de Groot, M. & Weisbeck, P. (1986) *Plant Mol. Biol.* **7,** 433–440.
35. Goloubinoff, P., Gatenby, A. A. & Lorimer, G. H. (1989) *Nature (London)* **337,** 44–47.
36. Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W. & Ellis, R. J. (1988) *Nature (London)* **333,** 330–334.
37. Wasmann, C. C., Ramage, R. T., Bohnert, H. J. & Ostrem, J. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1198–1202.