Mutations in the Small Subunit of Ribulosebisphosphate Carboxylase Affect Subunit Binding and Catalysis

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ABSTRACT: Fully functional *Synechococcus* PCC 6301 ribulose 1,5-bisphosphate carboxylase–oxygenase (k_{cat} = 11.8 s^{-1}) was assembled in vitro following separate expression of the large- and small-subunit genes in different *Escherichia coli* cultures. The small subunits were expressed predominantly as monomers, in contrast to the large subunits which have been shown to be largely octameric when expressed separately [Andrews, T. J. (1988) *J. Biol. Chem. 263*, 12213–12219]. This separate expression system was applied to the study of mutations in the amino-terminal arm of the small subunit, which is one of the major sites of contact with the large subunit in the assembled hexadecamer. It enabled the effects of a mutation on the tightness of binding of the small subunit to the large-subunit octamer to be distinguished from the effects of the same mutation on catalysis carried out by the assembled complex when fully saturated with mutant small subunits. This important distinction cannot be made when both subunits are expressed together in the same cell. Substitutions of conserved amino acid residues at positions 14 (Ala, Val, Gly, or Asp instead of Thr) and 17 (Cys instead of Tyr), which make important contacts with conserved large-subunit residues, were introduced by site-directed mutagenesis. All mutant small subunits were able to bind to large subunits and form active enzymes. A potential intersubunit hydrogen bond involving the Thr-14 hydroxyl group is shown to be unimportant. However, the binding of Gly-14, Asp-14, and Cys-17 mutant small subunits was weaker, and the resultant mutant enzymes had reduced catalytic rates compared to the wild type. All mutant enzymes had similar substrate affinities to the wild-type enzyme, except for the Gly-14 mutant enzyme, which had a 5-fold reduction in K_M(ribulose-P_2). Apparently, disruptions of intersubunit interactions at this highly conserved contact site have rather limited consequences.

Ribulose-P_2 carboxylase[1] (EC 4.1.1.39) catalyzes both the carboxylation and the oxygenation of ribulose-P_2, the initial reactions of the competing photosynthetic carbon reduction and photorespiratory cycles. In all eukaryotes and most prokaryotes, this enzyme is composed of eight large (53-kDa) and eight small (12–18-kDa) subunits (L_8S_8) (Andrews & Lorimer, 1987). Crystallographic structural studies (Chapman et al., 1988; Andersson et al., 1989; Knight et al., 1990) showed that a tetramer of large-subunit dimers [$(L_2)_4 = L_8$] forms the core of the molecule. The small subunits are arranged as tetramers (S_4) at the two poles of the molecule. Chemical cross-linking and site-directed mutagenesis studies demonstrated that the active site of the enzyme occurs at an interface between large subunits in the large-subunit dimer with residues of an α/β barrel domain of one large subunit and an N-terminal domain of its companion large subunit both contributing to the active site (Larimer et al., 1987; Lee et al., 1987). Thus, there are two active sites per dimer. The small subunits are remote from the active sites. Nevertheless, they are important in promoting catalytic competence. In their absence, the catalytic activity of the L_8 core is only 1% of that of the fully assembled L_8S_8 form (Andrews, 1988). However, the nature of the interactions between the large and small subunits responsible for this catalytic enhancement is unclear. Unraveling the mechanism(s) by which the small subunits enhance the catalytic competence of the L_8 core will contribute significantly to our understanding of this complex enzyme.

The genes for the large and small subunits of ribulose-P_2 carboxylase in higher plants are encoded on different genomes and do not direct the synthesis of properly folded and assembled subunits when expressed in *Escherichia coli* (Gatenby et al., 1987). By contrast, the *rbc*L and *rbc*S genes of cyanobacteria (e.g., *Synechococcus* PCC 6301) constitute a single operon and direct the synthesis of fully assembled and active enzyme when expressed in *E. coli* (Tabita & Small, 1985; Gatenby et al., 1985; Terzaghi et al., 1986). Various laboratories have attempted to use site-directed mutagenesis of the cyanobacterial ribulose-P_2 carboxylase, expressed in *E. coli*, to identify functional residues in the small subunit. Mutant small subunits, E13V,[2] W67(55)R,[3] P73(61)H, and Y98(86)N, which produced inactive holoenzymes when dually expressed in *E. coli* (i.e., *rbc*L and *rbc*S were carried on different plasmids but expressed in the same cell), have been reported by Fitchen et al. (1990). Using the coexpression system (i.e., *rbc*L and *rbc*S were carried on the same plasmid), small-subunit mutants with single-residue substitutions, W67(55)F, W70(58)F (Voordouw et al., 1987; referred to as W54F, W57F by these authors), L21E (Lee et al., 1991), and double-residue substitutions, P19A-P20A, S16A-Y17D,

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[1] Abbreviations: ribulose-P_2 carboxylase, D-ribulose 1,5-bisphosphate carboxylase–oxygenase; carboxypentitol-P_2, unresolved isomeric mixture of 2′-carboxy-D-arabinitol 1,5-bisphosphate and 2′-carboxy-D-ribitol 1,5-bisphosphate; PMSF, phenylmethanesulfonyl fluoride; Hepps, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

[2] The shorthand notation used for specifying a small subunit with an amino acid residue substitution uses the sequence number preceded by the single-letter representation for the wild-type residue at this position and followed by the single-letter representation for the replacement. Thus T14A represents that mutant small subunit where threonine-14 has been replaced by alanine.

[3] Where the sequence numbers for the spinach and *Synechococcus* differ, the spinach number is given followed by the number for the corresponding *Synechococcus* residue in parentheses [see Andrews & Lorimer (1987) for an alignment].

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T14A-S16A, R10G-R11G (McFadden & Small, 1988), have also been reported. In each case, the catalytic activity of the mutant holoenzymes in *E. coli* extracts was greatly reduced. However, the use of the dual-expression or coexpression systems made it impossible to determine if the changes introduced in the small subunits perturbed the binding of small subunits to large subunits, or if there was a direct effect on catalysis, or both. If the change caused a weakening in the binding of the small subunits to the large-subunit octamer, or decreased the stability or expression level of the small subunits, then the assembled enzyme may not have had a full complement of eight small subunits. This would, by itself, reduce the activity (Andrews & Ballment, 1983). Here we report the expression of functional small subunits of the cyanobacterial ribulose-P_2 carboxylase in *E. coli* in the absence of large subunits. This has also been reported recently by Lee and Tabita (1990). Reconstitution of the holoenzyme in vitro allows assembly to be forced even when the binding affinity between small and large subunits is reduced and provides a method for estimating the binding affinity. This methodology allows the relationship between small-subunit binding and catalytic enhancement to be investigated.

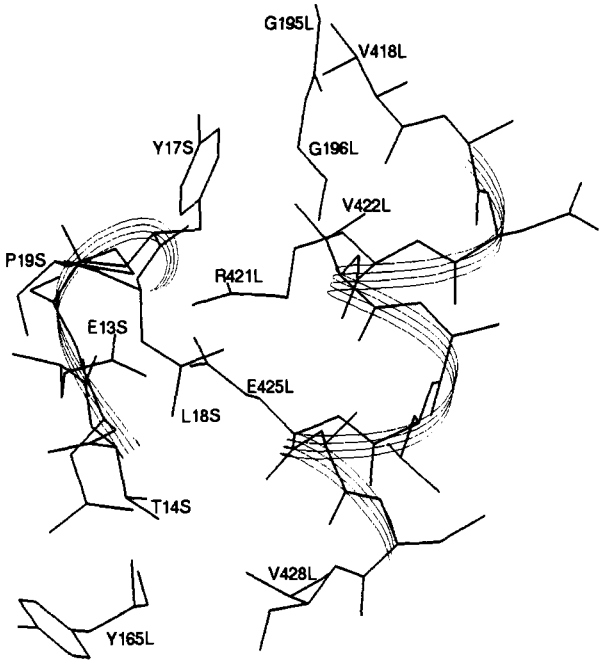
Crystallographic studies on the spinach ribulose-P_2 carboxylase (Andersson et al., 1989; Knight et al., 1990) have shown that the N-terminal arm of the small subunit (residues 1–21) interacts extensively with helix α8 of the α/β barrel domain of the large subunit. [We follow the nomenclature of Knight et al. (1990) for identifying the secondary structure elements.] Helix α8 is an important region of the α/β barrel since its position affects the orientation of loop 8 (the loop which joins the carboxyl end of strand β8 with the amino end of helix α8) which provides part of the binding site for the phosphate group attached to C-1 of the substrate or reaction intermediate. Comparison of the structure of spinach ribulose-P_2 carboxylase with that of the enzyme from *Rhodospirillum rubrum*, which is a dimer of large subunits lacking small subunits, shows that a major difference in the conformation of the large subunits between the two structures is the position of helix α8 (Schneider et al., 1990). Interactions between helix α8 and the small subunit, which are largely confined to the N-terminal arm of the small subunit, appear to alter the positions of helix α8 and loop 8 and may contribute to the observed differences in catalytic parameters between L_2 and L_8S_8 ribulose-P_2 carboxylases. The lack of these interactions may also be a cause of the 100-fold reduction in activity and concomitant weakening of substrate affinities which accompanies removal of the small subunits from the L_8S_8 enzyme (Andrews, 1988). Mutation of small-subunit residues which participate in these interactions may lead to a better understanding of the manner in which these interactions influence catalysis.

Within the N-terminal arm of the small subunit, interactions with helix α8 are concentrated in the region between residues 13 and 20, which is one of the most highly conserved regions of the small-subunit sequence (Figure 1). Such strong conservation reinforces the suspicion that this large-subunit contact site may be functionally important. Of these residues, Glu-13, Thr-14, and Tyr-17 of the spinach small subunit show the greatest decrease in accessibility to solvent on forming the small subunit–large subunit complex, becoming buried within the molecule (Knight et al., 1990). Substitution of Glu-13 of the small subunits of ribulose-P_2 carboxylase from *Anabaena* 7120 with Val resulted in small subunits which did not assemble significantly with *Anabaena* large subunits when dually expressed in *E. coli* (Fitchen et al., 1990). The use of the dual-expression system prevented further characterization of this mutation. We chose Thr-14 and Tyr-17 of the small subunit of *Synechococcus* ribulose-P_2 carboxylase as targets for mutagenesis. The three-dimensional structure of *Synechococcus* ribulose-P_2 carboxylase has not yet been reported. However, the primary structure of this enzyme resembles that of the plant enzymes very closely (Shinozaki & Suguira, 1983), and the subunit structures are similar enough that catalytically active subunit hybrids may be constructed between the *Synechococcus* and spinach enzymes (Andrews & Lorimer, 1985; Andrews, 1988). Therefore, the structure of the spinach enzyme is likely to provide quite an accurate guide to the structure of its cyanobacterial homologue, particularly in the highly conserved regions of intersubunit contact. Part of the structure of spinach ribulose-P_2 carboxylase (Knight et al., 1990) showing the interactions of Thr-14 and Tyr-17 of the small-subunit with large-subunit residues is represented in Chart I. The hydroxyl oxygen of Thr-14 is positioned appropriately with respect to the carbonyl oxygen of the conserved large-subunit residue, Tyr-165(162), to form a hydrogen bond (Knight et al., 1990). It also interacts with the hydrophobic part of the side chain of Arg-167(164) (not shown in Chart I), which is also conserved. Both of these residues are located in the loop of the large subunit between helix αE and strand β1 which closes the mouth of the α/β barrel on the opposite side to the active site. The hydroxyl oxygen of the side chain of Tyr-17 of the small subunit interacts with Gly-196(193), the aromatic portion interacts with the hydrophobic part of the side chain of Arg-421(418) (α8), and the β-carbon is close to a carboxyl oxygen of Glu-425(422) (α8). All three of these residues are conserved in all known large-subunit sequences of L_8S_8 ribulose-P_2 carboxylases (Knight et al., 1990). Gly-196(193) occurs in a totally conserved loop at the non-active-site side of the barrel connecting helix α1 with strand β2. The position of this loop may affect the orientation of the sequence Lys-201(198)–Asp-203(200) at the other end of strand β2 which binds the catalytically essential divalent metal.

	10									20
spinach	K	K	F	E	T	L	S	Y	L	P	P
soybean	.	.	.	.	.	.	.	.	.	.	D
tobacco	.	.	Y	.	.	.	.	.	.	.	D
wheat	.	.	.	.	.	.	.	.	.	.	.
Lemna gibba	.	.	.	.	.	.	.	.	F	.	L
Euglena gracilis	.	F	W	.	.	F	.	.	.	.	.
Chlamydomonas reinhardtii	.	M	.	.	.	F	.	.	.	.	.
Acetabularia mediterranea	.	M	.	.	.	F	.	F	.	.	.
Cyanophora paradoxa	R	.	.	.	.	F	.	.	.	.	.
Synechococcus PCC 6301	R	R	.	.	.	F	.	.	.	.	.
Anabaena PCC 7120	R	R	Y	.	.	.	.	.	.	.	.
Chromatium vinosum	R	.	.	.	.	F	.	.	.	.	A
Alcaligenes eutrophus	I	T	Q	G	.	F	.	F	.	.	E
Rhodobacter sphaeroides (form I)	I	T	Q	G	C	F	.	F	.	.	D
Fucus vesiculosus	L	T	Q	G	S	F	.	F	.	.	D
Cryptomonas Φ	L	T	Q	G	A	F	.	F	.	.	D
Porphyridium aerugineum	L	T	Q	G	.	F	.	F	.	.	D

FIGURE 1: Comparison of amino acid residues 10–20 in the N-terminal arm of the small subunits of ribulose-P_2 carboxylases from a variety of species. Residues identical to the spinach residue at that position are indicated by dots. The sequences shown are from the following sources: spinach, Martin (1979); soybean, Berry-Lowe et al. (1982); tobacco, Mazur and Chui (1985); wheat, Broglie et al. (1983); *Lemna gibba*, Stiekema et al. (1983); *Euglena gracilis*, Sailland et al. (1986); *Chlamydomonas reinhardtii*, Goldschmidt-Clermon and Rahire (1986); *Acetabularia mediterranea*, Schneider et al. (1989); *Cyanophora paradoxa*, Starnes et al. (1985); *Synechococcus* PCC 6301, Shinozaki and Sugiura (1983); *Anabaena* PCC 7120, Nierzwicki-Bauer et al. (1984); *Chromatium vinosum*, Viale et al. (1989); *Alcaligenes eutrophus*, Andersen and Caton (1987); *Rhodobacter sphaeroides* (form I), Wagner et al. (1988); *Fucus vesiculosus*, Keen et al. (1988); *Cryptomonas* Φ, Douglas and Durnford (1989); *Porphyridium aerugineum*, Valentin and Zetsche (1989).

Chart I: Interactions between the Large and Small Subunits in the Vicinity of the Small-Subunit Residues, Thr-14 and Tyr-17[a]



[a] The diagram is based on the crystallographic coordinates of activated spinach ribulose-P_2 carboxylase with 2'-carboxy-D-arabinitol 1,5-bisphosphate bound (Knight et al., 1990), kindly supplied by Professor C.-I. Brändén. The conserved region of the small subunit near the amino terminus (residues 13–19) is traced by the left ribbon. The right ribbon traces part of helix α8 of the large subunit. The positions of the large-subunit residues, Tyr-165, Gly-195, and Gly-196, are also shown. (For the large-subunit residues shown here, the *Synechococcus* numbers are three less than the spinach numbers shown.) The suffixes, L and S, of the labels refer to large- and small-subunit residues, respectively.

In this paper, we report the effects of substitutions at residues 14 and 17 of the small subunit of *Synechococcus* ribulose-P_2 carboxylase. By exploiting the separate expression of large and small subunits, followed by reconstitution in vitro, we separate the effects of the substitutions on the binding affinity of the small subunits from the effects on catalysis by the fully assembled complex.

Experimental Procedures

Materials. Ribulose-P_2 was synthesized according to Horecker et al. (1958) without exposure to alkaline pH and purified by Dowex 1-X8-Cl^- chromatography (Edmondson et al., 1990). [^{14}C]Carboxypentitol-P_2 was synthesized as previously described (Collatz et al., 1979). Radioisotopes ([α-^{35}S]dATP and $NaH^{14}CO_3$) were obtained from Amersham. PMSF and pepstatin A were from Sigma Chemical Co., leupeptin was from Calbiochem, and immunoblotting reagents were from Bio-Rad. *E. coli* strain JM101 (Messing, 1983) was used for mutagenesis experiments. *E. coli* strains HB101 (Boyer & Roulland-Dussoix, 1969) or CAG630 [*lac*Z(am), *trp*(am), *pho*(am), *sup*C^{ts}, *rps*L, *mal*(am), *rpoH165-Tn10*] (kindly provided by Dr. Carol A. Gross) were used for expression of wild-type and mutant small subunits. *Synechococcus* PCC 6301 ribulose-P_2 carboxylase was purified by the method of Andrews and Ballment (1983) from extracts of *E. coli* coexpressing the *Synechococcus rbc*L and *rbc*S genes on plasmid pSH1 (Andrews, 1988). *R. rubrum* ribulose-P_2 carboxylase was purified from extracts of *E. coli* expressing the *R. rubrum rbc*L gene on plasmid pRR1 (Morell et al., 1990) by a modification of the method of Schloss et al. (1982). To raise antibodies against the small subunit of *Synechococcus*

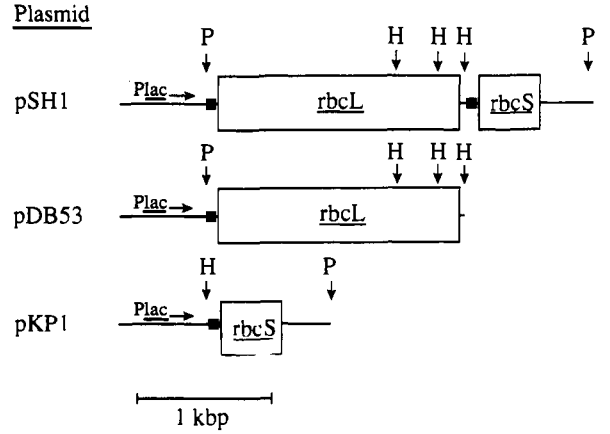


FIGURE 2: Expression vectors for *Synechococcus* PCC 6301 ribulose-P_2 carboxylase. Plasmid pSH1 (Andrews, 1988) contains a 2.2-kilobase *Pst*I fragment of the *Synechococcus* PCC 6301 genome which encodes both *rbc*L and *rbc*S genes. Plasmid pDB53 (Gatenby et al., 1985) contains a 1.7-kilobase *Pst*I/*Hin*dIII fragment harboring the *rbc*L gene alone. Plasmid pKP1, constructed in this study, contains a 663 bp *Hin*dIII/*Pst*I fragment encoding the *rbc*S gene alone. H, *Hin*dIII; P, *Pst*I; ■, *Synechococcus* ribosome binding site, P*lac*, β-galactosidase promoter region.

ribulose-P_2 carboxylase, the coding region of *rbc*S was inserted into the *Sma*I site of plasmid pGEX-2T (Smith & Johnson, 1988) which directed the synthesis of the small subunit fused to the glutathione-binding domain of glutathione *S*-transferase. The fusion protein was purified by absorption on glutathione–agarose as described by Smith and Johnson (1988). Antibodies against this purified fusion protein were then raised in rabbits.

Plasmid Construction. Plasmid pSH1 (Andrews, 1988), a derivative of pUC18, was digested to completion with *Hin*dIII and *Pst*I. The resulting 663 bp fragment containing the ribosome binding site and coding region of the *rbc*S gene of *Synechococcus* PCC 6301 was subcloned into the *Hin*dIII and *Pst*I sites of pTZ19R, downstream from the *lac*Z promoter. This plasmid was designated pKP1 (Figure 2). pKP1 expresses *rbc*S, but the gene is not in frame with the *lac*Z gene; therefore, fusions of β-galactosidase and the small subunit were not produced. Plasmid pDB53 (Figure 2), harboring *rbc*L alone (Gatenby et al., 1985), was a gift from Dr. S. van der Vies.

Oligonucleotide-Directed Mutagenesis. Mutations were introduced into the *Synechococcus rbc*S gene by the method of Taylor et al. (1985) using the oligonucleotide-directed in vitro mutagenesis kit supplied by Amersham. Oligonucleotides (5'GCGTCGTTTCGAGGNTTCTCGT3') and (5'TTTCTCGTNCCTGCCTCCCCTC3'), synthesized on a Pharmacia Gene Assembler Plus, were used as primers. An equimolar mixture of G, A, T, and C was incorporated into the oligonucleotides at the positions marked N. Altered *rbc*S genes were identified by DNA sequencing using the dideoxy termination method (Sanger et al., 1977). The entire coding region was sequenced in each case, confirming that only the directed sequence changes had occurred. Mutants containing codons GCT (Ala), GTT (Val), GGT (Gly), or GAT (Asp) in place of the wild-type codon ACT (Thr) at position 14 were obtained. In the case of residue 17, all of the mutations recovered contained the codon TGC (Cys), replacing the wild-type codon TAC (Tyr).

Growth and Extraction of E. coli Cultures. The growth of plasmid-bearing *E. coli* HB101 and CAG630 cells and preparation of extracts were carried out as previously described (Andrews, 1988) except that glycerol and isopropyl β-D-thiogalactopyranoside were omitted from the growth medium.

In addition to PMSF (2 mM), leupeptin (10 μM) and pepstatin A (22 μM) were also added to the extraction buffer immediately before sonication. The crude extract was clarified of cell debris by ultracentrifugation (100000*g*, 30 min, 4 °C) and stored at 4 °C.

Partial Purification of Wild-Type Small Subunits Expressed in E. coli HB101. All procedures were carried out at 4 °C. The crude extract (6 mL, derived from 2 g of cells) was passed through a Sephadex G-100 (coarse) column (40 × 3.2 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4), containing 1 mM EDTA. Fractions containing small subunits were pooled and loaded onto a Pharmacia Mono-Q HR 10/10 column equilibrated with the same buffer. The column was washed with 70 mL of the buffer and eluted with a 320-mL linear 0–0.5 KCl gradient in buffer. Fractions containing small subunits were detected by their ability to stimulate the carboxylase activity of large subunits (see later), pooled, and stored frozen at −20 °C.

Estimation of Molecular Size. The partially purified wild-type small subunits from above were subjected to gel permeation chromatography on a Pharmacia Superose-12 HR 10.30 column, equilibrated at 4 °C with 50 mM potassium phosphate buffer (pH 7.3), containing 1 mM EDTA (flow rate 0.5 ml·min^{-1}). The column was calibrated with the following proteins, which were detected by their absorbance at 280 nm: cytochrome *c* (horse heart, 12400 Da), soybean trypsin inhibitor (20100 Da), carbonic anhydrase (bovine erythrocytes, 30000 Da), bovine serum albumin (67000 Da), and purified *R. rubrum* ribulose-P_2 carboxylase (110000 Da). The calibration plot of log (molecular mass) vs elution volume was linear. The elution of small subunits from the column was detected by enzyme assay (see later).

SDS–Polyacrylamide Gel Electrophoresis and Immunoblotting. Samples (2 μg of protein) were dissociated with SDS and fractionated on a 0.45 mm thick, 20% (w/v) acrylamide gel using the Pharmacia PhastSystem. The buffer system in the gel, supplied by Pharmacia, was 0.112 M acetate and 0.112 M Tris, pH 6.5. The gel was run with PhastGel SDS buffer strips which contain 0.2 M tricine, 0.2 M Tris, and 0.55% (w/v) SDS, pH 8.1. The fractionated proteins were blotted onto nitrocellulose using the LKB Novablot electrophoretic transfer kit. The membrane was blocked with 1% (w/v) bovine serum albumin and incubated with rabbit antiserum raised against the purified glutathione *S*-transferase/small subunit fusion protein. Background reactivity of this antiserum toward *E. coli* proteins was suppressed by pretreating the antiserum with an extract of *E. coli* CAG630 cells which harbored plasmid pTZ19R. Immunoreactive bands were visualized using goat anti-rabbit antibodies conjugated to alkaline phosphatase.

Binding of [^{14}C]Carboxypentitol-P_2. The concentration of ribulose-P_2 carboxylase active sites in extracts containing large subunits was determined by measuring the irreversible binding of the reaction-intermediate analogue carboxypentitol-P_2 (Miziorko, 1979; Pierce et al., 1980), as described by Andrews (1988). No increase in carboxypentitol-P_2 binding was observed when the large subunits were pretreated with a saturating level of small subunits.

Carboxylase Assay. Carboxylase activity was measured at pH 7.8 as previously described (Andrews, 1988) with 1 mg·mL^{-1} bovine serum albumin included in the assay solution. Small subunits were detected by the stimulation they induced in the activity of large subunits. An aliquot of extract or column eluate was added to an assay solution which also contained a fixed volume of large-subunit extract. The usual preincubation and assay procedure was then carried out (Andrews, 1988). The trace of intrinsic activity due to the large subunits alone was measured in controls lacking small subunits and subtracted. In these assays, the molar ratio of small subunits to large subunits was never more than 0.5. This was verified by showing that the activity was never more than half of that obtained when an excess of small subunits was present. In this substoichiometric region, the activity is a nearlinear function of the small-subunit concentration (see Figure 3). When determining the binding affinity of small subunits and the maximum activity that they stimulated at saturation (Figure 3), assay mixtures contained a constant concentration of large subunits (determined by [^{14}C]-carboxypentitol-P_2 binding) and varying amounts of small subunit extract. The data for the activities observed were fitted to the equation:

$$v = k_{cat}\{L + (xS) + K_D - [L + (xS) + K_D]^2 - 4L(xS)\}^{1/2}/(2L) \quad (1)$$

where v is the turnover rate (s^{-1}) at a particular concentration of small subunits, L is the concentration of large subunits in the assay (nM), and x is the volume (mL) of small-subunit extract per milliliter of assay mixture. This allowed estimation of S, the concentration of small subunits in the small-subunit extract (nM), K_D, the binding constant for small subunits (nM), and k_{cat}, the maximum turnover rate (s^{-1}) when the large subunits are saturated by small subunits. This equation is a variant of that used by Andrews and Ballment (1983).

Substrate affinities were measured in assays containing saturating levels of small subunits. When ribulose-P_2 was varied, the concentration of $NaHCO_3$ was fixed at 25 mM. When CO_2 concentration was varied, two alterations were introduced into the assay procedure. First, O_2-free conditions were maintained by exhaustive sparging of the assay buffer solution with N_2 and the assays were carried out in septum-capped reaction vials under a N_2 atmosphere. Second, the

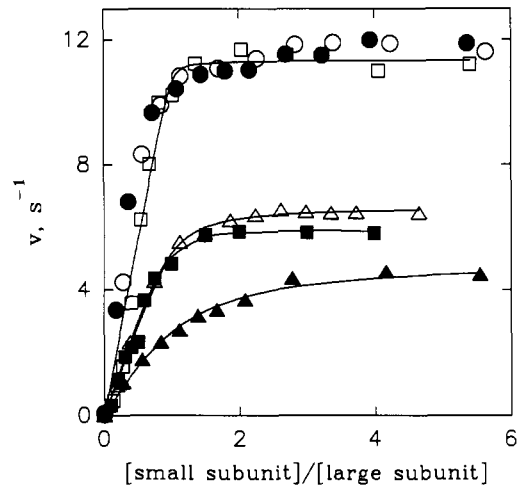


FIGURE 3: Activation of *E. coli* HB101 expressed large subunits of ribulose-P_2 carboxylase by wild-type and mutant small subunits expressed separately in *E. coli* CAG630. A fixed concentration of large subunits (0.4–1.0 nM as measured by [^{14}C]carboxypentitol-P_2 binding) was activated in the presence of increasing amounts of extracts containing either wild-type or mutant small subunits and assayed for carboxylase activity (see Experimental Procedures). The data were fitted to eq 1 as described under Experimental Procedures in order to estimate K_D, k_{cat}, and the concentration of small subunits in the extracts. These estimates are reported in Table I. Using the estimated concentration of small subunits, the data and the fitted lines were then replotted with the ratio between the small-subunit and large-subunit concentrations in the assay solution as the abscissa. The small subunit types are as follows wild type, ○; T14A, ●; T14V, □; T14G, ■; T14D, ▲; and Y17C, △.

Table I: Binding Data for Wild-Type and Mutant Small Subunits and Kinetic Data for Holoenzymes Assembled with These Small Subunits[a]

small subunit	K_D (pM)	k_{cat} (s^{-1})	$K_M(CO_2)$ (μM)	K_M(ribulose-P_2) (μM)	expression level (%)
wild type	1 ± 5	11.8 ± 0.1	251 ± 32	17.2 ± 1.3	0.75
T14A	5 ± 8	11.5 ± 0.2	280 ± 38	11.2 ± 1.3	0.09
T14V	1 ± 3	11.3 ± 0.3	226 ± 23	12.5 ± 1.7	0.07
T14G	17 ± 7	6.0 ± 0.2	207 ± 13	3.4 ± 0.8	0.04
T14D	25 ± 12	4.9 ± 0.2	240 ± 27	10.9 ± 1.4	0.01
Y17C	71 ± 31	6.7 ± 0.1	273 ± 36	11.2 ± 1.0	0.01

[a] K_D, k_{cat}, and the concentration of small subunits in the *E. coli* CAG630 extracts were estimated from the data presented in Figure 3 by fitting them to eq 1 as described under Experimental Procedures. Expression level refers to the percentage of the total protein content of crude extracts represented by small subunits capable of binding to large subunits and promoting activity.

mixture of extracts containing large and small subunits was preincubated for 30 min in the presence of 40 mM $MgCl_2$ and 30 mM $NaHCO_3$. The assay was then started by adding an aliquot of this mixture to the otherwise complete assay solution which contained 0.3 mM ribulose-P_2. K_M values were estimated by fitting the data to the Michaelis–Menten equation. The concentration of CO_2 was calculated using a pK' value for the CO_2/HCO_3^- equilibrium of 6.12 at 25 °C.

Protein Determination. Protein was determined by the Pierce BCA procedure.

Results

Authentic Small Subunits Were Expressed by E. coli. Wild-type small subunits expressed by *E. coli* HB101 showed two closely spaced immunoreactive bands on immunoblots of SDS gels (data not shown). The higher molecular weight band corresponded to the authentic small subunit dissociated from the purified holoenzyme. The lower molecular weight band may be the result of proteolysis, but it could not be eliminated by incorporating protease inhibitors in the extraction buffer. Furthermore, a combination of gel filtration and anion-exchange chromatography was not fully effective in separating it from the larger species (lane 1, Figure 4). However, when wild-type small subunits were expressed by *E. coli* strain CAG630, an *rpoH165* (*htpR165*) mutant with reduced proteolysis (Baker et al., 1984), the lower molecular weight band was absent (lane 2, Figure 4). Likewise, mutant small subunits expressed by *E. coli* HB101 also gave rise to two immunoreactive species (data not shown), but when expressed by *E. coli* CAG630, only the higher-molecular-weight species, corresponding to authentic small subunits, were produced (lanes 3–7, Figure 4). *E. coli* CAG630 was therefore used for expressing wild-type and mutant small subunits for kinetic studies.

Properties of Small Subunits Expressed by E. coli. Wild-type small subunits expressed by *E. coli* CAG630 were fully functional, as judged by their ability to assemble with large subunits (expressed in *E. coli* HB101) to form active enzyme. In the presence of saturating amounts of small subunits, the assembled enzyme had a k_{cat} of 11.8 s^{-1}, a turnover rate comparable to the highest reported for cyanobacterial ribulose-P_2 carboxylases (Andrews & Lorimer, 1985). The Michaelis constants for CO_2 and ribulose-P_2 were 251 μM and 17.2 μM, respectively (Table I). These kinetic parameters for *Synechococcus* PCC 6301 ribulose-P_2 carboxylase are slightly different from the analogous parameters for the enzyme from the marine cyanobacterium, *Synechococcus* ACMM 323 (Andrews & Lorimer, 1985). While the k_{cat} and $K_M(CO_2)$ values are similar for the two enzymes, the enzyme from the marine organism has a 4-fold higher K_M(ribulose-P_2).

The binding of wild-type small subunits to large subunits was very tight, the estimated K_D being 1 ± 5 pM (Table I). This estimate is lower than the estimate of Andrews and Lorimer (1985) for the enzyme from the marine *Synechococcus* (0.12 ± 0.27 nM). However, it is obviously difficult to obtain an accurate estimate of the K_D when its magnitude is so much lower than the large-subunit concentration used in the experiments. A much lower large-subunit concentration (0.9 nM) was used in this study than in that of Andrews and Lorimer (1985), and it is possible that further lowering of the large-subunit concentration would lower the K_D estimate still further. Therefore, the present estimate of 1 ± 5 pM should still be regarded as a maximum estimate. The large standard error is also a reflection of the magnitude of the estimated K_D, relative to the concentration of large subunits used.

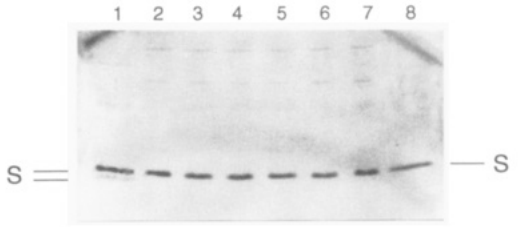


FIGURE 4: Immunoblot of an SDS electrophoresis gel of extracts of *E. coli* expressing the small subunits of ribulose-P_2 carboxylase from *Synechococcus* PCC 6301. See Experimental Procedures for details. Unless stated otherwise, the *E. coli* strain was CAG630. Lane 1, partially purified preparation of wild-type small subunits expressed in *E. coli* HB101 (eluate after Mono-Q column chromatography, see Experimental Procedures); lane 2, wild type; lane 3, T14A; lane 4, T14V; lane 5, T14G; lane 6, T14D; lane 7, Y17C; and lane 8, holoenzyme purified from *E. coli* expressing both *rbc*L and *rbc*S genes. The small-subunit bands are indicated (S).

The molecular mass of wild-type small subunits capable of assembling with large subunits was estimated by gel chromatography as described under Experimental Procedures using the stimulation that small subunits induce in the activity of large subunits as the method of detection. The estimate obtained, 12.6 kDa, is close to the value predicted from the nucleotide sequence (Shinozaki & Sugiura, 1983). This result demonstrates that the small subunits chromatograph as monomers in this system.

Binding Properties of Mutant Small Subunits. Five different variants of the small subunit (T14A, T14V, T14G, T14D, and Y17C) were obtained by oligonucleotide-directed mutagenesis (see Experimental Procedures) and expressed in *E. coli* CAG630. Extracts of these cells were used to stimulate the activity of large-subunit octamers derived from *E. coli* HB101 cells expressing the large-subunit gene using the in vitro reconstitution assay (Andrews & Ballment, 1983; Andrews & Lorimer, 1985; Andrews, 1988). All five mutant small subunits were able to assemble with large-subunit octamers to form active holoenzymes (Figure 3). However, T14G, T14D, and Y17C bound less tightly to the large subunit than did the wild-type small subunit, giving rise to higher K_D values (Table I). In spite of the large standard errors in the K_D estimates, the weaker binding of the T14G, T14D, and Y17C mutant small subunits is quite obvious from the more relaxed curvature in the region near the intersection of the two

near-linear arms of the saturation curves (Figure 3). The holoenzymes formed with these mutant small subunits also had 1.8- to 2.4-fold lower k_{cat} values than the wild-type enzyme (Table I). On the other hand, holoenzymes formed with T14A and T14V mutant small subunits were very similar to the wild type in terms of their k_{cat} and K_D values (Figure 3 and Table I).

Kinetic Properties of Holoenzymes with Mutant Small Subunits. The $K_M(CO)_2$ and K_M(ribulose-P_2) values of the reconstituted mutant enzymes were similar to those of the wild-type enzyme except when T14G mutant small subunits were present. In this case, the K_M(ribulose-P_2) was reduced 5-fold (Table I).

Expression Levels of Subunits. Large subunits (determined by [^{14}C]carboxypentitol-P_2 binding) represented 4% of the soluble protein of *E. coli* HB101 extracts. This is a much higher level of expression of large subunits than that obtained in a previous study (0.05–0.5%; Andrews, 1988) where the host strain was *E. coli* TG_2. Wild-type small subunits capable of assembling with large subunits and promoting activity constituted 0.75% of the soluble protein in the *E. coli* CAG630 extracts. However, all of the mutant small subunits were consistently expressed at significantly lower levels (Table I).

Discussion

Separately Expressed Small Subunits Are Fully Functional. We have demonstrated that fully active *Synechococcus* PCC 6301 ribulose-P_2 carboxylase (k_{cat} = 11.8 s^{-1}) can be produced following assembly in vitro of large and small subunits expressed separately in different *E. coli* cultures (Figure 3 and Table I). Lee and Tabita (1990) recently also reported stimulation of the activity of purified large-subunit octamers by extracts of *E. coli* cells expressing the *Synechococcus* small-subunit gene. However, the maximum k_{cat} they attained was only 0.35 s^{-1}, perhaps because of extensive denaturation of the large subunits during purification. We have also observed the instability of isolated large subunits during purification (Andrews, 1988) and, in this study, chose not to purify them but, rather, to measure their concentration in extracts by their ability to bind [^{14}C]carboxypentitol-P_2. Large-subunit octamers retain their ability to become carbamylated and to bind the analogue (Andrews & Ballment, 1984; Andrews, 1988). The stoichiometric nature of the binding of the analogue to isolated large subunits under the conditions used in this study was confirmed by showing that reconstitution of the holoenzyme by exposure of the large-subunit octamers to an excess of small subunits before addition of the analogue did not promote increased analogue binding (see Experimental Procedures). Christeller et al. (1985) failed to obtain activity by mixing extracts of *E. coli* cells harboring plasmids with large- and small-subunit genes. However, in their study, the large-subunit gene had six codons at the 3′ end of the coding region replaced by eight unrelated codons, and this may have resulted in inactive large subunits being produced.

Monomeric Nature of Small Subunits. E. coli expressed small subunits were predominantly monomeric, at least under the relatively dilute conditions of the gel filtration experiment. By contrast, large subunits expressed in *E. coli* are predominantly octameric (Andrews, 1988). Interactions between small subunits leading to tetramers might be expected from consideration of the crystallographically determined quaternary structure (Chapman et al., 1988; Andersson et al., 1989; Knight et al., 1990), but the present data show that such interactions must be relatively weak.

Consequences of Substitutions of Residues 14 and 17. Catalytically competent assembled enzymes were obtained with all of the small-subunit mutants created in this study. The hydrophobic residues, Ala and Val, were able to replace the polar Thr residue at position 14 with no significant differences in the binding affinity of the small subunits or the kinetic parameters of the holoenzymes containing them (Table I). Clearly, the potential hydrogen bond between the hydroxyl oxygen of Thr-14 and the carbonyl oxygen of the large subunit residue, Tyr-165(162), whose presence was inferred from crystallographic studies on the activated spinach enzyme (Knight et al., 1990), is of no significance. Likewise, the polarity of the residue at this position is also of little consequence. The size and charge of the residue at position 14 are likely to be more important. Since Val is of similar size and its preferred side-chain conformation within a protein is the same as for Thr (Janin et al., 1978), perhaps few or no perturbations were introduced by this substitution. However, the smaller residue, Ala, also replaced Thr without obvious consequences. Deletion of the side chain altogether (Gly-14) or introduction of a negative charge (Asp-14) loosened the binding of the small subunits by more than an order of magnitude and reduced the small-subunit-saturated turnover rate approximately 2-fold. It is likely that the packing of amino acids in this region might have been perturbed by the greater flexibility of the main chain (in Gly-14) and the introduction of a charged side chain (in Asp-14). These conclusions are supported by the natural variation known at this position, where Ser, Cys, or Ala occur in the small subunits of some bacterial or non-green algal ribulose-P_2 carboxylases (Figure 1). However, it should be noted that the latter class of ribulose-P_2 carboxylases is rather different, particularly in the N-terminal arm of the small subunits, and seems to constitute a separate line of evolutionary descent from those of cyanobacterial and higher plant origin.

Only one mutant substitution was obtained at residue 17, which is Tyr in the small subunits of ribulose-P_2 carboxylases from cyanobacteria and higher plants or Phe in some bacterial and non-green algal enzymes. Cys was substituted, a quite radical replacement which removes both the bulk and the aromaticity of the side chain. The binding of this Y17C small subunit was some 70-fold looser than that of the wild-type and the k_{cat} of the holoenzyme fully saturated with these small subunits was 60% of that of the wild type (Table I). These seem to be relatively minor consequences for such a gross change in the size and character of this side chain which has extensive, and highly conserved, interactions at an important interface between the large and small subunits [see introduction and Knight et al. (1990)].

In every case where the intersubunit binding (K_D) was weakened, the k_{cat} of the holoenzyme when fully saturated with small subunits also suffered (Table I). A similar increase in K_D and decrease in k_{cat} was observed when heterologous spinach small subunits were hybridized to the *Synechococcus* ACMM 323 large-subunit octamer (Andrews & Lorimer, 1985). Although the correlation between k_{cat} and K_D does not appear to be a simple linear one, it is possible that the tightness with which the small subunit binds may influence its ability to induce the necessary changes in conformation at the active site on the large subunit. More radical alterations to the subunit interfaces than those that we have currently effected may elucidate this possibility further.

The affinities of the mutant holoenzymes for CO_2 were not very different from that of the wild type. However, the 5-fold reduction in the K_M(ribulose-P_2) of T14G is particularly notable (Table I).

Expression Levels of Wild-Type and Mutant Small Subunits. It was particularly apparent that all of the mutant small subunits created in this study, even those that had no effect kinetically or on the binding affinity, were expressed in *E. coli* CAG630 at considerably lower levels than were wild-type small subunits (Table I). Perhaps, the mutant small subunits had a greater propensity for improper folding, and subsequent degradation, than did the wild-type small subunits. This emphasizes that, in addition to the necessity for appropriately matched interactions between large and small subunits, the structural prerequisites for production of a stable monomeric small subunit must also be satisfied.

Advantages of the Separate Expression System. The separate expression system used in this study has a distinct advantage over the coexpression or dual-expression systems for working with mutant small subunits. When both large and small subunits are expressed together in the same cell, a serious difficulty in interpretation is encountered if the mutation weakens the small subunit's binding affinity or if, as is frequently the case (Table I), the mutant small subunit is expressed at low levels. In either case, the assembled enzyme may be undersaturated with small subunits, leading to an underestimate of its activity. These potential problems make it impossible to determine whether the small-subunit mutations studied by Voordouw et al. (1987), McFadden and Small (1988), Fitchen et al. (1990), and Lee et al. (1991) affected the small subunit's activity-promoting capabilities, its binding affinity, or, merely, its expression level relative to large subunits. Lee et al. (1991) showed that some of their mutant small subunits [S16D, L21E, and T66(54)S] were less tightly bound to large subunits by observing that these mutant small subunits dissociated from large subunits during nondenaturating gel electrophoresis and that activity was lost during sucrose density gradient centrifugation. However, because of the use of the coexpression system, these authors were unable to quantitate the reduction in binding tightness of their mutant small subunits. Fitchen et al. (1990) showed by sucrose density gradient centrifugation followed by SDS gel electrophoresis and immunodetection that their small-subunit mutants did not assemble into active holoenzymes. However, poor expression of mutant small subunits coupled with weak binding could have resulted in inability to detect activity. The separate expression system overcomes these problems by allowing the ratio of small to large subunits to be varied during reconstitution in vitro (Figure 3). Provided a large enough ratio of small-subunit preparation to large-subunit preparation is used, full saturation is guaranteed regardless of how weakly the small subunits are expressed or bound. The in vitro reconstitution approach also allows the binding affinity to be measured.

Necessity for Structural Studies with Mutants. Crystallographic studies have presented us with penetrating insights into the intricate network of interactions between the subunits of ribulose-P_2 carboxylase. However, while interactions between atoms and residues of the two subunits may be inferred from their proximity, the significance of a particular interaction to subunit binding and consequent conformation changes is more difficult to assess. The present studies demonstrate that not all of the interactions that are perceived from the structure to be significant necessarily are so. An iterative procedure where the significance of interactions is tested by mutagenic substitution, followed by structural studies with the mutant holoenzymes, is likely to be an informative approach. Hence, crystallographic studies would be required to identify the subtle differences between the structures of the large subunits in the holoenzymes formed with wild-type, Gly-14, Asp-14, and Cys-17 small subunits. These differences may reveal the structural basis for the apparent correlation between the tightness of binding of the small subunits and their ability to induce the conformational changes at the active site that are necessary to promote catalysis.

Acknowledgments

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References

Andersen, K., & Caton, K. (1987) *J. Bacteriol. 167*, 4547–4558.

Andersson, I., Knight, S., Schneider, G., Lindqvist, Y., Lundqvist, T., Brändén, C.-I., & Lorimer, G. H. (1989) *Nature 337*, 229–234.

Andrews, T. J. (1988) *J. Biol. Chem. 263*, 12213–12219.

Andrews, T. J., & Ballment, B. (1983) *J. Biol. Chem. 258*, 7514–7518.

Andrews, T. J., & Ballment, B. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3660–3664.

Andrews, T. J., & Lorimer, G. H. (1985) *J. Biol. Chem. 260*, 4632–4636.

Andrews, T. J., & Lorimer, G. H. (1987) in *The Biochemistry of Plants* (Hatch, M. D., & Boardman, N. K., Eds.) Vol. 10, pp 131–218, Academic Press, New York.

Baker, T. A., Grossman, A. D., & Gross, C. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6779–6783.

Berry-Lowe, S. L., McKnight, T. D., Shah, D. M., & Meagher, R. B. (1982) *J. Mol. Appl. Genet. 1*, 483–498.

Boyer, H. W., & Roulland-Dussoix, D. (1969) *J. Mol. Biol. 41*, 459–472.

Broglie, R., Coruzzi, G., Lamppa, G., Keith, B., & Chua, N.-H. (1983) *Bio/Technology 1*, 55–61.

Chapman, M. S., Suh, S. E., Curmi, P. M. G., Cascio, D., Smith, W. W., & Eisenberg, D. S. (1988) *Science 241*, 71–74.

Christeller, J. T., Terzaghi, B. E., Hill, D. F., & Laing, W. A. (1985) *Plant Mol. Biol. 5*, 257–263.

Collatz, G. J., Badger, M. R., Smith, C., & Berry, J. A. (1979) *Year Book—Carnegie Inst. Washington 78*, 171–175.

Douglas, S. E., & Durnford, D. G. (1989) *Plant Mol. Biol. 13*, 13–20.

Edmondson, D. L., Badger, M. R., & Andrews, T. J. (1990) *Plant Physiol. 93*, 1376–1382.

Fitchen, J. H., Knight, S., Andersson, I., Brändén, C.-I., & McIntosh, L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5768–5772.

Gatenby, A. A., van der Vies, S. M., & Bradley, D. (1985) *Nature 314*, 617–620.

Gatenby, A. A., van der Vies, S. M., & Rothstein, S. J. (1987) *Eur. J. Biochem. 168*, 227–231.

Goldschmidt-Clermont, M., & Rahire, M. (1986) *J. Mol. Biol. 191*, 421–432.

Horecker, B. L., Hurwitz, J., & Weissbach, A. (1958) *Biochem. Prep. 6*, 83–90.

Janin, J., Wodak, S., Levitt, M., & Maigret, B. (1978) *J. Mol. Biol. 125*, 357–386.

Keen, J. N., Pappin, D. J. C., & Evans, L. V. (1988) *J. Phycol. 24*, 324–327.

Knight, S., Andersson, I., & Brändén, C.-I. (1990) *J. Mol. Biol. 215*, 113–160.

Larimer, F. W., Lee, E. H., Mural, R. J., Soper, S. T., & Hartman, F. C. (1987) *J. Biol. Chem. 262*, 15327-15329.
Lee, B., & Tabita, F. R. (1990) *Biochemistry 29*, 9352-9357.
Lee, B., Berkas, R. M., & Tabita, F. R. (1991) *J. Biol. Chem. 266*, 7417-7422.
Lee, E. H., Soper, T. S., Mural, R. J., Stringer, C. D., & Hartman, F. C. (1987) *Biochemistry 26*, 4599-4604.
Martin, P. G. (1979) *Aust. J. Plant Physiol. 6*, 401-408.
Mazur, B. J., & Chui, C.-F. (1985) *Nucleic Acids Res. 13*, 2373-2386.
McFadden, B. A., & Small, C. L. (1988) *Photosynth. Res. 18*, 245-260.
Messing, J. (1983) *Methods Enzymol. 101*, 20-78.
Miziorko, H. M. (1979) *J. Biol. Chem. 254*, 270-272.
Morell, M. K., Kane, H. J., & Andrews, T. J. (1990) *FEBS Lett. 265*, 41-45.
Nierzwicki-Bauer, S. A., Curtis, S. E., & Haselkorn, R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5961-5965.
Pierce, J., Tolbert, N. E., & Barker, R. (1980) *Biochemistry 19*, 934-942.
Sailland, A., Amiri, I., & Freyssinet, G. (1986) *Plant Mol. Biol. 7*, 213-218.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Schloss, J. V., Phares, E. F., Long, M. V., Norton, L. I., Stringer, C. D., & Hartman, F. C. (1982) *Methods Enzymol. 90*, 522-528.
Schneider, G., Knight, S., Andersson, I., Brändén, C.-I., Lindqvist, Y., & Lundqvist, T. (1990) *EMBO J. 9*, 2045-2050.
Schneider, S. U., Leible, M. B., & Yang, X.-P. (1989) *Mol. Gen. Genet. 218*, 445-452.
Shinozaki, K., & Sugiura, M. (1983) *Nucleic Acids Res. 11*, 6957-6964.
Smith, D. B., & Johnson, K. S. (1988) *Gene 67*, 31-40.
Starnes, S. M., Lambert, D. H., Maxwell, E. S., Stevens, S. E., Porter, R. D., & Shively, J. M. (1985) *FEMS Microbiol. Lett. 28*, 165-169.
Stiekema, W. J., Wimpee, C. F., & Tobin, E. M. (1983) *Nucleic Acids Res. 11*, 8051-8061.
Tabita, F. R., & Small, C. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6100-6103.
Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8764-8785.
Terzaghi, B. E., Laing, W. A., Christeller, J. T., Petersen, G. B., & Hill, D. F. (1986) *Biochem. J. 235*, 839-846.
Valentin, K., & Zetsche, K. (1989) *Curr. Genet. 16*, 203-209.
Viale, A. M., Kobayashi, H., & Akazawa, T. (1989) *J. Bacteriol. 171*, 2391-2400.
Voordouw, G., de Vries, P. A., van den Berg, W. A. M., & de Clerk, E. P. J. (1987) *Eur. J. Biochem. 163*, 591-598.
Wagner, S. J., Stevens, S. E., Jr., Nixon, B. T., Lambert, D. H., Quivey, R. G., Jr., & Tabita, F. R. (1988) *FEMS Microbiol. Lett. 55*, 217-222.

Diastereotopic Covalent Binding of the Natural Inhibitor Leupeptin to Trypsin: Detection of Two Interconverting Hemiacetals by Solution and Solid-State NMR Spectroscopy†

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ABSTRACT: The naturally occurring peptidyl protease inhibitor leupeptin (*N*-acetyl-L-leucyl-L-leucyl-L-argininal) has been prepared labeled with ^{13}C at the argininal carbonyl. ^{13}C chemical shift data for the trypsin–leupeptin inhibitor complex in the pH range 3.0–7.6 reveal the presence of two pH-dependent covalent complexes, suggestive of two interconverting diastereomers at the new asymmetric tetrahedral center created by covalent addition of Ser195 to either side of the ^{13}C-enriched aldehyde of the inhibitor. At pH 7 two signals are observable at δ 98.8 and δ 97.2 (84:16 ratio), while at pH 3.0 the latter signal predominates. In the selective proton ^{13}C-edited NOE spectrum of the major diastereomer at pH 7.4, a strong NOE is observed between the hemiacetal proton of the inhibitor and the C2 proton of His57 of the enzyme, thus defining the stereochemistry of the high pH complex to the *S* configuration in which the hemiacetal oxygen resides in the oxyanion hole. pH titration studies further indicate that the ^{13}C chemical shift of the *S* diastereomer follows a titration curve with a pK_a of 4.69, the magnitude of which is consistent with direct titration of the hemiacetal oxygen. Similar pH-dependent chemical shifts were obtained by using CPMAS ^{13}C NMR, providing evidence for the existence of the same diastereomeric equilibrium in the solid state.

Peptide aldehydes are known to be efficient reversible inhibitors of serine proteases (Thompson, 1973). It has been suggested that the tight binding of the aldehyde results in the formation of a covalent complex between the nucleophilic hydroxyl of the serine protease and the aldehyde carbonyl. One such inhibitor is the slow-binding natural aldehyde leupeptin (*N*-acetyl-L-leucyl-L-leucyl-L-argininal), which reacts with the active site serine (Ser195-OH) of trypsin by a two-step mechanism beginning with formation of a weakly bound complex (K_i = 1.2 mM) followed by covalent bonding (K_d = 1.3 nM) (Aoyagi et al., 1969; Kuramochi et al., 1979). Since the resultant tetrahedral hemiacetal represents a transition

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