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An Intersubunit Interaction at the Active Site of D-Ribulose-1,5-bisphosphate Carboxylase/Oxygenase As Revealed by Cross-Linking and Site-Directed Mutagenesis†

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ABSTRACT: For measurement of distances between active-site residues of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* (a homodimer of 50.5-kDa subunits), the reaction of the enzyme with 4,4′-difluoro-3,3′-dinitrodiphenyl sulfone (which spans 9 Å) has been explored. Inactivation of the enzyme by the bifunctional reagent is not associated with an increase in apparent molecular weight, thereby excluding intermolecular cross-linking. However, in the presence of urea, gel filtration of the inactivated enzyme reveals a prominent dimeric species attributed to intersubunit cross-linking. The major chromophoric peptide has been isolated from a tryptic digest of the dimer; sequence analysis of this peptide reveals that the intersubunit cross-link occurs between Cys-58 and active-site Lys-166. In contrast to previous substitutions for Lys-166 introduced by site-directed mutagenesis, replacement by aspartic acid prevents association of the two subunits. In addition to identifying an intersubunit contact, these observations suggest that the catalytic site of the carboxylase is positioned at an interface between subunits and that segments of both subunits may be required for catalytic competence.

Because of its pivotal role in establishing the overall efficiency of CO_2 utilization by plants, ribulose-P_2 carboxylase[1] (EC 4.1.1.39) has been subjected to close scrutiny in recent years [for thorough discussions about the structure, mechanism, and regulation of the enzyme, see Lorimer (1981), Miziorko and Lorimer (1983), and Ellis and Gray (1986)]. On the basis of affinity labeling and species invariance, two different lysyl residues (Lys-166 and Lys-329 of the carboxylase from *Rhodospirillum rubrum*) have been assigned to the active site (Hartman et al., 1984). Enhanced acidities and nucleophilicities of these lysyl ϵ-amino groups suggest that both participate in catalysis (Hartman et al., 1985), a postulate supported by site-directed mutagenesis studies (Hartman et al., 1987; Soper et al., 1987). The essential base that initiates enolization of ribulose-P_2 may be the ϵ-amino group of Lys-166, as its pK_a (7.9) determined from chemical modification (Hartman et al., 1985) approximates the pK_a (7.5) observed from the pH dependence of V_{max} with [3-^{2}H]ribulose-P_2 as substrate (Van Dyk & Schloss, 1986).

The unusual reactivities of Lys-166 and Lys-329 of *R. rubrum* ribulose-P_2 carboxylase, a homodimer (Tabita & McFadden, 1974; Hartman et al., 1984), provide an opportunity to probe the active-site environment with chemical cross-linking reagents. Proximity of Lys-166 and Lys-329, as dictated by their purported catalytic roles, has been confirmed by their propensity to undergo efficient intrasubunit bridging with 4,4′-diisothiocyanostilbene-2,2′-disulfonate, a bifunctional

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[1] Abbreviations: ribulose-P_2 carboxylase, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; FNPS, 4,4′-difluoro-3,3′-dinitrodiphenyl sulfone; carboxyarabinitol-P_2, 2-carboxyarabinitol 1,5-bisphosphate; carboxyribitol-P_2, 2-carboxyribitol 1,5-bisphosphate; PTH, phenylthiohydantoin; kDa, kilodalton(s); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

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reagent that spans 12 Å (Lee et al., 1986). To continue mapping residues in the immediate vicinity of Lys-166 and Lys-329, we have explored the reaction of the carboxylase with FNPS, a bifunctional arylating reagent that spans 9 Å. In this paper, we show that FNPS forms an intersubunit cross-link between active-site Lys-166 and Cys-58, thereby revealing a subunit–subunit contact and raising the possibility that each catalytic site of the dimeric enzyme is comprised of polypeptide regions from both subunits. Such a contact is supported by site-directed mutagenesis, whereby replacement of Lys-166 by aspartic acid prevents the association of the carboxylase subunits.

Materials and Methods

Materials. Commonly used chemicals, reagents, and substrates were procured from Sigma. Other commercial materials and vendors were as follows: FNPS was from Pierce; buffers and ninhydrin for amino acid analyses were from Beckman; reagents for peptide sequencing were from Applied Biosystems. Reagents and procedures for oligonucleotide synthesis have been reported and cited previously (Hartman et al., 1987). Ribulose-P_2, carboxyarabinitol-P_2, and carboxyribitol-P_2 were synthesized according to published procedures (Horecker et al., 1958; Pierce et al., 1980).

Ribulose-P_2 carboxylase from *R. rubrum* was purified as described earlier (Schloss et al., 1982) and was stored at −70 °C as a concentrated stock at 45 mg/mL in pH 8.0 activation buffer (50 mM Bicine, 66 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM EDTA) that contained 20% (v/v) glycerol and 10 mM 2-mercaptoethanol. The enzyme had a specific activity of 5.5 units/mg in the spectrophotometric assay (Racker, 1963; Schloss et al., 1982). For chemical modification studies, aliquots of the stock carboxylase solution were dialyzed at 24 °C against either the activation buffer just described or a pH 8.0 deactivation buffer (50 mM Bicine, 1 mM EDTA) to which Chelex-100 resin (Bio-Rad) (10 g/L of buffer) was added to ensure the absence of trace metal ions. Carboxylase concentration was based on the absorbency at 280 nm and an $\epsilon_{1cm}^{1\%}$ of 12.0 (Schloss et al., 1982).

The Asp-166 mutant of ribulose-P_2 carboxylase was expressed in *Escherichia coli* JM107 [a strain described by Yanisch-Perron et al. (1985)] under conditions previously reported and was purified by immunoaffinity chromatography (Niyogi et al., 1986).

Construction of the Asp-166 Mutant of Ribulose-P_2 Carboxylase. The mutant that encodes Asp-166 was introduced into the carboxylase gene by bandaid mutagenesis as previously described (Mural & Foote, 1986; Hartman et al., 1987). A single-stranded deoxyoligonucleotide (dAGCTTCGGATCGATGATCGTAC) was ligated into a gap formed in the plasmid vector, pFL70, by digestion with the restriction endonucleases *Kpn*I and *Hin*dIII, which leave protruding 3′ and 5′ ends, respectively. The mutagenic oligonucleotide that introduces the position 166 mutation also abolishes the *Kpn*I restriction site and thus provides a convenient screen for potential mutants. The desired mutation was confirmed by sequencing the appropriate region of the double-stranded plasmid DNA (Chen & Seeburg, 1985; Zagursky et al., 1985).

Cross-Linking of Ribulose-P_2 Carboxylase with FNPS. Modifications of the enzyme with FNPS were carried out at room temperature in the dark and examined in the presence and absence of CO_2 and Mg^{2+}. In the absence of these two ligands, ribulose-P_2 carboxylase exists in a catalytically inactive form. Activation entails reaction of CO_2 with a specific lysyl ϵ-amino group to form a carbamate that is stabilized by Mg^{2+} (Miziorko & Lorimer, 1983).

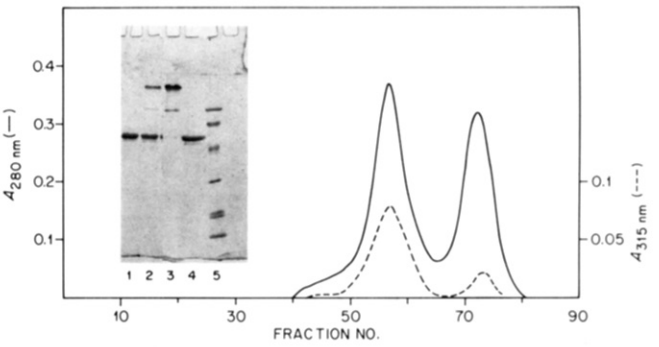

FIGURE 1: Gel filtration of FNPS cross-linked, carboxymethylated ribulose-P_2 carboxylase (10 mg) on a 2.5 × 100 cm column of Ultrogel AcA 34, equilibrated and eluted with 6 M urea/50 mM Hepes/50 mM NaCl/10 mM EDTA, pH 6.8. Inset: Polyacrylamide gel electrophoretogram (in the presence of sodium dodecyl sulfate) of untreated enzyme (lane 1), cross-linked enzyme before gel filtration (lane 2), first peak from column (lane 3), last peak from column (lane 4), and a mixture of molecular weight standards (Bio-Rad) in which the largest protein is phosphorylase B (92.5 kDa) (lane 5). Electrophoretic conditions were essentially those of Laemmli (1970) and result in a running pH of ~9.5. The acrylamide concentration was 15% in the separating gel and 4.5% in the stacking gel. Electrophoresis was performed with a microslab apparatus (Idea Scientific, Corvallis, OR); gel dimensions were 100 mm in width × 90 mm in height × 0.5 mm in thickness. Proteins were visualized with Coomassie blue (0.1%) in methanol/water/acetic acid, 5:5:1 (v/v/v), and destained in the same solvent.

Four 30-μL portions of freshly prepared 0.02 M FNPS (final concentration in reaction mixture = 400 μM) in acetonitrile were added at 40-min intervals to a gently stirred solution (6 mL) of 100 μM carboxylase (5 mg/mL) in activation buffer (see Materials) that contained 2% (v/v) acetonitrile. Prior to each addition of reagent, 10-μL aliquots of the reaction mixture were mixed with 190 μL of activation buffer for immediate determination of enzymic activity. Separate 50-μL aliquots were quenched with 10 μL of 2 M glycine (pH 8.0) for electrophoretic analysis. Forty minutes after the final addition of reagent, 45% of the initial enzymic activity remained; the entire reaction mixture was then quenched with 1 mL of 2 M glycine (pH 8.0).

A portion of the quenched reaction mixture (~5 mg of protein) was subjected to gel filtration on a 2.5 × 100 cm column of Ultrogel AcA 34 (LKB) equilibrated and eluted with activation buffer. Another portion of the quenched reaction mixture (~20 mg of protein) was carboxymethylated with 15 mM iodoacetate in the presence of buffered 4 M guanidine (pH 8.0) and was then dialyzed exhaustively against 0.02 M NH_4HCO_3 (pH 8.0), during which time the protein precipitated. Half of the suspension (~10 mg of protein) was digested at 37 °C with 200 μg of trypsin for 12 h. The protein (~10 mg) in the other half of the suspension was collected by centrifugation, dissolved in 1.5 mL of 6 M urea (pH 6.8), and subjected to gel filtration under denaturing conditions (Figure 1). Fractions comprising the two major peaks were pooled, dialyzed, and digested with trypsin.

Smaller samples (0.5 mL) of the carboxylase (5 mg/mL) in activation buffer, containing either 1 mM carboxyribitol-P_2 or 1 mM carboxyarabinitol-P_2, and in deactivation buffer (absence of CO_2 and Mg^{2+}, see Materials) were treated with FNPS as described above. In each case, the modified protein was carboxymethylated and digested with trypsin.

Results

Gross Characteristics of Ribulose-P_2 Carboxylase Modified by FNPS. Treatment of the fully activated carboxylase (i.e.,

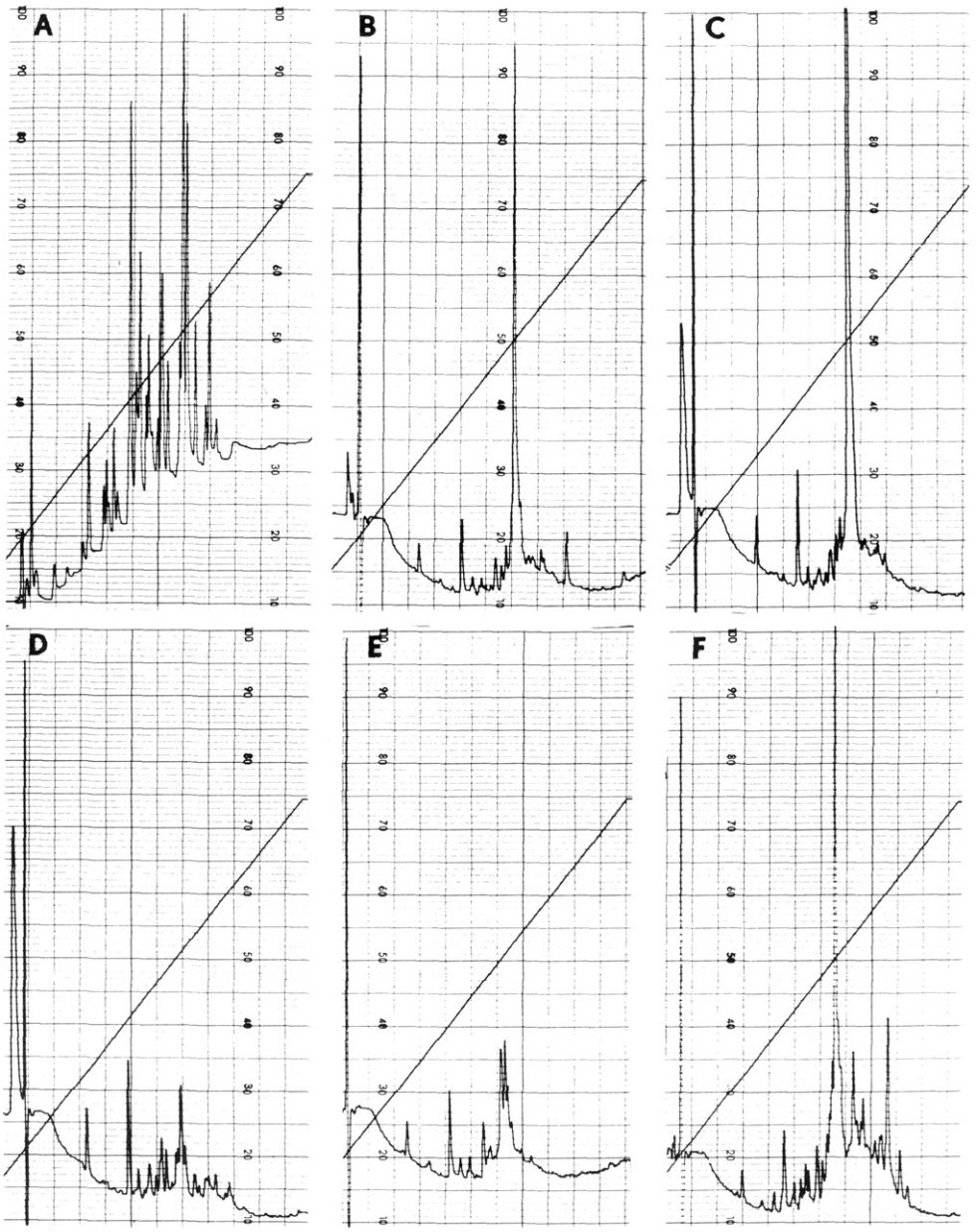

FIGURE 2: HPLC profiles of tryptic digests of cross-linked, carboxymethylated ribulose-P_2 carboxylase. The solvent system for the column (250 × 4.6 mm) of Lichrosorb RP8 (Laboratory Data Control) consisted of 0.1% (v/v) aqueous trifluoroacetic acid (equilibration) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (limit) as described previously (Mahoney & Hermodson, 1980). Except for (A) in which all peptides detected at 215 nm are shown, only FNPS-labeled peptides (which absorb at 315 nm) are illustrated. Panels A and B represent a tryptic digest of the carboxylase that had been 55% inactivated by FNPS. Panels C and D represent tryptic digests of the dimeric and monomeric species, respectively, of the cross-linked carboxylase isolated by gel filtration (see Figure 1). Panels E and F represent tryptic digests of the carboxylase that had been treated in the presence of carboxyarabinitol-P_2 or in the absence of CO_2 and Mg^{2+}, respectively.

in the presence of CO_2 and Mg^{2+}) with only a fourfold molar excess of FNPS as described under Materials and Methods leads to a 55% loss of the initial activity; inclusion of 1 mM carboxyribitol-P_2, a simple competitive inhibitor in contrast to the epimeric, tight-binding carboxyarabinitol-P_2 (Pierce et al., 1980), in the reaction mixture provided substantial protection, whereby only 10% inactivation occurred during the same time period. The inactivation profile was similar under conditions that favor the deactivated form of the enzyme (i.e., in the absence of CO_2 and Mg^{2+}).

After treatment of the activated carboxylase with FNPS, the derivatized enzyme was subjected to gel filtration on Ultrogel AcA 34 (data not shown). The derivatized enzyme emerged at the same position as untreated native carboxylase, proving that intermolecular cross-linking had not occurred. In contrast, if the derivatized enzyme (after carboxymethylation of sulfhydryls, see Materials and Methods) was subjected to gel filtration under denaturing conditions, a major peak was observed ahead of the position at which the carboxylase subunit eluted, consistent with the formation of intersubunit cross-links (Figure 1). Confirmation of the dimeric nature of the first peak was provided by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 1, inset). The carboxylase monomer ran as a 54-kDa peptide, and the dimer ran as a 133-kDa peptide. The anomalous electrophoretic behavior of the dimer may be due to the cross-link.

To ascertain the specificity of modification of the carboxylase by FNPS, tryptic digests were inspected by HPLC (Figure 2). The digest of the inactivated carboxylase, prior to fractionation by gel filtration, revealed only one major chomophoric peptide (panel B). This peptide also predominated the A_{315nm} profile of the digest of the dimeric fraction of the carboxylase (panel C) that was isolated by gel filtration

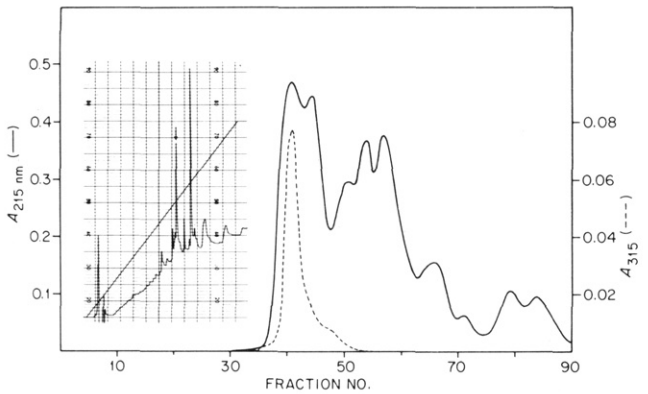

FIGURE 3: Purification of the cross-linked peptide from a tryptic digest of FNPS-treated ribulose-P_2 carboxylase. The digest (4.6 mg, 92 nmol) of dimeric polypeptide (see Figure 1) was subjected to gel filtration on a 1.7 × 220 cm column of Sephadex G-25 and equilibrated and eluted with 0.02 M NH_4HCO_3 (pH 8.0). Excluding its trailing edge, the single major peak observed at 315 nm represented 50 nmol (54%) of labeled peptide. A portion of this peptide (20 nmol) was subjected to further purification by HPLC under conditions described in the legend to Figure 2 (the absorbance profile at 215 nm is shown in the inset). Fractions (0.5 mL) of the FNPS-labeled peptide (denoted with arrow) that emerges at 50% acetonitrile were collected for amino acid and sequence analyses.

under denaturing conditions (see Figure 1). Little of this peptide was observed in digests of the monomeric fraction (panel D) from the gel filtration column or in digests of the carboxylase that was treated with FNPS in the presence of carboxyarabinitol-P_2 (panel E). Hence, the chromophoric peptide that emerges at 50% acetonitrile correlated with intersubunit cross-linking and with inactivation. The exclusion of CO_2 and Mg^{2+} from the reaction mixture during derivatization of the carboxylase by FNPS did not appreciably alter the labeling pattern (panel F).

Purification and Characterization of the Cross-Linked Peptide. The major chromophoric peptide in tryptic digests of FNPS-inactivated carboxylase was purified by preparative gel filtration (Figure 3) followed by HPLC (Figure 3, inset). The amino acid composition agreed with that predicted for an equimolar mixture of two tryptic peptides representing regions 34–65 and 149–168 of the intact subunit, excepting the absence of (carboxymethyl)cysteine and one less residue of lysine (Table I). Cysteine and lysine were thus assumed to be involved in the cross-link.

Edman degradation[2] of the chromophoric peptide confirmed that it consists of two chains derived from positions 34–65 and 149–168 with the sequences [see Hartman et al. (1984) for the sequence of the entire subunit]

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35        40        45        50        55        60        65
A-G-Y-G-Y-V-A-T-A-A-H-F-A-A-E-S-S-T-G-T-N-V-E-V-C-T-T-D-D-F-T-R

V-L-G-R-P-E-V-D-G-G-L-V-V-G-T-I-I-K-P-K
150       155       160       165
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Two residues were released at each cycle through 20 cycles (the end of one chain), except at cycle 18 where only PTH-Thr was observed. The absence of PTH-Lys at this position identified Lys-166 as one of the sites of the cross-link. A gap was also observed at cycle 25, which identified Cys-58 as the other site of the cross-link.

Table I: Amino Acid Composition of Cross-Linked Peptide[a]

amino acid	found (nmol)	no. of residues	expected[b]
Cys(Cm)	0.10	0.05	1
Asx	2.2	3.9	4
Thr	3.5	6.4	7
Ser	1.1	2.0	2
Glx	2.0	3.6	3
Pro	1.1	2.0	2
Gly	3.5	6.4	7
Ala	3.0	5.4	6
Val	3.4	6.2	7
Ile	0.81	1.5	2
Leu	1.3	2.3	2
Tyr	0.94	1.7	2
Phe	1.1	2.1	2
His	0.55	1	1
Lys	0.59	1.1	2
Arg	0.95	1.8	2

[a] Total acid hydrolysis of peptides was achieved in evacuated (<50 μmHg) sealed tubes with 6 M HCl/0.01 M 2-mercaptoethanol at 110 °C for 21 h. Hydrolysates were dried on a Speed Vac concentrator (Savant) and subjected to chromatography on a Beckman 121M amino acid analyzer using Beckman's 3-h, single-column system. [b] Based on an equimolar mixture of tryptic fragments encompassing positions 34–65 and 149–168 of the *R. rubrum* carboxylase [see Hartman et al. (1984) for the complete primary structures].

Size of the Asp-166 Mutant of Ribulose-P_2 Carboxylase. The purified Asp-166 mutant protein and extracts prepared from *E. coli*, in which the mutant protein was expressed, were completely devoid of detectable carboxylase activity. Subsequent to its purification by immunoaffinity chromatography, the Asp-166 mutant of the carboxylase coeluted from a Sephadex G-150 column with horseradish peroxidase (M_r 45 000), clearly resolved from wild-type ribulose-P_2 carboxylase (M_r 100 000) (data not shown). To determine whether the mutant protein remained as a monomer in *E. coli* after expression or had undergone dissociation during purification, it was examined in whole-cell extracts by Western blotting (Figure 4A) of a polyacrylamide gel (Figure 4B) run under nondenaturing conditions. Although the observed electrophoretic mobility of the Asp-166 mutant in the extract was the same as for the purified protein, its mobility was considerably greater than that for wild-type enzyme. In contrast, other position 166 mutants (e.g., His-166) comigrated with wild-type enzyme as reported earlier (Hartman et al., 1987). The broadness of the band for the Asp-166 mutant was not due to heterogeneity, because this mutant displayed a sharp major band on denaturing gels (Figure 4C).

Discussion

In principle, the reaction of a cross-linking agent with a dimeric protein can be intrasubunit, intersubunit, or intermolecular. However, the modification of *R. rubrum* ribulose-P_2 carboxylase by FNPS proceeds almost exclusively via intersubunit cross-linking, as shown by gel filtration of the inactivated enzyme under nondenaturing and denaturing conditions (Figure 1). Because of the high degree of selectivity of modification (only one major labeled peptide found in tryptic digests by HPLC; Figure 2), identification of the intersubunit cross-link between Lys-166 and Cys-58 was straightforward and unequivocal. Clearly, the ε-amino group of Lys-166 of one subunit can be positioned within 9 Å (the span of FNPS) of the sulfhydryl group of Cys-58 of the adjacent subunit. Whether or not this accurately reflects the interresidue distance in the catalytically competent enzyme depends in part on side-chain flexibility. If rotation of these two side chains is unrestricted, 9 Å could represent the mean between their

[2] The peptide was subjected to automated Edman degradation by using a gas-phase sequencer (Applied Biosystems, Model 470A) according to the manufacturer's instructions. Phenylthiohydantoin amino acids were identified and quantified by HPLC (Laboratory Data Control, Riviera Beach, FL) on a C_{18} column (Spherisorb ODS 5 μm, 4.6 × 250 mm) run at room temperature with a flow rate of 1.5 mL/min. Two different solvent systems were used: (i) an isocratic solvent of Lottspeich (1980) containing 68.5% (v/v) aqueous sodium acetate (0.01 M, pH 5.20), 31% (v/v) acetonitrile, and 0.5% (v/v) 1,2-dichloroethane; (ii) a gradient of 10–50% acetonitrile in 0.1% aqueous phosphoric acid.

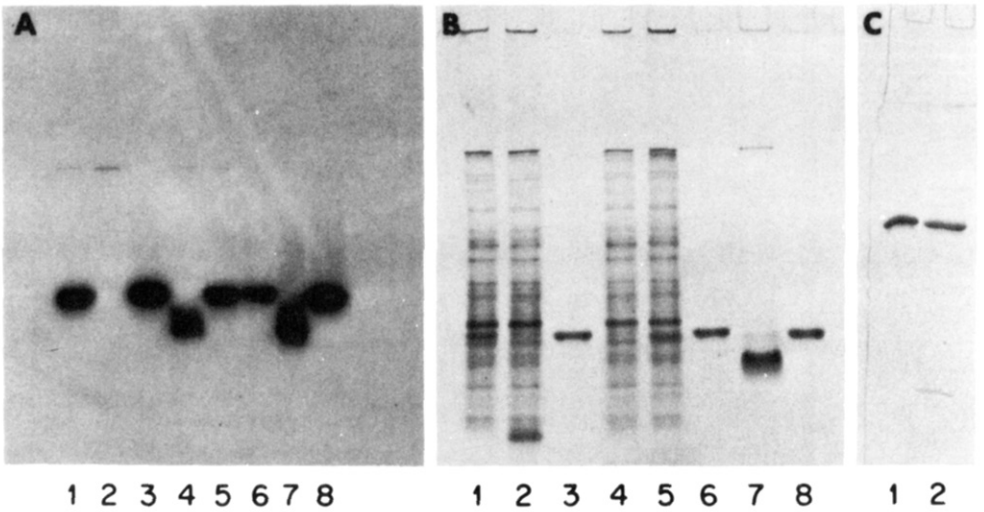

FIGURE 4: Autoradiogram (panel A) of a Western blot prepared from a gel identical with that shown in panel B except for the omission of protein staining. The Western blotting was accomplished by the procedure of Burnette (1981) with slight modification (Johnson et al., 1984); polyclonal rabbit antibody to *R. rubrum* carboxylase has been described (Niyogi et al., 1986). In panels A and B, samples are as follows: lane 1, extract of *E. coli* in which wild-type carboxylase was expressed; lane 2, extract of *E. coli* which lacked the cloned carboxylase gene; lanes 3 and 6, purified *R. rubrum* carboxylase; lane 4, extract of *E. coli* in which the Asp-166 mutant carboxylase was expressed; lane 5, extract of *E. coli* in which the His-166 mutant carboxylase was expressed; lane 7, purified Asp-166 mutant carboxylase; lane 8, purified His-166 mutant carboxylase. The nondenaturing polyacrylamide gel shown in panel B was prepared as described in the legend to Figure 1 except sodium dodecyl sulfate was omitted and the acrylamide concentration in the separating gel was reduced to 9.5%. A sodium dodecyl sulfate–polyacrylamide gel of *R. rubrum* carboxylase (lane 1) and Asp-166 mutant carboxylase (lane 2) is shown in panel C.

minimal separation of 4 Å, the constraint dictated by van der Waals' radii (Robson & Platt, 1986), and their maximal separation of 13.5 Å, the distance attainable without repositioning the respective α-carbons.

A limitation in the use of bifunctional reagent to measure interresidue distances is the possibility that the initial modification (i.e., reaction of a given residue with one of the two reactive groups of the reagent) distorts the protein conformation and thereby influences the site of modification that completes the cross-link. If Cys-58 and Lys-166 of opposing subunits do provide an intersubunit contact as indicated by cross-linking, subunit association may be sensitive to the nature of the amino acid that occupies either of these two positions. We have explored this possibility by site-directed mutagenesis.

In previous studies of the catalytic function of Lys-166, it was replaced by site-directed mutagenesis with Gly, Ala, Ser, Gln, Cys, His, or Arg (Hartman et al., 1987). In each of these cases, the mutant protein existed as a dimer (like the wild-type enzyme) and exhibited an electrophoretic mobility, under nondenaturing conditions, very similar to that of wild-type enzyme. Substitution of Lys-166 with aspartic acid, however, prevents the association of the carboxylase subunit. The increased electrophoretic mobility of the Asp-166 mutant compared to wild-type enzyme and the other position 166 mutants, observed by Western blots of gels of whole-cell extracts (Figure 4), is greater than can be explained by charge difference and must reflect a monomeric structure. As expected, the purified Asp-166 mutant behaved as a monomer of ~50000 Da during gel filtration. If Lys-166 and Cys-58 are in close proximity at an interface between subunits, failure of the Asp-166 mutant to form a dimer could be due to electrostatic repulsion between the carboxylate and thiolate anions. Another possible explanation is that the replacement of Lys-166 by aspartic acid alters the polypeptide conformation in the region of an intersubunit contact and thereby prevents their association. A disruption of conformation might explain why the Asp-166 mutant protein runs as a rather diffuse band on nondenaturing gels (Figure 4). In either case, both the cross-linking and mutagenesis studies support the interpretation that Lys-166 of one subunit is close to Cys-58 of the other subunit.

In addition to locating an intersubunit contact of ribulose-P_2 carboxylase, our studies imply that the catalytic site is located at this interface. The presence of Lys-166 at the active site and its probable participation in catalysis have been thoroughly documented (Hartman et al., 1984, 1985, 1987; Lee et al., 1986). Furthermore, Cys-58 has been assigned to the active-site region as a consequence of its being targeted by the affinity label 2-[4-(bromoacetamido)anilino]-2-deoxypentitol 1,5-bisphosphate (Herndon & Hartman, 1984). Because it is not species invariant, Cys-58 does not appear to be essential. In retrospect, one can envision reversible anchoring of the affinity label to one subunit at the interface, through electrostatic interaction with the reagent's phosphate groups, and subsequent modification of the adjacent subunit at the interface. The distance between C2 of the reagent's ribitol chain and the α-carbon of the bromoacetyl extender group is ~10 Å, very similar to the bridging distance of FNPS that links Lys-166 and Cys-58 covalently.

Although binding studies have shown that both dimeric and hexadecameric forms of ribulose-P_2 carboxylase contain the same number of catalytic sites as large subunits (Miziorko & Sealy, 1980; Badger & Lorimer, 1981; Donnelly et al., 1983; Jordan & Chollet, 1983), the issue of whether a completely functional catalytic site comprises a single subunit or requires polypeptide regions from more than one subunit has not been previously addressed. Our studies certainly favor the latter possibility, which would be consistent with the fact that a catalytically active monomer of the carboxylase has never been observed. Even if residues from only one subunit at the interface participate directly in catalysis, the subunit–subunit interaction could be required for proper active-site conformation.

The recently published three-dimensional structure of *R. rubrum* ribulose-P_2 carboxylase (in the deactivated form) at 2.9-Å resolution does show an interaction between the NH_2-terminal domain of one subunit and a region of the adjacent subunit that includes active-site Lys-166 (Schneider et al., 1986). Unfortunately, the distance between Lys-166 and Cys-58 of the adjacent subunit is not revealed, because fitting of amino acid side chains to the electron density map

has not been completed and because the electron density is weak in portions of the NH_2-terminal domain. Schneider et al. (1986) concluded that the intersubunit distance is too long for direct involvement in catalysis by residues from the NH_2-terminal domain. They stressed, however, that the intersubunit distance may depend upon the activation state of the enzyme. In this connection, we observed the same cross-linking pattern with FNPS irrespective of whether the carboxylase was treated in the absence or presence of the essential activators, i.e., CO_2 and Mg^{2+} (Figure 2).

In conclusion, two diverse approaches—chemical cross-linking and site-directed mutagenesis—identify Lys-166 and Cys-58 or *R. rubrum* ribulose-P_2 carboxylase at an interface between subunits and suggest that a functional catalytic site requires, directly or indirectly, regions of both subunits at this interface.

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