Distance between two active-site lysines of ribulose biphosphate carboxylase from *Rhodospirillum rubrum*

(active-site geometry/chemical cross-linking/4,4'-diisothiocyano-2,2'-disulfonate stilbene)

EVA H. LEE, CLAUDE D. STRINGER, AND FRED C. HARTMAN*

Biology Division, Oak Ridge National Laboratory and University of Tennessee—Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge, TN 37831

Communicated by N. Edward Tolbert, August 27, 1986

ABSTRACT In the absence of a three-dimensional structure of ribulose-biphosphate carboxylase/oxygenase [3-phospho-D-glycerate carboxy-lyase(dimerizing), EC 4.1.1.39], we have probed the distance between two active-site lysyl residues (Lys-166 and Lys-329) of the *Rhodospirillum rubrum* enzyme with 4,4'-diisothiocyano-2,2'-disulfonate stilbene, a covalent cross-linking reagent that spans 12 Å. The reagent rapidly inactivated the carboxylase, and a competitive inhibitor provided substantial protection. To remove products arising from intersubunit or intermolecular cross-linking, the inactivated enzyme was subjected to gel filtration in the presence of urea. Inspection of a tryptic digest of the isolated monomeric fraction revealed that more than half of the incorporated reagent was associated with a single peptide. This peptide was purified by gel filtration, followed by high HPLC. Compositional and sequence analyses of the purified peptide established that it was composed of two chains, encompassing positions 149–168 and 314–337 of the original protein subunit and connected by a cross-link between Lys-166 and Lys-329. Thus, the two active-site lysines of the carboxylase can be juxtaposed within 12 Å, a finding that is consistent with their purported proximity to ribulose biphosphate in the enzyme–substrate complex. The cross-link was not formed when the carboxylase was treated with the reagent either in the presence of a transition-state analogue (carboxyarabinitol biphosphate) or in the absence of CO₂ and Mg²⁺, conditions under which the enzyme exists in a deactivated form.

D-Ribulose-1,5-bisphosphate carboxylase/oxygenase [ribulose-*P*₂ carboxylase; 3-phospho-D-glycerate carboxy-lyase (dimerizing), E.C. 4.1.1.39] plays a pivotal role in plant growth and yield because it catalyzes competing reactions of ribulose-*P*₂ with CO₂ and O₂—the respective initial steps in photosynthetic fixation of CO₂ and in photorespiration (see refs. 1 and 2 for extensive reviews on the structure, mechanism, and regulation of the enzyme). Our laboratory has focused on active-site characterization of the carboxylase and elucidation of the function of implicated residues. Two different active-site lysines of the carboxylase have been identified by affinity labeling and comparative sequence studies (3–6). These essential lysines occupy positions 166 and 329 in the *Rhodospirillum rubrum* enzyme and positions 175 and 334 in the spinach enzyme. Enhanced acidities and nucleophilicities displayed by both active-site lysyl ϵ -amino groups of the *R. rubrum* carboxylase suggest catalytic functionalities, perhaps in discrete proton-transfer steps (7). Such an involvement in the reaction pathway dictates proximity of these essential ϵ -amino groups to ribulose-*P*₂ within the enzyme–substrate complex and, hence, proximity to each other. This supposition cannot be evaluated directly because a three-dimensional structure of ribulose-*P*₂ carboxylase

from any source has not been solved despite considerable effort (8–13). Thus, we were led to examine the spatial relationship between the two active-site lysines by use of bifunctional reagents. In this paper, we report that Lys-166 and Lys-329 of the *R. rubrum* carboxylase are efficiently cross-linked by 4,4'-diisothiocyano-2,2'-disulfonate stilbene (DIDS), a reagent capable of bridging 12 Å.

MATERIALS AND METHODS

Materials. Commercial materials and vendors were as follows: Bicine, Hepes, glutathione, NADH, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate phosphokinase, glycerophosphate dehydrogenase/triose phosphate isomerase, and D-ribose 5-phosphate were from Sigma; tosylphenylalanyl chloromethyl ketone-treated trypsin was from Millipore; Chelex 100 resin was from Bio-Rad; iodoacetic acid was from Aldrich and was recrystallized from ether/petroleum ether; sequential grade trifluoroacetic acid and DIDS were from Pierce; HPLC-grade acetonitrile was from Burdick and Jackson Laboratories (Muskegon, MI); buffers and ninhydrin for amino acid analyses were from Beckman; and reagents for peptide sequencing were from Applied Biosystems (Foster City, CA). Ribulose-*P*₂, 2-carboxyarabinitol 1,5-bisphosphate (carboxyarabinitol-*P*₂), and 2-carboxyribitol 1,5-bisphosphate (carboxyribitol-*P*₂) were prepared as described (14, 15).

Ribulose-*P*₂ carboxylase from *R. rubrum* was purified as described (16) and stored at –70°C as a concentrated stock (>40 mg/ml) in activation buffer (50 mM Bicine/66 mM NaHCO₃/10 mM MgCl₂/1 mM EDTA, pH 8.0) containing 20% (vol/vol) glycerol and 10 mM 2-mercaptoethanol. The enzyme had a specific activity of 5.5 units per mg in the assay described below. As needed, aliquots of the stock were dialyzed at room temperature against either the activation buffer or deactivation buffer (50 mM Bicine/1 mM EDTA, pH 8.0) to which was added Chelex 100 resin (10 g/liter of buffer) to ensure complete removal of trace metal ions. Protein concentrations were based on absorbancies at 280 nm with molar absorptivity $\epsilon^{1\%} = 12.0 \text{ M}^{-1}\text{cm}^{-1}$ (16). *R. rubrum* ribulose-*P*₂ carboxylase is a homodimer of 52 kDa subunits (6, 16).

Assays for Ribulose-*P*₂ Carboxylase. Slight modification (16) of the spectrophotometric method of Racker (17) was used to monitor carboxylase activity during treatments with DIDS. The absorbance changes were followed with a Perkin-Elmer λ 3B spectrophotometer equipped with a 3600 data station. The temperature of the sample compartment was maintained at $25 \pm 0.1^\circ\text{C}$ with an external circulator.

Abbreviations: ribulose-*P*₂ carboxylase, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; DIDS, 4,4'-diisothiocyano-2,2'-disulfonate stilbene; carboxyarabinitol-*P*₂, 2-carboxyarabinitol 1,5-bisphosphate; carboxyribitol-*P*₂, 2-carboxyribitol 1,5-bisphosphate; >PhNCS, phenylthiohydantoin.

*To whom reprint requests should be addressed.

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.

Amino Acid and Sequence Analyses. Total acid hydrolysis of peptides was achieved in evacuated ($<50 \mu\text{m Hg}$) sealed tubes with 6 M HCl/0.01 M 2-mercaptoethanol at 110°C for 21 hr. Hydrolysates were dried on a Speed Vac concentrator (Savant) and subjected to chromatography on a Beckman 121M amino acid analyzer using Beckman's "3-hour, single-column system." The analyzer was hard-wired to a PDP data acquisition system (Real Time Peripherals, Fort Lauderdale, FL). Computer programs used in data collection and analysis were provided by S. S. Stevens and J. T. Holderman of the Oak Ridge National Laboratory.

Peptides were subjected to automated Edman degradation by using a gas-phase sequencer (Applied Biosystems, model 470A) according to the manufacturer's instructions. Phenylthiohydantoin ($>\text{PhNCS}$)-conjugated amino acids were identified and quantified by HPLC (Laboratory Data Control, Riviera Beach, FL) on a C_{18} column (Spherisorb ODS $5 \mu\text{m}$, $4.6 \times 250 \text{ mm}$) 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 (18) containing 68.5% aqueous sodium acetate (0.01 M, pH 5.20), 31% (vol/vol) acetonitrile, and 0.5% 1,2-dichloroethane; and (ii) a gradient of 10–50% acetonitrile in 0.1% aqueous phosphoric acid.

Cross-Linking of Ribulose- P_2 Carboxylase with DIDS. All protein modifications with DIDS were conducted in the dark. To a 20-ml, gently stirred solution of the carboxylase (5 mg/ml, 96 μM subunit) in activation buffer at room temperature were added five 100- μl portions of freshly prepared 0.01 M DIDS in methanol at 20-min intervals (final DIDS concentration = 245 μM). Before each addition of reagent, 10- μl aliquots of the reaction mixture were diluted with 190 μl of activation buffer for immediate determination of enzymic activity. Separate 50- μl aliquots of the reaction mixture were quenched with 25 μl of 1 M glycine (pH 8.0) for subsequent analysis by NaDodSO₄/PAGE. Only 10% of the initial enzymic activity remained after the final addition of reagent. The entire reaction mixture was then quenched with 10 ml of 1 M glycine (pH 8.0) and treated by standard procedures (19) with 15 mM iodoacetate in the presence of 4 M guanidine in order to carboxymethylate protein sulfhydryl groups. After carboxymethylation, the protein was dialyzed exhaustively against 0.02 M NH_4HCO_3 (pH 8.0), during which time precipitation occurred. Half of the suspension ($\approx 45 \text{ mg}$ of protein) was digested with 900 μg of trypsin at 37°C for 6 hr. The protein in the other half of the suspension was collected by centrifugation, dissolved in 3.5 ml of 6 M urea (buffered to pH 6.8), and subjected to gel filtration (Fig. 1). Fractions comprising the three major peaks were pooled, dialyzed, and digested with trypsin as described above for the unfractionated protein.

Smaller samples of the carboxylase (2 ml at 5 mg/ml) in the presence of 0.3 mM carboxyarabinitol- P_2 or in the absence of CO_2 and Mg^{2+} were also treated with DIDS as described above for the $\text{CO}_2/\text{Mg}^{2+}$ -activated enzyme. In both cases, reactions were terminated by the addition of glycine; subsequent to carboxymethylation of sulfhydryl groups, the DIDS-treated proteins were digested with trypsin (see preceding paragraph).

RESULTS

General Features of the Reaction of DIDS with Ribulose- P_2 Carboxylase. The carboxylase was quite sensitive to DIDS, with the competitive inhibitor carboxyarabinitol- P_2 ($K_i = 2 \times 10^{-6} \text{ M}$; ref. 16) affording substantial protection against inactivation (Fig. 2). Deviation from pseudo-first-order kinetics is not surprising in view of more than one site of modification and the formation of both intra- and intermolecular cross-links as documented below.

Progression of the cross-linking reaction could be monitored by NaDodSO₄/PAGE; samples of the carboxylase at

FIG. 1. Gel filtration of cross-linked, carboxymethylated ribulose- P_2 carboxylase (45 mg) on a $2.5 \times 100 \text{ cm}$ column of Ultrogel AcA 34 (LKB), equilibrated and eluted with 6 M urea/50 mM Hepes/50 mM NaCl/10 mM dithiothreitol/1 mM EDTA, pH 6.8. The peaks labeled aggregate, dimer, and monomer contained 6.4 mg, 12.3 mg, and 20.5 mg of protein, respectively.

various levels of inactivation (prepared as described under *Materials and Methods*) revealed small amounts of aggregates ($\geq 150 \text{ kDa}$), 110-kDa dimers (which would result from either intersubunit or intermolecular cross-linking), and a new monomeric species with an apparent size of 60 kDa, in contrast to 52 kDa as with the untreated enzyme (Fig. 3, lanes 2–5). The peaks that were resolved preparatively by gel filtration of the cross-linked enzyme in the presence of urea (denoted aggregate, dimer, and monomer in Fig. 1) correspond to the bands separated by NaDodSO₄/PAGE (Fig. 3, lanes 6–8). Intramolecular cross-linking, resulting in retention of some tertiary structure even in the presence of NaDodSO₄, presumably accounts for the anomalous mobility of the derivatized monomer (Fig. 3, lane 8). When the deactivated form of the carboxylase (i.e., in the absence of CO_2 and Mg^{2+}) or the carboxylase in the presence of the transition-state analogue carboxyarabinitol- P_2 was treated with DIDS, the 60-kDa monomeric band was not seen on gels (Fig. 3, lanes 9–10).

FIG. 2. Inactivation of ribulose- P_2 carboxylase (0.3 mg/ml, 5.8 μM subunit) by DIDS (50 μM) with (○) and without (●) carboxyarabinitol- P_2 (100 μM) at 25°C in 50 mM Bicine/66 mM MgCl_2 /10 mM MgCl_2 /1 mM EDTA, pH 8.0. The control (□) contained neither DIDS nor carboxyarabinitol- P_2 .

FIG. 3. NaDodSO₄/PAGE of cross-linked ribulose-*P*₂ carboxylase. Electrophoretic conditions were essentially those of Laemmli (20) 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 $\times$ 90 mm in height $\times$ 0.5 mm in thickness. Proteins were visualized with Coomassie blue (0.1%) in methanol/water/acetic acid, 5:5:1 (vol/vol), and destaining was achieved with the same solvent. Protein samples in lanes: 1, untreated control enzyme; 2-5, enzyme treated with DIDS until 67% (lane 2), 34% (lane 3), 10% (lane 4), and 1% (lane 5) of the original activity remained; 6-8, aggregated (lane 6), dimer (lane 7), and monomer (lane 8) fractions isolated by gel filtration (see Fig. 1); 9, enzyme treated with DIDS in the presence of carboxyarabinitol-*P*₂; 10, enzyme treated with DIDS in the absence of CO₂ and Mg²⁺; 11, a mixture of molecular weight standards (Bio-Rad) containing phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) as denoted by the arrows.

Based on the extinction coefficient at 342 nm of DIDS (21), the inactivated enzyme contains 1.5 mol of incorporated reagent per mol of subunit. To evaluate the specificity of incorporation, tryptic digests of the DIDS-modified enzyme were subjected to HPLC. More than half of the total reagent

FIG. 4. HPLC profiles of tryptic digests of cross-linked, carboxymethylated ribulose-*P*₂ carboxylase. The column (250 $\times$ 4.6 mm) of Lichrosorb RP8 was from Laboratory Data Control. The solvent system consisted of 0.1% aqueous trifluoroacetic acid (equilibration) and 0.1% trifluoroacetic acid in acetonitrile (limit) as described (22). Except for A in which all peptides detected at 215 nm are shown, only DIDS-labeled peptides (which absorb at 342 nm) are illustrated. (A and B) A tryptic digest of the carboxylase that had been 90% inactivated by DIDS. (C) A tryptic digest of the monomeric, cross-linked carboxylase isolated by gel filtration (see Fig. 1). (D and E) Tryptic digests of the carboxylase that had been treated in the presence of carboxyarabinitol-*P*₂ or in the absence of CO₂ and Mg²⁺ (D); arrows denote the elution position of the cross-linked peptide.

incorporated was associated with a single peptide that was eluted at 46% acetonitrile; the digest of the monomeric fraction (Fig. 4C) isolated by gel filtration (Fig. 1) was slightly enriched with respect to this labeled peptide in comparison to the digest of unfractionated enzyme (Fig. 4B). Digests of the carboxylase that were modified with DIDS in the presence of carboxyarabinitol-*P*₂ (Fig. 4E) or in the absence of CO₂ and Mg²⁺ (Fig. 4D) contained little, if any, of the labeled peptide

FIG. 5. Purification of the crosslinked peptide from a tryptic digest of DIDS-treated ribulose-*P*₂ carboxylase. The digest (41 mg, 788 nmol) of monomeric polypeptide (see Fig. 1) was subjected to gel filtration on a 1.7 $\times$ 220 cm column of Sephadex G-25, equilibrated, and eluted with 0.02 M NH₄HCO₃ (pH 8.0). Excluding its trailing edge, the single major peak observed at 342 nm represented 456 nmol (58%) of labeled peptide. Aliquots (20 nmol) from the peak fraction were subjected to further purification by HPLC under conditions described in the legend to Fig. 4 (profiles are shown in the *Inset* of duplicate samples monitored at either 215 nm or 342 nm). Fractions of the labeled peptide were collected for characterization.

at 46% acetonitrile despite total incorporations of 1.2 and 1.7 mol of reagent per mol of subunit, respectively. Furthermore, little specificity was exhibited by DIDS when CO₂ and Mg²⁺ were excluded from the modification reaction mixture.

Purification and Characterization of Cross-Linked Peptide.

The major DIDS-labeled peptide found in tryptic digests of the monomeric carboxylase (Fig. 4C) was purified by successive gel filtration and HPLC. Fractionation of the total digest by gel filtration gave the desired peptide (represented in Fig. 5 by the single major peak observed at 342 nm) at a sufficient level of enrichment whereby it could be obtained in pure form by subsequent HPLC (Fig. 5 *Inset*).

Amino acid analysis of the purified, labeled peptide revealed a composition in close agreement with that predicted for an equimolar mixture of two tryptic peptides representing regions 149–168 and 314–337 of the total subunit (Table 1). One of these two peptides could only arise provided Lys-329 was derivatized, thereby preventing attack by trypsin; the peptide containing Lys-166 would be an end-product of digestion, irrespective of its derivatization, because Lys-166 is followed by a prolyl residue. The low recovery of lysine is consistent with only partial regeneration of lysine from its putative thiocarbamoyl derivative during acid hydrolysis of the peptide. A small ninhydrin-positive peak, which we attribute to the lysine derivative, was observed on the chromatograms just ahead of the elution position for tryptophan.

Edman degradation of the purified, labeled peptide confirmed that it consists of two chains that encompass positions 149–168 and 314–337 with the following sequences (Table 2) (see ref. 6 for the sequence of the entire subunit):

Two residues were released at each cycle of the degradation except when the same amino acid occurred at a given position of both chains and in cycles 21–24, which are beyond the COOH terminus of one chain. For example, only >PhNCS-

Table 1. Amino acid composition of cross-linked peptide*

Amino acid	Residues, no.	Theory [†]
Asx	2.1	2
Thr	2.8	3
Ser	2.8	3
Glx	4.3	4
Pro	2.4	2
Gly	10.4	10
Ala	1.1	1
Val	3.6	4
Met	1.7	2
Ile	2.7	3
Leu	2.6	3
Phe	1.1	1
His	0.9	1
Lys	2.6	3
Arg	2.0 [‡]	2

*Based on the A_{342} value, 2.2 nmol of peptide hydrolysate were subjected to analysis.

[†]Predicted composition for an equimolar mixture of tryptic fragments encompassing positions 149–168 and 314–337 of the *R. rubrum* carboxylase (see ref. 6 for the complete primary structure).

‡Arbitrary assignment.

Table 2. Sequence analysis of cross-linked peptide*

Cycle	>PhNCS- amino acid	Yield, [†] pmol	Cycle	>PhNCS- amino acid	Yield, [†] pmol
1	Val	1466	13	Val	668
	Leu	1419		Gly	337
2	Leu	1197	14	Gly	367
	Gln	1219		Phe	337
3	Gly	1679	15	Thr	— [‡]
				Gly	309
4	Arg	285	16	Ile	220
	Ala	1454		Lys	— [§]
5	Pro	1242	17	Ile	179
	Ser	— [‡]		Met	363
6	Glu	1029	18	Lys	— [§]
	Gly	850		Glu	201
7	Val	1014	19	Pro	246
	Ile	1068		Gly	170
8	Asp	512	20	Lys	109
	His	206		Glu	111
9	Gly	527	21	Ser	— [‡]
	Thr	— [‡]			
10	Gly	801	22	Ser	— [‡]
11	Leu	648	23	Asp	62
	Thr	— [‡]			
12	Val	671	24	Arg	44
	Met	669			

*Sample size: 2060 pmol.

[†]Repetitive yield: 91%.

[‡]Due to decomposition of >PhNCS-Ser and >PhNCS-Thr, their yields are not meaningful.

[§]A derivatized lysyl residue is presumed to occupy this position on the basis of the observed gap.

glycine was found at cycles 3 and 10, as anticipated from the known sequence of the enzyme. The location of the covalent cross-link between Lys-166 and Lys-329 was established by the complete absence at cycles 16 and 18 of the >PhNCS derivative of lysine, which would have appeared if the two lysines were not covalently modified.

DISCUSSION

We first suggested proximity of two essential lysyl residues of ribulose- P_2 carboxylase based on the observation that both were reactive toward the affinity label 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate, but within a given catalytic subunit, only one residue was modified (23). A similar situation arose with the arylating reagent trinitrobenzenesulfonate, whose specificity for either of the two active-site lysines was dependent on the species of ribulose- P_2 carboxylase examined (7). Having observed a high degree of active-site specificity with a general lysyl reagent containing an anionic aromatic nucleus (trinitrobenzenesulfonate), we were prompted to evaluate DIDS (a bifunctional phenylisothiocyanate also possessing the anionic sulfonate substituent) as a cross-linking agent for the two active-site lysines.

DIDS was first introduced as a probe of membrane proteins involved in anion permeability (21, 24). As a stilbene derivative, DIDS undergoes light-catalyzed *cis-trans* isomerization (21); since this process could be monitored by HPLC, we

were able to demonstrate that the commercial preparation used in the present study was the pure trans isomer, whose span is 12 Å (24). To avoid isomerization, chemical modifications with DIDS were carried out in the dark with freshly prepared solutions.

The specificity of DIDS in its reaction with ribulose-*P*₂ carboxylase is rather remarkable in view of the enzyme's numerous nucleophilic sidechains, including 19 residues of lysine per molecule of subunit. At a 2.6-fold molar excess of DIDS over subunit, the carboxylase is almost completely inactivated with the concomitant incorporation of only 1.5 molar equivalents of reagent. More than half of the incorporated reagent, visualized by HPLC of tryptic digests (Fig. 4 *B* and *C*), is associated with a cross-link between Lys-166 and Lys-329. This cross-link must be intrasubunit, as opposed to intersubunit or intermolecular, because the pertinent peptide was isolated from the monomeric fraction of the inactivated carboxylase obtained by gel filtration under denaturing conditions (Fig. 1). Thus, the ϵ -amino groups of these two active-site lysines are clearly within 12 Å of each other.

In the absence of CO₂ and Mg²⁺, ribulose-*P*₂ carboxylase exists in a catalytically inactive form. Activation entails chemical reaction of CO₂ with a specific lysyl ϵ -amino group—Lys-201 with the spinach enzyme (25) and Lys-191 with the *R. rubrum* enzyme (26)—to form a carbamate, which requires a divalent cation for stabilization (27). A variety of data suggests conformational differences between the activated and deactivated forms of ribulose-*P*₂ carboxylase (28–32). Hence, it was of interest to ascertain whether the covalent bridge between Lys-166 and Lys-329 could be formed in the deactivated enzyme. When CO₂ and Mg²⁺ were excluded from the buffer in which the DIDS-derivatization of the carboxylase was carried out, reagent specificity was diminished, and the cross-linked peptide was not observed in significant amount (Fig. 4*D*). The simplest interpretation of these results is that the two essential lysines are farther apart in the deactivated enzyme and that the conformational change induced by carbamate formation is crucial to proper alignment of catalytic groups. Possible influence of the carbamate on active-site conformation is quite plausible because the activator site and the catalytic site are contiguous (33). Not surprisingly, the transition-state analogue carboxy-arabinitol-*P*₂, which forms a very tight exchange-inert complex with the carboxylase (2, 15), also prevents cross-linking of the two active-site lysines (Fig. 4*E*).

A 12-Å distance between essential ϵ -amino groups at the active site of the carboxylase may be compared to the experimentally measured distance of 6 Å between the essential divalent metal cation and the phosphorus atoms of enzyme-bound ribulose-*P*₂ (33); also, the distance between phosphorus atoms in the fully-extended conformation of ribulose-*P*₂ is 11 Å. We stress that covalently linking Lys-166 and Lys-329 with a reagent that spans 12 Å does not prove that this distance prevails within the catalytically competent enzyme. Because of the length and flexibility of lysyl side chains, two ϵ -amino groups of a protein that can approach van der Waals' contact radius of ≈ 4 Å (34) can also separate by ≈ 18 Å without movement of their respective α -carbon atoms. As the mean of this range is very close to the distance between the two active-site lysines of the carboxylase as measured with DIDS, we conclude that the essential groups could be as close as 4 Å. Irrespective of the precise distance, the results of the present study are compatible with proximity

of Lys-166 and Lys-329 as dictated by their purported catalytic functionalities.

This research was sponsored jointly by the Office of Health and Environmental Research, U.S. Department of Energy under Contract DE-AC05-84OR21400 with the Martin Marietta Energy Systems, Inc., and by Grant PCM-8207516 from the National Science Foundation.

1. Lorimer, G. H. (1981) *Annu. Rev. Plant Physiol.* **32**, 349–383.
2. Mizioroko, H. M. & Lorimer, G. H. (1983) *Annu. Rev. Biochem.* **52**, 507–535.
3. Stringer, C. D. & Hartman, F. C. (1978) *Biochem. Biophys. Res. Commun.* **80**, 1043–1048.
4. Herndon, C. S., Norton, I. L. & Hartman, F. C. (1982) *Biochemistry* **21**, 1380–1385.
5. Fraij, B. & Hartman, F. C. (1983) *Biochemistry* **22**, 1515–1520.
6. Hartman, F. C., Stringer, C. D. & Lee, E. H. (1984) *Arch. Biochem. Biophys.* **232**, 280–295.
7. Hartman, F. C., Milanez, S. & Lee, E. H. (1985) *J. Biol. Chem.* **260**, 13968–13975.
8. Johal, S., Bourque, D. P., Smith, W. W., Suh, S. W. & Eisenberg, D. (1980) *J. Biol. Chem.* **255**, 8873–8880.
9. Barcena, J. A., Pickersgill, R. W., Adams, M. J., Phillips, D. C. & Whatley, F. R. (1983) *EMBO J.* **2**, 2363–2367.
10. Andersson, I. & Branden, C.-I. (1984) *J. Mol. Biol.* **172**, 363–366.
11. Janson, C. A., Smith, W. W., Eisenberg, D. & Hartman, F. C. (1984) *J. Biol. Chem.* **259**, 11594–11596.
12. Meisenberger, O., Pilz, I., Bowien, B., Pal, G. P. & Saenger, W. (1984) *J. Biol. Chem.* **259**, 4463–4465.
13. Schneider, G., Branden, C.-I. & Lorimer, G. (1986) *J. Mol. Biol.* **187**, 141–143.
14. Horecker, B. L., Hurwitz, J. & Weissbach, A. (1958) *Biochem. Prep.* **6**, 83–90.
15. Pierce, J., Tolbert, N. E. & Barker, R. (1980) *Biochemistry* **19**, 934–942.
16. Schloss, J. V., Phares, E. F., Long, M. V., Norton, I. L., Stringer, C. D. & Hartman, F. C. (1982) *Methods Enzymol.* **90**, 522–528.
17. Racker, E. (1973) in *Methods of Enzymatic Analysis*, ed. Bergmeyer, H. U. (Academic, New York), pp. 188–190.
18. Lottspeich, F. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1829–1834.
19. Schloss, J. V., Stringer, C. D. & Hartman, F. C. (1978) *J. Biol. Chem.* **253**, 5707–5711.
20. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
21. Cabantchik, Z. I. & Rothstein, A. (1974) *J. Membr. Biol.* **15**, 207–226.
22. Mahoney, W. C. & Hermodson, M. A. (1980) *J. Biol. Chem.* **255**, 11199–11203.
23. Norton, I. L., Welch, M. H. & Hartman, F. C. (1975) *J. Biol. Chem.* **250**, 8062–8068.
24. Cabantchik, Z. I. & Rothstein, A. (1972) *J. Membr. Biol.* **10**, 311–330.
25. Lorimer, G. H. (1981) *Biochemistry* **20**, 1236–1240.
26. Donnelly, M. I., Stringer, C. D. & Hartman, F. C. (1983) *Biochemistry* **22**, 4346–4352.
27. Lorimer, G. H. & Mizioroko, H. M. (1980) *Biochemistry* **19**, 5321–5328.
28. Siegel, M. I. & Lane, M. D. (1972) *Biochem. Biophys. Res. Commun.* **48**, 508–516.
29. Grebanier, A. E., Champagne, D. & Roy, H. (1978) *Biochemistry* **17**, 5150–5155.
30. Mizioroko, H. M. & Sealy, R. C. (1980) *Biochemistry* **19**, 1167–1171.
31. Jordan, D. B. & Chollet, R. (1983) *J. Biol. Chem.* **258**, 13752–13758.
32. Jordan, D. B., Chollet, R. & Ogren, W. L. (1983) *Biochemistry* **22**, 3410–3418.
33. Pierce, J. & Reddy, G. S. (1986) *Arch. Biochem. Biophys.* **245**, 483–493.
34. Robson, B. & Platt, E. (1986) *J. Mol. Biol.* **188**, 259–281.