Demonstration of a Functional Requirement for the Carbamate Nitrogen of Ribulosebisphosphate Carboxylase/Oxygenase by Chemical Rescue†

Harry B. Smith and Fred C. Hartman*

Biology Division, Oak Ridge National Laboratory, and University of Tennesee—Oak Ridge Graduate School of Biomedical Sciences, P.O. Box 2009, Oak Ridge, Tennessee 37831-8077

Received October 10, 1990; Revised Manuscript Received January 18, 1991

ABSTRACT: Ribulosebisphosphate carboxylase/oxygenase is reversibly activated by the reaction of CO_2 with a specific lysyl residue (Lys191 of the *Rhodospirillum rubrum* enzyme) to form a carbamate that coordinates an essential Mg^{2+} cation. Surprisingly, the Lys191 → Cys mutant protein, in the presence of CO_2 and Mg^{2+}, exhibits tight binding of the reaction intermediate analogue 2-carboxyarabinitol bisphosphate [Smith, H. B., Larimer, F. W., & Hartman, F. C. (1988) *Biochem. Biophys. Res. Commun. 152*, 579–584], a property normally equated with effective coordination of the Mg^{2+} by the carbamate. Catalytic ineptness of the Cys191 mutant protein, despite its ability to coordinate Mg^{2+} properly, might be due to the absence of the carbamate nitrogen. To investigate this possibility, we have evaluated the ability of exogenous amines to restore catalytic activity to the mutant protein. Significantly, the Cys191 protein manifests ribulose bisphosphate dependent fixation of $^{14}CO_2$ when incubated with aminomethanesulfonate but not ethanesulfonate. This novel activity reflects a K_m value for ribulose bisphosphate which is not markedly perturbed relative to wild-type enzyme, a K_m for Mg^{2+} which is in fact decreased 10-fold, and rate saturation with respect to aminomethanesulfonate (K_d = 8 mM). Chromatographic and spectrophotometric analyses reveal the product of CO_2 fixation to be D-3-phosphoglycerate, while turnover of [1-^{3}H]ribulose bisphosphate into [^{3}H]phosphoglycolate confirms oxygenase activity. We conclude that aminomethanesulfonate restored ribulosebisphosphate carboxylase/oxygenase activities to the Cys191 mutant protein by providing a nitrogenous function which satisfies a catalytic demand normally met by the carbamate nitrogen of Lys191.

Toney and Kirsch (1989) have described a new approach, which they designate "chemical rescue", to discern the roles of active-site residues. A putative active-site residue is replaced by site-directed mutagenesis, thereby rendering the enzyme deficient in catalysis. In some cases, the crippled mutant protein can be partially rehabilitated merely through noncovalent interaction with an exogenously added compound. For example, the virtually inactive K258A[1] mutant of aspartate aminotransferase is stimulated by primary amines; the degree of stimulation, after correcting for steric effects, correlates with the pK_a of the amine in accordance with the Brønsted relationship. These observations provide strong, direct evidence that the ϵ-amino group of Lys258 is the catalytic base that abstracts the α-carbon proton from the aldimine intermediate as postulated from earlier crystallographic studies (Kirsch et al., 1984).

In the present investigation, we have applied chemical rescue to a long-standing issue concerning the mechanism of ribulosebisphosphate carboxylase/oxygenase (EC 4.1.1.39), namely, the basis of the requirement for protein carbamylation. Both activities are absolutely dependent on nonenzymic derivatization of an active-site lysyl residue (Lys191 of the *Rhodospirillum rubrum* enzyme) by CO_2 to form a carbamate, which is stabilized by the catalytically essential Mg^{2+} cation (Laing & Christeller, 1976; Lorimer et al., 1976; Lorimer & Miziorko, 1980; Lorimer, 1981; Donnelly et al., 1983). As predicted by EPR and NMR[2] spectroscopy (Styring & Brändén, 1985; Pierce & Reddy, 1986) and recently proven by crystallography (Andersson et al., 1989; Knight et al., 1990), the oxyanion of the carbamate provides one ligand for the Mg^{2+} ion. If the only role of the carbamate is to assist in coordination of Mg^{2+}, the obligate dependence of catalytic competence on carbamate formation appears enigmatic, because equivalent coordination of the metal cation could conceivably have been achieved more directly by means of an aspartyl or glutamyl side chain. Invoking a catalytic demand for the carbamate nitrogen directly, however, argues that a protein carboxylate would provide inadequate in supporting catalysis.

An investigation of the properties of K191C *R. rubrum* ribulose-P_2 carboxylase provides indirect evidence of a role for the carbamate that transcends metal ion binding (Smith et al., 1988). Surprisingly, the K191C mutant protein, lacking the carbamate-forming lysyl residue, retains the capacity to bind tightly the reaction intermediate analogue carboxyarabinitol-P_2 in a CO_2/Mg^{2+}-dependent manner normally indicative of carbamate formation and proper coordination of metal ion (Miziorko & Sealy, 1980; Hall et al., 1981). Despite its manifestation of the ligand-binding properties which closely mimic those of the wild-type enzyme, the mutant protein is devoid of detectable carboxylase activity, thereby implicating the carbamate nitrogen per se in catalysis. To explore this

† This research was sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under Contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

* Address correspondence to this author at the Biology Division, Oak Ridge National Laboratory.

[1] The single-letter code for amino acids is used to designate mutants. The first letter denotes the amino acid present in the wild-type enzyme at the numbered position. The final letter denotes the amino acid present at the corresponding position in the mutant.

[2] Abbreviations: AMS, aminomethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; carboxyarabinitol-P_2, 2-carboxyarabinitol 1,5-bisphosphate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DEAE-cellulose, (diethylaminoethyl)cellulose; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; Pipes, 2,2′-piperazine-1,4-diylbis(2-ethanesulfonic acid); ribulose-P_2, D-ribulose 1,5-bisphosphate; ribulose-P_2 carboxylase, ribulosebisphosphate carboxylase/oxygenase; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/91/0430-5172$02.50/0

possibility more fully, we have evaluated the ability of exogenous amines to "rescue" the mutant protein. Here, we describe a specific interaction of the K191C mutant of ribulose-P_2 carboxylase with a single exogenous amine, viz., AMS, which partially restores catalytic activity in a manner fully consistent with a specific role for the carbamate nitrogen.

Experimental Procedures

Materials. Commonly used chemicals, reagents, substrates, and enzymes were of the highest purity commercially available. AMS and other chemicals tested for stimulatory effects upon catalytic activities of mutant forms of ribulose-P_2 carboxylase were procured from Aldrich. AMS was also purchased from Sigma and, irrespective of source, eluted in a single, well-resolved peak between cysteic acid and (carboxymethyl)cysteine when subjected to amino acid analysis (with the single-column system of the Beckman 121M amino acid analyzer). Ribulose-P_2, [1-^{3}H]ribulose-P_2, and carboxyarabinitol-P_2 were synthesized according to published procedures (Horecker et al., 1958; Kuehn & Hsu, 1978; Pierce et al., 1980). Na$H^{14}CO_3$ was obtained from ICN. Ribulose-P_2 carboxylase from *R. rubrum* and mutant proteins expressed in *Escherichia coli* were purified by DEAE-cellulose chromatography as reported elsewhere (Smith & Hartman, 1988); construction and expression of the gene encoding the K191C and K191A mutant proteins have been described previously (Smith et al., 1988; Larimer et al., 1990).

Protein and Enzyme Assays. The dye-binding method of Bradford (1976), with the required reagent obtained from Bio-Rad, was used to determine protein concentration; the *R. rubrum* carboxylase served as the standard.

Carboxylase activity was determined by a modification (Niyogi et al., 1986) of the $^{14}CO_2$ fixation procedure (Lorimer et al., 1977). Typical assay mixtures (final volume = 125 μL; pH = 8.0) contained 13 mM Na$H^{14}CO_3$ (23 mCi/mmol), 25 mM Bicine, 5 mM $MgCl_2$, 0.7 mM ribulose-P_2, 80 μg of bovine serum albumin, and 0.24 μg of purified native *R. rubrum* ribulose-P_2 carboxylase or 50–200 μg of the purified K191C mutant protein; assays of mutant protein additionally contained 100 mM AMS unless noted otherwise. In instances where carboxylase activity was examined as a function of pH, 25 mM Bicine was replaced by 100 mM Pipes (pH 6.5–7.0), 100 mM Tris (pH 7.5), 100 mM Bicine (pH 8.0–8.5), or 100 mM Ches (pH 9.0–9.5). Reactions were initiated by addition of ribulose-P_2 to the otherwise complete solutions which had been incubated at room temperature for 1 h. Fixation of $^{14}CO_2$ by the wild-type enzyme was then monitored by acid quenching of 30-μL aliquots of reaction mixtures during 0.5–1.5 min. Carboxylase activity was similarly detected in reaction mixtures of the K191C protein during 1–3 h.

To assess oxygenase activity of the K191C protein in the presence of AMS, production of [^{3}H]phosphoglycolate from [1-^{3}H]ribulose-P_2 was examined. Mixtures of 13 mM $NaHCO_3$, 25 mM Bicine, 5 mM $MgCl_2$, 156 μg of purified K191C mutant protein, and 100 mM AMS (final volume = 200 μL; pH = 8.0) were incubated at room temperature for 1 h before addition of 0.4 mM [1-^{3}H]ribulose-P_2 (12 200 cpm/nmol); reactions were quenched 8.5 h later with sodium borohydride (2.2 mM). Excess borohydride was consumed 5 min later by addition of glucose (4.9 mM). To reduce [^{3}H]phosphoglycerate to [^{3}H]phosphoglycerol, which can be readily separated from [^{3}H]phosphoglycolate by DEAE-cellulose chromatography according to an established procedure (Smith et al., 1990), quenched reaction mixtures were added to pH 8.0 buffered solutions of glyceraldehydephosphate dehydrogenase (15 units/mL), phosphoglycerate kinase (70 units/mL), phosphoglycerol dehydrogenase (3 units/mL), and triosephosphate isomerase (28 units/mL) in 50 mM Bicine, 10 mM ATP, 66 mM $NaHCO_3$, 10 mM $MgCl_2$, and 0.25 mM NADH. After 25 min at room temperature, the resulting solutions were diluted with water to a final volume of 2 mL and filtered through a Centricon-10 apparatus. The deproteinated filtrates were then applied to 5-mL columns of DEAE-cellulose equilibrated in 25 mM NH_4CO_3 (pH 8.0). Isotopically labeled material was then eluted from the columns with a 120-mL linear gradient of 25–275 mM NH_4HCO_3 (pH 8.0). Column fractions (1 mL) were collected and analyzed radiometrically.

Identification of the K191C/AMS Carboxylase Reaction Product. To confirm that observed fixation of $^{14}CO_2$ reflected production of D-3-phosphoglycerate, reaction mixtures were subjected to DEAE chromatography with and without prior reduction through the NADH-coupled enzyme system that converts phosphoglycerate to phosphoglycerol (see preceding paragraph). Carboxylase assay mixtures (400 μL) were divided into two equal portions after reaction times of 8.5 h. To each of these was then added 1 mL of a solution (pH 8.0) containing 10 mM ATP, glyceraldehydephosphate dehydrogenase (22 units), phosphoglycerate kinase (100 units), phosphoglycerol dehydrogenase (5 units), and triosephosphate isomerase (40 units). One of the two portions additionally received NADH to a final concentration of 0.25 mM. After 30 min, both samples were acidified with 100 mM HCl and dried in vacuo to remove volatile $^{14}CO_2$; the residues were redissolved in 1 mL of H_2O. A portion (100 μL) of each resulting solution (~5000 cpm) was diluted to 10 mL with 25 mM NH_4CO_3 (pH 8.0) containing a trace quantity of [^{3}H]ribulose-P_2 as a chromatographic standard and subjected to DEAE-cellulose chromatography as detailed in the preceding paragraph. In separate experiments, oxidation of NADH, concomitant with conversion of 3-phosphoglycerate to phosphoglycerol, was confirmed by direct monitoring at 340 nm. Carboxylase reaction mixtures of 1.5 mL were first assayed for $^{14}CO_2$ fixation and then dried in vacuo as before after an 8.5-h reaction period. The resulting dry residues were redissolved in 200 μL of H_2O and transferred to cuvettes containing the appropriate coupling enzymes as above and 0.25 mM NADH in a final volume of 400 μL. After establishing an absorbance base line at 340 nm, 0.25 mM ATP was added to the cuvette and absorbance was monitored thereafter. As a negative control, a 1.5-mL "carboxylase reaction" mixture which contained all ingredients (including AMS) except for protein was similarly dried and analyzed.

Additional methods may be found in figure legends.

Results

The K191C mutant protein remains catalytically inept under standard conditions which fully activate the wild-type enzyme (5 mM Mg^{2+}, 13 mM Na$H^{14}CO_3^-$, pH 8.0). When AMS, irrespective of commercial vendor, is included during preincubation and assay, however, significant levels of $^{14}CO_2$-fixation activity are restored to the mutant protein (Figure 1). AMS cannot promote such fixation of $^{14}CO_2$ in the absence of either the K191C mutant protein or ribulose-P_2. The novel activity is totally inhibited by carboxyarabinitol-P_2 and is reminiscent of wild-type carboxylation in its absolute dependence on divalent metal cation. Mn^{2+} will sustain the fixation at 40% of the rate supported by Mg^{2+}, whereas the rate of the wild-type activity is reduced to 15% of the Mg^{2+}-supported rate (data not shown). Restoration of activity to the mutant protein appears to require the amino moiety of AMS, because ethanesulfonate or methanesulfonate are not stimulatory. By

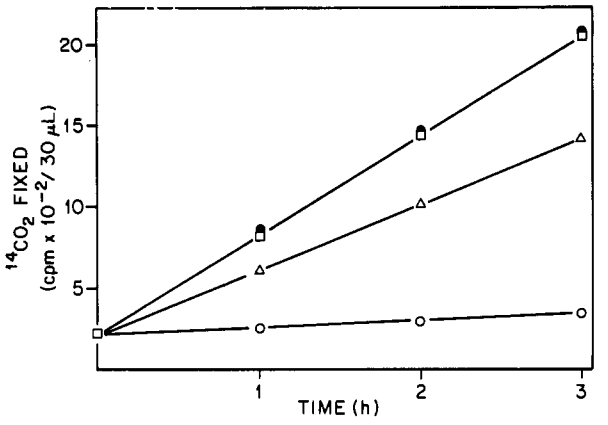


FIGURE 1: Time course of $^{14}CO_2$ fixation as promoted by the K191C mutant protein (0.78 mg/mL) in the presence of 100 mM (□), 10 mM (△), or 0 mM (○) AMS. The background rate of fixation represented by open circles (○) is identically observed in the presence of 100 mM AMS provided that (1) carboxyarabinitol-P_2 (20 μM) is present, (2) the K191C protein is absent, or (3) Mg^{2+} is absent. The rate of $^{14}CO_2$ fixation is also shown as promoted by K191C protein which had been preincubated at higher concentration (13.5 mg/mL) with 100 mM AMS prior to dilution into a complete assay mixture (final protein concentration = 0.78 mg/mL; final AMS concentration = 5 mM) (●). Under the standard assay conditions, 1 nmol of CO_2 fixed per 30 μL of the reaction mixture is equivalent to 15000 cpm. (See text for further details.)

contrast, ammonia can endow K191C with the ability to fix $^{14}CO_2$, although at only one-fourth the rate detected with an equivalent concentration of AMS (data not shown). In addition, the cysteinyl residue at position 191 seems necessary for the acquisition of $^{14}CO_2$-fixation activity, because the K191A mutant protein is refractory to the stimulatory effects of AMS.

The requirements for the fixation of $^{14}CO_2$, as outlined above, prove to be kinetically characteristic of an enzymatic activity. While the rate of fixation is a linearly dependent upon the concentration of the K191C protein, hyperbolic kinetics are observed with respect to AMS concentation (Figure 2). Lineweaver–Burk analysis yields an apparent K_d value for AMS of 8 mM and a k_{cat} which is $\sim 10^{-5}$ times that of wild-type enzyme. This latter value reflects a specific activity of 3×10^{-5} units/mg in comparison to 3 units/mg for the wild-type enzyme. Similarly, the K191C/AMS protein displays a K_m for ribulose-P_2 (35 μM) that is not drastically altered relative to the wild-type value (10 μM) and a K_m for Mg^{2+} that is indeed 10-fold lower (0.3 mM vs 3 mm). In contrast to stimulation of the K191C protein, AMS is a mixed-type inhibitor of the wild-type enzyme with a K_i of about 50 mM.

Although the AMS-induced stimulation of K191C protein is highly reproducible, the order of addition of AMS vs ribulose-P_2 is crucial to the observed CO_2-fixation activity. When ribulose-P_2 is included during the preincubation of protein under activation conditions (i.e., 5 mM Mg^{2+}, 13 mM $H^{14}CO_3^-$, pH 8.0), subsequent addition of AMS does not restore enzyme activity. Furthermore, once the K191C protein has been allowed to interact with AMS during the preincubation period, the specific activity of $^{14}CO_2$ fixation during assay is no longer dependent upon the concentration of the exogenous amine. For example, when K191C mutant protein (13.5 mg/mL) and AMS (100 mM) were preincubated together and then diluted into otherwise complete carboxylase reaction mixtures (final [AMS] = 5 mM, [protein] = 0.78 mg/mL), the observed rate of fixation was equivalent to that for the standard assay reaction (final [AMS] = 100 mM, [protein] = 0.78 mg/mL) as shown in Figure 1.

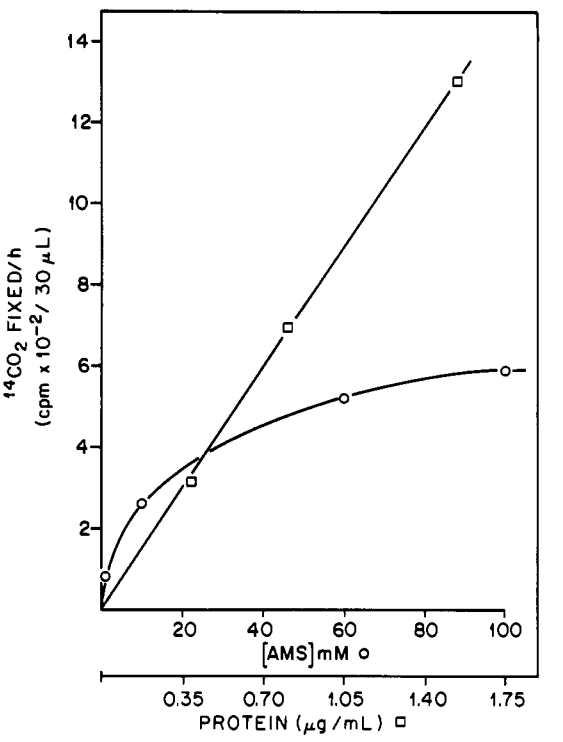


FIGURE 2: Rate of K191C/AMS-promoted $^{14}CO_2$ fixation as a function of AMS (○) and protein (□) concentrations. Carboxylase activity was monitored as described in the text, except that protein concentration was varied (0.35–1.5 mg/mL) at a constant concentration of AMS (100 mM) or the AMS concentration was varied (1–100 mM) at a constant concentration of protein (0.78 mg/mL).

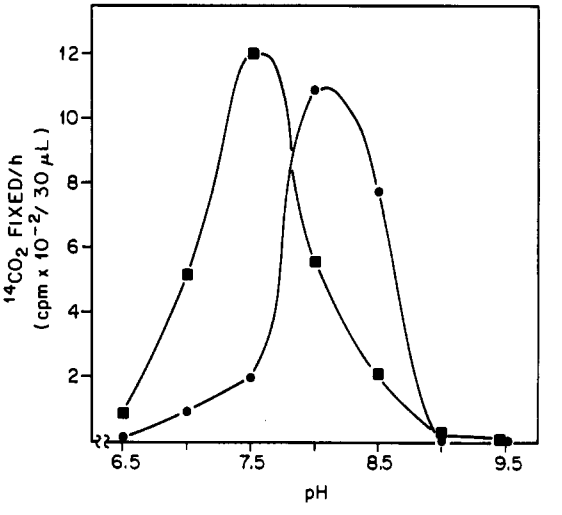


FIGURE 3: Rates of $^{14}CO_2$ fixation supported by K191C/AMS (0.78 mg/mL) (●) and wild-type enzyme (0.2 μg/mL) (■) as a function of pH.

The profiles of the K191C/AMS and wild-type carboxylase activities at varied pH values are shown in Figure 3. A bell-shaped curve, typifying an enzyme activity, is observed in the analysis of the K191C protein. The pH optimum for the K191C/AMS system (~8.1) is somewhat higher than that observed for the wild-type enzyme (~7.7), and major differences between the two profiles are observed in the alkaline range. For example, activity of the K191C protein in the presence of AMS drops precipitously at pH values greater than 8.5.

Fixation of $^{14}CO_2$ resulting from the coincubation of the K191C mutant protein and AMS reflects an authentic ribulose-P_2 carboxylase activity, inasmuch as ^{14}C-labeled D-3-phosphoglycerate is produced. Identification of the product

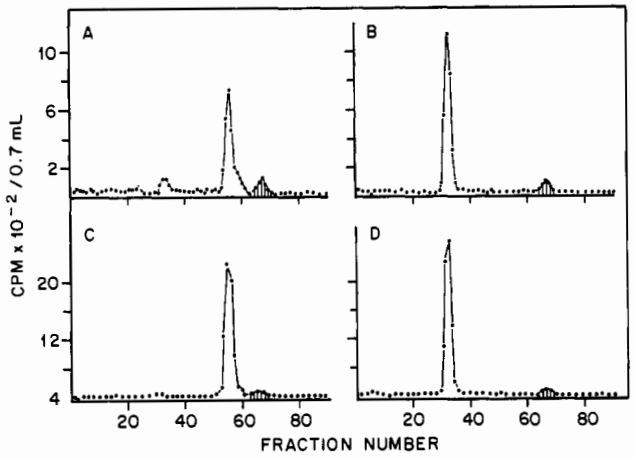


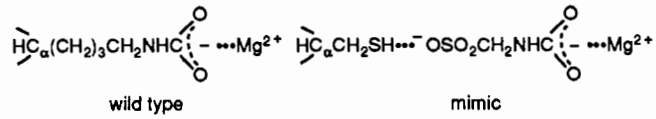
FIGURE 4: DEAE chromatographic identification of the labeled product of $^{14}CO_2$ fixation as catalyzed by K191C/AMS and wild-type carboxylase before (panels A and C, respectively) and after (panels B and D, respectively) enzyme-coupled reduction by NADH as described in the text. The cross-hatched areas represent tritiated ribulose-P_2 added to the samples as markers.

as phosphoglycerate is based on chromatographic analysis and on its enzyme-catalyzed reduction to phosphoglycerol (see Experimental Procedures). As can be seen in Figure 4, radioactivity fixed by the K191C protein in the presence of AMS elutes from DEAE-cellulose (panel A) at the same position as that fixed by the wild-type enzyme (i.e., D-3-phosphoglycerate, panel C). After enzyme-coupled reduction of the reaction mixture with NADH, however, elution of the K191C/AMS product (panel B) coincides with similarly treated product of wild-type $^{14}CO_2$ fixation (i.e., phosphoglycerol, panel D). The observed shift in the position of elution truly reflects enzymatic reduction of the product, for the appropriate decrease in absorption at 340 nm, indicative of the concomitant oxidation of NADH, is detected spectrophotometrically (data not shown). It is noteworthy that the phosphoglycerate produced by the K191C/AMS incubation consists entirely of the D-epimer, as revealed by its total enzymatic conversion into phosphoglycerol. This demonstrates a stereospecific protonation of the terminal intermediate in the overall reaction pathway (a C-2 carbanion of 3-phosphoglycerate) as occurs during normal wild-type enzymatic turnover.

Detection of [^{3}H]phosphoglycolate in reaction mixtures of [1-^{3}H]ribulose-P_2 with K191C/AMS confirms ribulose-P_2 oxygenase activity (Figure 5). DEAE chromatographic analysis of [^{3}H]phosphoglycolate (peak III) is feasible, because the NADH-coupled enzymatic treatment of reaction mixtures converts phosphoglycerate (see previous paragraph), which would otherwise coelute with phosphoglycolate, to phosphoglycerol (peak II). Appearance of peak I is not dependent on AMS and is not suppressed even in the presence of carboxyarabinitol-P_2. This peak, as well as a portion of peak II, can be attributed to a contaminating phosphatase activity detectable with *p*-nitrophenyl phosphate as substrate according to an established assay procedure (Worthington, 1988). Peak IV represents nonutilized [1-^{3}H]ribulose-P_2.

Discussion

Despite the elucidation of the three-dimensional structure of the carboxylase (Andersson et al., 1989; Knight et al., 1990), the only role heretofore ascribed to the carbamate is participation of its oxyanion in coordination of Mg^{2+}. The purpose of our present investigation was to assess the possibility of a catalytic role for the carbamate nitrogen of ribulose-P_2 carboxylase as suggested indirectly by the catalytically incompetent K191C mutant (Smith et al., 1988). A possible explanation for the observed ligand-binding properties of K191C would be for Cys191 to interact via hydrogen bonding with carbonate (or bicarbonate) anion, whereby a necessary ligand for proper coordination of the Mg^{2+} cation is introduced. The standard length of a hydrogen bond linking a sulfhydryl group to an oxygen atom of the bound carbonate species would place the Mg^{2+} in a position approximating that in the activated form of the wild-type enzyme. In preliminary studies, ^{13}C NMR spectroscopy has indeed suggested such an interaction (G. Lorimer and S. Gutteridge, personal communication). Irrespective of the precise mode of interaction, the accommodation of a molecule of carbonate (or bicarbonate) suggests that other anionic species might be similarly anchored at the active site of K191C so as to provide the potential for restoration of catalytic competence by the method of chemical rescue (Toney & Kirsch, 1989). Given this rationale, we have evaluated the catalytic consequences of incubating the K191C mutant protein, under conditions which would otherwise promote carboxylation of ribulose-P_2 by the wild-type enzyme, with an anionic species which bears an amine function, i.e., AMS. We assumed that restoration of activity would require (1) interaction of AMS with the protein and (2) carbamylation of the amino group of the bound reagent to generate a mimic of the wild-type carbamate:



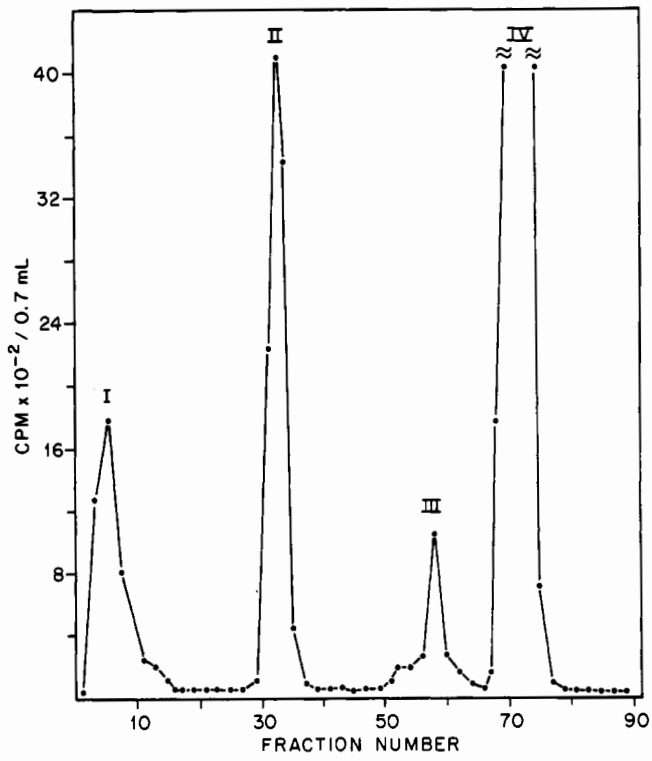


FIGURE 5: DEAE chromatographic detection of [^{3}H]phosphoglycolate (peak III) produced from [^{3}H]ribulose-P_2 (peak IV) in the presence of K191C/AMS. [^{3}H]Phosphoglycerate had undergone NADH-coupled reduction to [^{3}H]phosphoglycerol (peak II) prior to chromatography as detailed in the text.

Several lines of evidence support the conclusion that AMS-induced restoration of carboxylase activity is dependent on discrete complexation of the amine with mutant protein. Chief among these is the observed saturation of the $^{14}CO_2$-fixation rate with respect to amine concentration (an apparent

K_d of 8 mM). Credence to the notion that complex formation entails hydrogen bonding to the Cys191 sulfhydryl is provided by the failure of AMS to stimulate the catalytically inactive K191A protein and the failure of several aliphatic amines to stimulate the K191C protein. The former finding would appear to exclude the possibility that AMS mediates its effect indirectly through conformational changes. Formation of a protein–AMS complex is also strongly suggested by two distinct order-of-addition effects. First, the apparent V_{max} of fixation, sustained at 100 mM AMS with initiation of the reaction by addition of ribulose-P_2, is also achieved by dilution of a mixture of protein–AMS–Mg^{2+}–CO_2 into a solution of ribulose-P_2, whereby the final concentration of AMS is only 5 mM. Second, if AMS is added to a complete assay solution which includes ribulose-P_2, carboxylase activity is not observed. Presumably, a stable quaternary complex (K191C·CO_3^{2-}·Mg^{2+}–ribulose-P_2) precludes formation of the productive complex containing AMS. This interpretation is consistent with the known CO_2/Mg^{2+}-dependent tight binding of carboxyarabinitol-P_2 by the K191C and wild-type proteins (Smith et al., 1988; Pierce et al., 1980; Schloss, 1988) and by the virtual inaccessibility of the active site to solvent revealed by the crystallographic structure of the activated wild-type enzyme with bound carboxyarabinitol-P_2 (Andersson et al., 1989; Knight et al., 1990).

The a priori knowledge that activation of wild-type carboxylase requires carbamylation prompts the supposition that the catalytic activity displayed by the K191C mutant also requires carbamylation of AMS (either before or after its complexation with protein). Further evidence of obligatory carbamylation of the exogenous appendage includes the inability of alkanesulfonates, devoid of the amino groups, to rescue the crippled protein. The pH profile of the AMS-induced $^{14}CO_2$-fixation activity also provides a basis for suggesting that the active-site appendage undergoes carbamate formation. At moderately high pH (>8.5), where the concentration of CO_2 is depleted through equilibrium with carbonic acid, the weak nucleophilicity of the AMS amino group (pK_a = 5.8) (Jencks & Regenstein, 1976) would limit carbamylation and thereby explain the obliteration of catalytic activity.

In several respects, the K191C/AMS system faithfully mimics the properties of wild-type enzyme. Both proteins exhibit similar K_m values for ribulose-P_2 and for Mg^{2+}. Like wild-type enzyme, the semisynthetic system is more efficient with Mg^{2+} than with Mn^{2+} (Robinson et al., 1979; Christeller, 1981), is stereospecific in phosphoglycerate formation, and is active as an oxygenase as well as a carboxylase. We have been unable to determine the specificity factor for gaseous substrates of K191C/AMS due to the extremely low oxygenase activity, a complication compounded by trace contamination of the mutant protein by a phosphatase.

The amount of carboxylase activity restored to the K191C protein by AMS is slight. However, more significance should be attached to the qualitative finding that any carboxylase activity can be restored than to the failure of that activity to approach wild-type levels. Since the K191C mutant is devoid of detectable carboxylase activity (the threshold for detection is 10^{-6} of wild type even at the high protein concentrations used in the assay) and since the total rate enhancement provided by the wild-type enzyme is unknown due to the absence of a suitable nonenzymic model, the approximate 10^{-5} of wild-type k_{cat} displayed by the K191C protein at saturating levels of AMS could represent stimulation by multiple orders of magnitude. Furthermore, the full potential of AMS may not be apparent, as this compound is a mixed-type inhibitor of the wild-type enzyme. In the initial demonstration of the feasibility of chemical rescue as an approach to establishing structure–activity relationships (Toney & Kirsch, 1989), 100 mM methylamine (the most effective among those examined) resulted in a 8000-fold stimulation of the measurable residual activity ($\sim 10^{-6}$ of wild-type) of the K258A mutant aspartate aminotransferase. Direct comparison of the effectiveness of the two proteins to be chemically rescued is difficult, because rate saturation with respect to amine concentration was not observed with the aminotransferase in contrast to the carboxylase. The differences in quantitative features of the two systems may be due, in part, to the more demanding requirements for restoration of carboxylase activity in that the rescuer must not only interact with the protein but also undergo carbamylation as well.

The slow rate of $^{14}CO_2$ fixation supported by AMS as a substitute for the activator lysyl residue is also deemed dramatic in light of the exquisite sensitivity of catalytic activity even to extremely modest structural perturbations at position 191. When Lys191 was substituted with (aminoethyl)cysteine (Smith et al., 1988)—effectively, the mere replacement of the γ-methylene group of the lysyl residue by a sulfur atom achieved by covalent aminoethylation of the K191C mutant protein—k_{cat} was reduced 15- to 25-fold. The much lower catalytic efficiency of the K191C protein upon rescue by AMS is not surprising in view of reliance on noncovalent interaction to provide a site for carbamylation and the obvious differences in geometry between this mimic and the wild-type carbamate.

Data presented herein provide rather compelling evidence of a catlytic role for the carbamate nitrogen which may be partially fulfilled by a carbamylated exogenous amine. Our study thus explains the unique dependence of carboxylase activity on carbamate formation. Lys191 emerges as an active-site residue of multifaceted catalytic importance. Its role in enzyme activation and coordination of the essential metal ion is clear; its precise role or roles in the catalytic mechanism await determination.

ACKNOWLEDGMENTS

We are indebted to Drs. George Lorimer and Steven Gutteridge of Du Pont, Wilmington, DE, for providing data concerning the interaction of carbonate with the K191C protein prior to publication.

REFERENCES

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

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Christeller, J. T. (1981) *Biochem. J. 193*, 839–844.

Donnelly, M. I., Stringer, C. D., & Hartman, F. C. (1983) *Biochemistry 22*, 4346–4352.

Hall, N. P., Pierce, J., & Tolbert, N. E. (1981) *Arch. Biochem. Biophys. 212*, 115–119.

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

Jencks, W. P., & Regenstein, J. (1976) in *Handbook of Biochemistry and Molecular Biology* (Fasman, G. D., Ed.) Vol. 1, 3rd ed., p 322, CRC Press, Cleveland.

Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., & Jansonius, J. N. (1984) *J. Mol. Biol. 174*, 497–525.

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

Kuehn, G. D., & Hsu, T.-C. (1978) *Biochem. J. 175*, 909–912.

Laing, W. A., & Christeller, J. T. (1976) *Biochem. J. 159*, 563–570.

Larimer, F. W., Mural, R. J., & Soper, T. S. (1990) *Protein Eng. 3*, 227–231.
Lorimer, G. H. (1981) *Biochemistry 20*, 1236–1240.
Lorimer, G. H., & Miziorko, H. M. (1980) *Biochemistry 19*, 5321–5328.
Lorimer, G. H., Badger, M. R., & Andrews, T. J. (1976) *Biochemistry 15*, 529–536.
Lorimer, G. H., Badger, M. R., & Andrews, T. J. (1977) *Anal. Biochem. 78*, 66–75.
Miziorko, H. M., & Sealy, R. C. (1980) *Biochemistry 19*, 1167–1171.
Niyogi, S. K., Foote, R. S., Mural, R. J., Larimer, F. W., Mitra, S., Soper, T. S., Machanoff, R., & Hartman, F. C. (1986) *J. Biol. Chem. 261*, 10087–10092.
Pierce, J., & Reddy, G. S. (1986) *Arch. Biochem. Biophys. 245*, 483–493.
Pierce, J., Tolbert, N. E., & Barker, R. (1980) *Biochemistry 19*, 934–942.
Robinson, P. D., Martin, M. N., & Tabita, F. R. (1979) *Biochemistry 18*, 4453–4458.
Schloss, J. V. (1988) *J. Biol. Chem. 263*, 4145–4150.
Smith, H. B., & Hartman, F. C. (1988) *J. Biol. Chem. 263*, 4921–4925.
Smith, H. B., Larimer, F. W., & Hartman, F. C. (1988) *Biochem. Biophys. Res. Commun. 152*, 579–584.
Smith, H. B., Larimer, F. W., & Hartman, F. C. (1990) *J. Biol. Chem. 265*, 1243–1245.
Styring, S., & Brändén, R. (1985) *Biochemistry 24*, 6011–6019.
Toney, M. D., & Kirsch, J. F. (1989) *Science 243*, 1485–1488.
Worthington, C. C., Ed. (1988) *Worthington Enzyme Manual*, p 265, Worthington Biochemical Corporation, Freehold, NJ.

Polypeptides and Bacteriochlorophyll Organization in the Light-Harvesting Complex B850 of *Rhodobacter sphaeroides* R-26.1†

P. Braun‡ and A. Scherz*,§

Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

Received December 14, 1990

ABSTRACT: The light-harvesting complex (LHC) B850 from *Rhodobacter sphaeroides* was dissociated into several fragments by treatment with sodium dodecyl sulfate. The molecular weight of each fragment was determined by using transverse polyacrylamide gel electrophoresis under nondenaturing conditions and gel filtration techniques. Four B850 LHCs were observed, having molecular weights of 60 000, 72 000–75 000, 105 000, and 125 000–145 000, and two small bacteriochlorophyll (Bchl)–polypeptide complexes having molecular weights of 6000–8000 and 12 000–14 000. Each of the B850 complexes contains ca. one Bchl *a* for each 6.5-kDa protein. The optical absorption and circular dichroism of the B850 LHCs recorded directly from the gels are similar to those measured previously for a 22–24-kDa B850 LHC by Sauer and Austin [(1978) *Biochemistry 17*, 2011–2019]. These data, combined with studies of other groups, indicate that the smallest LHC in LH1 and LH2 is a Bchl–polypeptide tetramer. Each tetramer contains two Bchl dimers that probably have the structure of P-860, the primary electron donor in *Rhodobacter sphaeroides*, and two α–β-polypeptide pairs. Interactions among the paired Bchls shift their individual Q_y transitions from 780–800 to 850–860 nm, and interactions among two such pairs induce the circular dichroism signal of the LHCs. Three Bchl–polypeptide tetramers probably form a dodecamer having C_3 symmetry, and six such dodecamers organize into a large hexagon that can accommodate one or two reaction center complexes.

Biological photosynthesis converts electromagnetic radiation into useful chemical energy by the joint action of two membrane-bound complexes termed "antennas" and "reaction centers" (RCs) (Clayton, 1980). The antennas consist of light-harvesting complexes (LHCs) made of polypeptide networks that bind small clusters of chromophores, mostly chlorophylls in oxygenic photosynthetic organisms and bacteriochlorophylls (Bchls) in nonoxygenic bacteria. Purple non-sulfur bacteria contain three types of LHCs: LH1 or B875, whose pigment centers have maximum optical absorption at 870–890 nm; LH2 or B800–850, whose pigment centers absorb at 800 and 850– nm, and LH3 or B800–820, whose pigment centers absorb at 800 and 820 nm [for leading reviews, see Cogdell and Thornber (1980), Zuber (1985), and Hunter et al. (1989)]. In recent studies, van Grondelle et al. (1988) suggested a fourth kind of pigment center with optical absorption at 895 nm.

The LHCs are packed around the RCs in order of increasing wavelength, and as energy migrates from short-wavelength-absorbing complexes to long-wavelength-absorbing ones, a unidirectional energy flow is established. Captive photons are finally trapped by the long-wavelength-absorbing primary donors in the RCs, where they promote charge separation across the photosynthetic membrane.

Each of the above-mentioned pigment centers is formed by Bchl *a* molecules, which have their lowest energy absorption at 780 nm when isolated as monomers in vitro. Two basically different concepts have been developed to explain the large

† This work was supported by U.S.–Israel Binational Science Foundation Grant 84-00144.
* Correspondence should be addressed to this author.
‡ In partial fulfillment of an M.S. Thesis.
§ Incumbent of the Recaneti Career Development Chair.

0006-2960/91/0430-5177$02.50/0