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^{13}C nuclear magnetic resonance study of the CO_2 activation of ribulosebisphosphate carboxylase from *Rhodospirillum rubrum*

(photosynthesis/enzymology)

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ABSTRACT **Ribulosebisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] from *Rhodospirillum rubrum* is activated by CO_2 and Mg^{2+}. ^{13}C NMR spectra were determined for the unactivated enzyme and for enzyme that had been activated by $^{13}CO_2$ and Mg^{2+}. In addition to the expected resonance for $H^{13}CO_3^-/CO_3^{2-}$ at 161.8 ppm downfield from tetramethylsilane, the spectrum of the activated enzyme shows a broad resonance at 164.9 ppm. Analogy with previous NMR studies of $^{13}CO_2$ binding to hemoglobin [Morrow, J. S., Keim, P., Visscher, R. B., Marshall, R. C. & Gurd, F. R. N. (1973) *Proc. Natl. Acad. Sci. USA* 70, 1414–1418], to myoglobin, and to amino acids [Morrow, J. S., Keim, P. & Gurd, F. R. N. (1974) *J. Biol. Chem.* 249, 7484–7494] suggests that the CO_2 activation of ribulosebisphosphate carboxylase involves formation of a carbamate between an enzyme amino group and CO_2.**

Ribulose 1,5-bisphosphate (Rbu-P_2) carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] catalyzes the first step in CO_2 fixation in plants, the reaction of Rbu-P_2 with CO_2 to form two molecules of 3-phosphoglyceric acid (1, 2). In the presence of oxygen the enzyme also catalyzes the oxygenation of Rbu-P_2 to form 3-phosphoglyceric acid and phosphoglycollic acid. Maximum activity in both the carboxylase and oxygenase reactions is observed only when the enzyme is activated by incubation with CO_2 and Mg^{2+} prior to assay. The activation is a slow process, requiring as long as several minutes under some conditions (3). Kinetic studies reveal that the carbon substrate for activation is CO_2 rather than HCO_3^- (3). The activation also requires Mg^{2+}, and it appears that CO_2 binds first, followed rapidly by metal (3–5). Activation is slower and less complete at lower pH values (3). The chemical nature of this CO_2 binding has not been established. Based on the pH dependence of the activation process, Lorimer *et al.* (3) suggested that CO_2 might form a carbamate by reaction with an enzyme amino group.

^{13}C NMR spectroscopy is a useful tool in studies of protein structure (6, 7). Although some work has been done with natural abundance materials, most of the striking advances in the field have made use of isotopically enriched coenzyme (8), protein, or substrate (6, 7). Carbon dioxide has long been known to bind to hemoglobin and myoglobin, and NMR studies with $^{13}CO_2$ have revealed that in both cases the bound form is a carbamate (9, 10).

Concentration and molecular weight are important limitations on the use of the NMR technique. ^{13}C concentrations near 1 mM are required in order to obtain reasonable signals within 1–2 days. For a nucleus rigidly bound to a protein, the linewidth of the NMR signal increases with increasing molecular weight, and studies of ^{13}C NMR spectra have largely been limited to proteins having molecular weights below 100,000. The Rbu-P_2 carboxylase from higher plants is a multisubunit enzyme with a molecular weight greater than 500,000 (1); thus, it is not a logical candidate for ^{13}C NMR studies. However, the Rbu-P_2 carboxylase from the photosynthetic bacterium *Rhodospirillum rubrum* is a simple dimer of 56,000-dalton subunits (11) and is thus amenable to ^{13}C NMR studies. The CO_2 activation of the enzyme from *R. rubrum* (5) is like that observed with the enzyme from spinach (4).

MATERIALS AND METHODS

Rbu-P_2 carboxylase from autotrophically grown *R. rubrum* strain S-1 was isolated by the procedure of Schloss *et al.* (12). The enzyme was homogeneous by the criterion of disc-gel electrophoresis; its specific activity was 3.3 μmol of Rbu-P_2 consumed per min per mg of protein at 25°C when assayed as described (13).

Prior to use, enzyme that had been stored in 20% (wt/vol) glycerol in phosphate buffer (pH 7.6) at −70°C was dialyzed against 10 mM Tris·HCl/0.1 mM EDTA at pH 8.6. The enzyme was then concentrated to about 90 mg/ml by placing the dialysis bag in contact with Sephadex G-25 that had been carefully freed of organic solvents by several days of evacuation. The concentrated enzyme was further dialyzed against several changes of degassed buffer. The last dialysis was against buffer containing 15% ^2H_2O, and the final pH was 8.3. To the dialyzed enzyme was added sufficient $KH^{13}CO_3$ solution [97% ^{13}C, prepared by acidification of $Ba^{13}CO_3$ (Mound Laboratories) with H_2SO_4 and trapping the evolved $^{13}CO_2$ with aqueous KOH] to bring the final HCO_3^- concentration to 6.6 mM and sufficient aqueous $MgCl_2$ to make the final concentration 10.8 mM. Bicarbonate concentrations were monitored by use of phospho*enol*pyruvate carboxylase coupled to malate dehydrogenase. All concentrations given are for total CO_2 plus HCO_3^-.

After determination of its ^{13}C NMR spectrum, the enzyme was dialyzed against the same buffer to remove $H^{13}CO_3^-$ and Mg^{2+}, and the NMR spectrum was determined again. After this procedure the enzyme had specific activity 2.2 μmol of Rbu-P_2 consumed per min per mg of protein.

NMR Spectroscopy. ^{13}C NMR spectra were determined at 25.16 MHz on a Varian XL-100-15 NMR spectrometer interfaced with a Varian 620/L computer operating in the Fourier transform mode with a deuterium lock. Spectra were obtained with 12-mm tubes with coherent broad-band proton decoupling. Chemical shifts are given ppm downfield from tetramethylsilane. External standard dioxane was used as a reference. All samples were run at a probe temperature of 20–25°C with ^{13}C excitation pulse of 37 μsec (54°C), spectral widths of 5 kHz, and an acquisition time of 0.333 sec. The spectra rep-

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Abbreviation: Rbu-P_2, ribulose 1,5-bisphosphate.

resent data accumulations in excess of 200,000 transients with a 3.18-Hz digital broadening.

RESULTS AND DISCUSSION

In Fig. 1 are shown ^{13}C NMR spectra of Rbu-P_2 carboxylase (70 mg/ml) at pH 8.3 in the presence of 6 mM $H^{13}CO_3^-$ and 10 mM Mg^{2+} (*B*) and after dialysis to remove $H^{13}CO_3^-$ and Mg^{2+} (*A*). In the absence of $H^{13}CO_3^-$ the spectrum is similar to other ^{13}C NMR spectra of proteins that have been reported (6–8), with the addition of the standard dioxane resonance at 67.4 ppm and a sharp resonance for the Tris buffer at 62.4 ppm. The identities of the major peaks in such a spectrum have been given (8). According to Christeller and Laing (5), under the conditions used to obtain the spectrum in Fig. 1*B*, more than 95% of the enzyme should be in the activated state. In the presence of $H^{13}CO_3^-$, two additional signals are visible: the first, at 161.8 ppm, is due to free $H^{13}CO_3^-$ and $^{13}CO_3^{2-}$. Because interchange of these two species is rapid on the NMR time scale, only a single, sharp resonance is observed for the two (10). The second (indicaded by the arrow) is a broad resonance centered at 164.9 ppm. The width of this resonance indicates that it is not derived from a freely diffusing small molecule. The linewidth (approximately 26 Hz) is appropriate, however, for a carbon nucleus rigidly bound to a protein of molecular weight near 100,000.

The CO_2 activation of Rbu-P_2 carboxylase is pH dependent (3). When the spectrum is obtained at pH 7.3 in phosphate buffer under conditions otherwise identical to those of Fig. 1*B*, the $H^{13}CO_3^-$ resonance appears as expected, but the resonance at 164.9 ppm is barely visible above the base line. Previous studies (3, 5) indicate that less than 20% of the enzyme should be in the activated state under these conditions.

Although uncertainties arising from unknown nuclear Overhauser effects and from relaxation effects invalidate any quantitative discussion of signal intensities, qualitative comparison of the intensity of the peak at 164.9 ppm with other peaks in the spectrum is instructive. This signal is relatively intense; if it were due to some contaminant, then unless this contaminant were isotopically enriched, its concentration would have to approach 50 mM. If the contaminant were isotopically enriched, then it would have to constitute 20% or more of the total HCO_3^- present. Further, the width of the peak indicates that the nucleus in question must be protein bound. The peak is removable upon dialysis and is not observed under conditions where CO_2 binding to the protein is not expected.

On the other hand, the intensity of the peak at 164.9 ppm can be compared with the intensities of the amide carbonyl peak near 174 ppm and the peak for guanidino carbons and tyrosine ζ carbons at 157.8 ppm by use of amino acid analysis (12). The intensity corresponds satisfactorily to that expected for binding of one enriched carbon to each subunit [molecular weight 56,000 (11)] of the enzyme.‡

Thus we conclude that the broad resonance at 164.9 ppm in

‡ Although our results are most consistent with one bound carbon per 56,000 daltons, our results do not allow us to convincingly eliminate the possibility that the stoichiometry is one per 112,000 daltons.

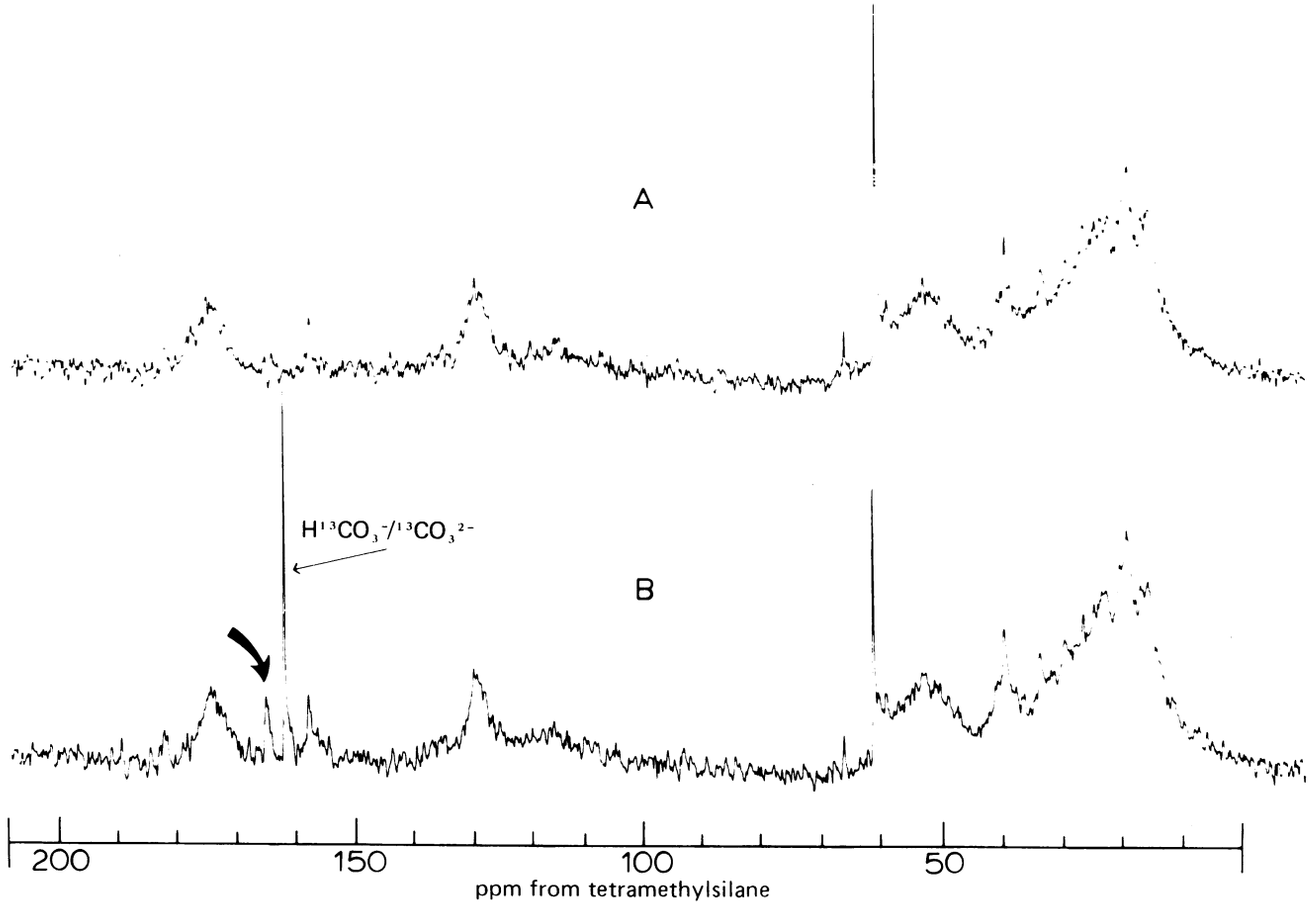

FIG. 1. (*A*) ^{13}C NMR spectrum of Rbu-P_2 carboxylase (70 mg/ml) from *R. rubrum* in 10 mM Tris buffer (pH 8.3). (*B*) Same, with the addition of 6.6 mM $H^{13}CO_3^-$ and 10.8 mM Mg^{2+}. Arrow indicates the position of the resonance attributed to enzyme-bound $^{13}CO_2$ (in whatever state).

Fig. 1*B* is due to carbon dioxide (in whatever chemical state) bound to the activating site of Rbu-P_2 carboxylase. In the following paragraphs we consider the chemical nature of this bound species.

Kinetically (3), the activation of Rbu-P_2 carboxylase involves CO_2 rather than HCO_3^-. However, the chemical shift of the bound carbon is not consistent with the existence of noncovalently bound CO_2, which should appear near the resonance position for free CO_2, 125.4 ppm (9). The possibility that the bound form is actually noncovalently bound HCO_3^- is unlikely on kinetic grounds, in view of the fact that CO_2 is the activating species. We would also expect that bound HCO_3^- should show very nearly the same chemical shift as free HCO_3^-.

The spectra shown in Fig. 1 are strikingly like those observed by Morrow *et al.* (9, 10), for $^{13}CO_2$ bound to myoglobin and hemoglobin. In those cases CO_2 is bound as a carbamate anion formed by reaction of CO_2 with an amino group. The chemical shifts of those carbamates (163.8–164.3 ppm) are quite similar to that found with Rbu-P_2 carboxylase (164.9 ppm), and our results are most simply explained if CO_2 activates Rbu-P_2 carboxylase by formation of a carbamate with a particular amino group of the protein. The effect of Mg^{2+} might be to complex this carbamate or in some other way to serve as a "lock" to increase the stability of the carbamate.

In addition to amino groups, proteins contain a number of other functional groups that might, at least in theory, be capable of forming covalent adducts with carbon dioxide. The possibility of forming such adducts has been considered in detail by Morrow *et al.* (10), who failed to find evidence by NMR for adducts of CO_2 with sulfhydryl, imidazolyl, or hydroxyl groups. Those arguments apply equally well here. The presumably low thermodynamic stability of such compounds makes it unlikely that they should be involved in CO_2 binding to proteins, although the failure of chemists to prepare and study them makes all arguments about their properties somewhat speculative.

Assuming that enzyme activation occurs by formation of a carbamate with an amino group of the enzyme, it is interesting to consider whether this is the amino-terminal amino group of the peptide chain, as is observed with hemoglobin (9) and myoglobin (10), or whether this is an ϵ-amino group of a lysine residue. The catalytic subunits of several Rbu-P_2 carboxylases appear to have a blocked amino terminus, (12, 14, 15) making it likely that carbamate formation involves a lysine amino group.

The lysine residue involved in carbamate formation would not be identical with those detected by affinity labeling techniques (13, 16, 19) because carbon dioxide either afforded no protection against modification by affinity labeling reagents or in some cases actually stimulated the modification rate.

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