An investigation of ribulosebisphosphate carboxylase activity by high resolution ^1H NMR

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Received 17 February 1984

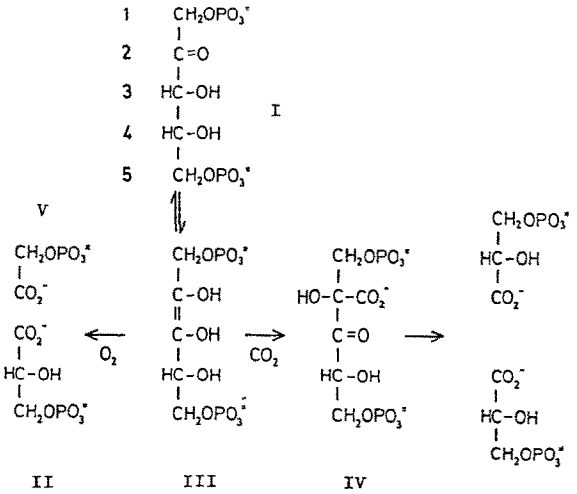
The reactions catalysed by ribulosebisphosphate carboxylase/oxygenase have been studied by high resolution proton NMR spectroscopy. The protons of the substrate ribulose-P_2 and the products of the carboxylase and oxygenase reactions are readily assigned to the various carbon centres. Half of the 3-phosphoglycerate molecules generated during carboxylase turnover of ribulose-P_2 in saturating CO_2 concentrations acquire deuterium from the solution at the C_2 position, whereas the other 3-phosphoglycerate molecules and also the 3-phosphoglycerate produced as a result of the oxygenase activity contain no deuterium of this type. However, the addition of activated enzyme (i.e., in the presence of an effector) to a solution of ribulose-P_2 in the near absence of either CO_2 or O_2 catalyses the exchange of the C_3 proton with the deuterium of the solution.

Ribulosebisphosphate carboxylase/oxygenase *NMR* *Enediol* *Proton/deuterium exchange* *Ribulose bisphosphate*

1. INTRODUCTION

Ribulose-P_2 carboxylase catalyses the carboxylation or the oxygenation of ribulose-P_2. The first of these reactions between CO_2 and the bisphosphate produces two molecules of 3-phosphoglycerate. The other reaction, with molecular oxygen, generates 2-phosphoglycollate and 3-phosphoglycerate (see scheme 1). Studies of the inhibition of both reactions and the direct competition between CO_2 and O_2 for ribulose-P_2 [1] show that the two activities occur at the same site on the enzyme.

The steps leading to product formation in the carboxylase reaction are now known in some detail [2]. The reaction involves the formation of a 6 carbon intermediate, 2-carboxy,3-keto arabinital bisphosphate (IV) which is hydrolytically cleaved to produce two 3-phosphoglycerate (II) molecules. In contrast, little is known about the oxygenase reaction. One hypothesis suggests that a 2-peroxy,3-keto derivative of the pentose bisphosphate would lead to phosphoglycollate (V)



Scheme 1.

and phosphoglycerate [3].

The form of ribulose-P_2 (I) which most likely reacts with either CO_2 or O_2 and over which they compete is an enediol (III) generated by enzymic

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00145793/84/$3.00

abstraction of the proton from the C_3 of the bisphosphate. Confirmation that an enediol does exist during carboxylation has been obtained by observing the exchange of tritium from ribulose-P_2 labelled at position C_3 [4,5] and also by investigations of the effects of tetranitromethane [6] (which can react with enediols) on the course of the carboxylase reaction.

What is not clear is the order in which the substrates bind to the enzyme or at what stage in the catalytic cycle an enediol might be generated. These questions are particularly important because there is no evidence that the substrates CO_2 or O_2 are required to bind at the active site of the enzyme for either carboxylation or oxygenation to proceed. Thus the enzyme may present a reactive intermediate, namely the enediol of ribulose-P_2, for the second substrates to carboxylate or oxygenate. We have obtained information on this and other aspects of the reactions of ribulose-P_2 carboxylase using high resolution ^1H NMR spectroscopy.

2. MATERIALS AND METHODS

Ribulose-P_2 carboxylase was purified from wheat as in [7] and stored as a freeze-dried powder. For the NMR experiments, the powder was dissolved in bis–Tris buffers made up in D_2O. The pH of solution was read directly from a pH meter without correction for deuterium isotope effects. The enzyme was activated by addition of small amounts of a concentrated solution of HCO_3^- and $MgCl_2$ in D_2O, to give final concentrations of 10 mM and 20 mM, respectively, and by warming the solution to 40°C for 40 min before use [7,8]. In those experiments using enzyme activated with sub-saturating concentrations of CO_2, 10 mM phosphate was included with 2 mM HCO_3^- and 20 mM $MgCl_2$. The specific activities of the enzyme solutions activated with or without phosphate were determined after the NMR experiments to ensure that the enzyme had remained stable. Ribulose-P_2 was of the highest purity available from Sigma and was lyophilised from D_2O before use.

2.1. *NMR spectroscopy*

The proton spectra were obtained at 25°C using a Bruker 270 or 500 MHz instrument. The assignment of the protons to individual carbon centres of the substrate or products of the carboxylase reactions was achieved by consideration of the chemical shift data and by using spin decoupling techniques to connect coupled resonances.

3. RESULTS

There are two reactions involving CO_2 and the carboxylase. The enzyme not only catalyses the fixation of CO_2 but also requires one molecule of CO_2 at the active site to catalyse carboxylation or oxygenation [9,10]. This activating molecule of CO_2 provided by the addition of HCO_3^-, is quite distinct from the substrate CO_2 [11] and exists as a carbamate of the ϵ-NH_2 group of a lysine residue of the enzyme [12]. The carbamate associates with Mg^{2+} to give an activated ternary complex of enzyme–CO_2–Mg^{2+} (ECM) that can catalyse the two reactions. However, this complex can readily dissociate in the absence of CO_2 or Mg^{2+} and therefore both activating co-factors are usually included to maintain the active state. Unfortunately, the necessity of adding HCO_3^- for this purpose applies a number of constraints in studies that may require its omission; i.e., the enzyme simply generates products of the carboxylase reaction. Fig.1 shows the progress of the carboxylation of ribulose-P_2 as followed by NMR at 270 MHz. In fig.1a the proton spectrum of the bisphosphate in D_2O is shown with the resonances assigned to the protons of each carbon centre except C_1 (see below) of the ketose molecule. The addition of activated enzyme to these solutions in the presence of CO_2 (in the form of HCO_3^-) and Mg^{2+} results in the consumption of the bisphosphate, which is observed most readily by the loss of the C_3 proton and the appearance of a new spectrum due to the formation of 3-phosphoglycerate (fig.1b–d). Spin decoupling experiments involving selective irradiation at the different resonant frequencies allowed the signal at δ 4.22 ppm (fig.1d) to be identified as that from the C_2 proton of 3-phosphoglycerate. A comparison of the intensities of the C_2 doublet and the C_3 signal positioned at δ 4.06 ppm indicated that half of the 3-phosphoglycerate product molecules must have acquired deuterium at C_2 from the solvent during catalysis.

Fig.1e shows the spectrum resulting from ribulose-P_2 being allowed to react with the carboxylase in solutions gassed with pure oxygen before

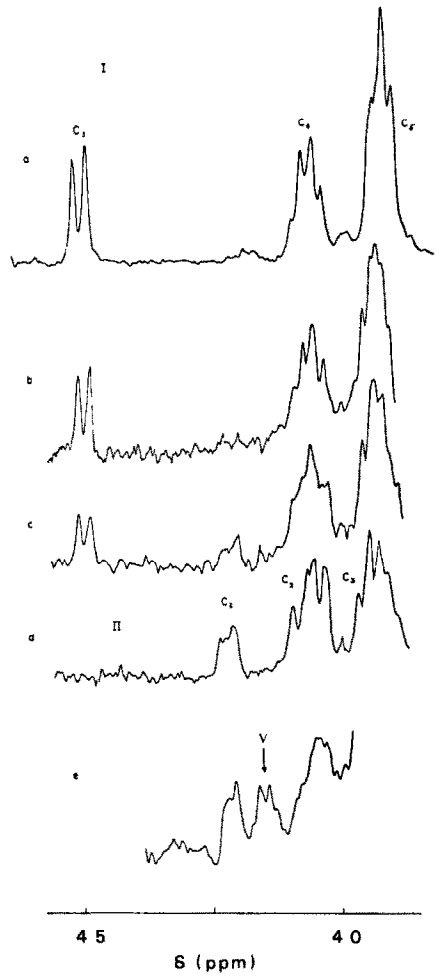

Fig.1. ^1H NMR spectra at 270 MHz and 25°C monitoring the formation of products from ribulose bisphosphate by ribulose bisphosphate carboxylase. The bisphosphate substrate I (5 mM) freeze-dried from D_2O was dissolved in D_2O containing 5 mM bis–Tris, 10 mM HCO_3^- and 20 mM $MgCl_2$. In (a) the ^1H spectrum is shown before the addition of activated carboxylase with the various resonances assigned to the carbon centres. (b–d) The effect over approx. 1 h of the addition of the enzyme (final concentration 1 mg/ml). The spectra (the sum of 400 pulses) were recorded after: (b) 2 min; (c) 30 min; (d) 60 min. Note that the two C_3 protons of 3-phosphoglycerate II reside in different environments and hence give rise to two distinct multiplets. Addition of the enzyme to solutions without HCO_3^- (carryover HCO_3^- concentrations 1 mM) and gassed with pure oxygen generated the spectrum shown in (e). The position of the phosphoglycollate product (V) is arrowed.

collecting the spectrum, and in low CO_2 concentrations (~0.4 mM HCO_3^-). The phosphoglycollate (δ 4.17 ppm) as well as phosphoglycerate products of the reaction are readily identifiable. In this case the intensity of the C_2 resonance of phosphoglycerate is approximately the same as the C_3 proton multiplets (allowing for a small amount of carboxylase activity) indicating that deuterium is not incorporated into 3-phosphoglycerate as a result of oxygenation of the ribulose-P_2.

The effect of active carboxylase on the bisphosphate without significant interference from CO_2 can be studied by activating the enzyme in suboptimal HCO_3^-. Phosphate was chosen as the effector because it stabilises the carboxylase, does not interfere with the ^1H NMR measurements and also provides stimulation of the enzyme activity [14]. In fig.2, the addition of the phosphate activated enzyme to a CO_2 free solution of ribulose-P_2 causes a significant change to the bisphosphate proton spectrum by catalysing the exchange of the C_3 proton with the deuterium of the medium. This process was followed with time and in two ways using the 500 MHz instrument. Fig.2a–c shows the loss of the C_3 resonance relative to the unchanging C_1 multiplets indicating that there was no significant conversion of substrate to products. In fig.2d–f the exchange process was recorded as both a loss of the C_3 resonance and a change to the adjacent C_4 multiplet at 4.06 ppm due to the increasing effect of deuterium at C_3. A small amount of the substrate has been converted to product in fig.2e and f as a result of contaminating oxygen during mixing of the enzyme with the substrate. The time course of the exchange reactions was within the period required to mix the enzyme with the bisphosphate substrate, retune the instrument and collect enough FIDS to give spectra of acceptable signal:noise ratio. The turnover number of wheat carboxylase is less than 2 s^{-1} at 25°C for the best preparations when activated with phosphate present [14]. Addition of this enzyme to the substrate dilutes the phosphate to concentrations that no longer inhibit ribulose-P_2 binding. However, there is also a progressive loss of activity as the amount of 'carryover' CO_2 and effector (phosphate) are no longer able to maintain the activated form of the enzyme. The carboxylase deactivates exponentially with a $t_{0.5}$ of ~60 s. Furthermore, there may be a significant deuterium isotope

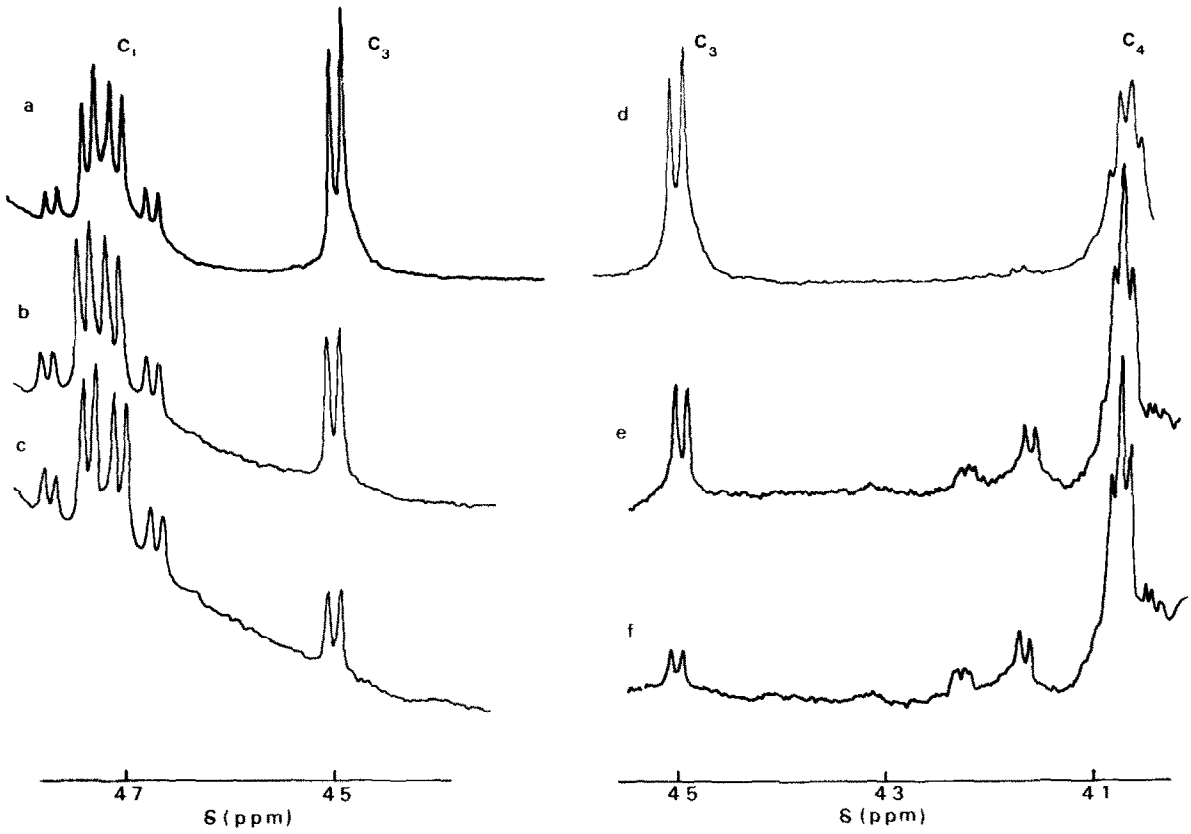

Fig.2. ^{1}H NMR spectra at 500 MHz monitoring the deuterium exchange of the C_3 proton from ribulose-P_2 in the presence of the carboxylase. The bisphosphate was dissolved in D_2O containing 5 mM bis–Tris and 10 mM $MgCl_2$. In (a) the C_1 and C_3 protons of the substrate are shown before the addition of activated carboxylase. (b,c) The depletion of the C_3 proton relative to C_1 at 10 and 20 min, respectively, after addition of the enzyme (5 mg/ml final concentration). (d–f) The C_3 and C_4 proton resonances were monitored during the exchange process; (d) was the starting spectrum, (e) collected 5 min, and (f) 9 min after addition of activated carboxylase. The spectra in (a–c) were recorded at 15°C to ensure adequate separation of the C_1 multiplets from the HOD peak. The spectra (d–f) were recorded at 25°C.

effect for the exchange reaction involving those substrate molecules already deuterated. Therefore, complete exchange of the C_3 protons within 10 min with these enzyme concentrations suggests that the process may occur within the normal catalytic cycle of the enzyme.

4. DISCUSSION

The NMR data provide further insight into the reaction mechanisms of ribulose-P_2 carboxylase and confirm some aspects that have been determined by other methods for other species of the enzyme. For example, with reference to scheme 1 it is possible to identify the 3-phosphoglycerate originating from C_1 and C_2 of the bisphosphate as the molecule that becomes deuterated during carboxylation. This is because the 3-phosphoglycerate generated during oxygenation, i.e., from C_3, C_4 and C_5 of ribulose-P_2 contains hydrogen. Although this is fully consistent with the accepted reaction mechanisms for the enzyme, the result leads to two conclusions. Firstly, the proton abstracted from the C_3 of the bisphosphate does not protonate the 'top' 3-phosphoglycerate product of carboxylation. It is not known at what stage in the reaction the top 3-phosphoglycerate molecule acquires deuterium, but probably when it

is an enzyme bound intermediate [16,17] to ensure that the correct stereoisomer is released [18]. Secondly, the reactivity of the enzyme bound bisphosphate to molecular oxygen could be due to a mechanism involving abstraction of the C_4 proton [15]. If this is the case, then this proton is not exchangeable with the medium.

Although the data do not allow further conclusions to be drawn about the reaction between the bisphosphate and CO_2 or O_2 they do indicate that the enzyme does not simply form a Michaelis complex with the ribulose-P_2. In the almost complete absence of either second substrate the carboxylase can effectively catalyse the abstraction and subsequent exchange of the C_3 proton from the bisphosphate. This is a necessary step in the formation of an enediol intermediate that would then be able to react with CO_2 or molecular O_2. The competitive inhibition of CO_2 on oxygenation and vice versa observed from steady-state kinetic analysis may not arise from competition for the same binding site on the enzyme but competition for the bisphosphate intermediate generated by the carboxylase.

A mechanism which apparently involves the direct reaction of the enediol without the intervention of the enzyme has important consequences. It argues against the prospect of being able to manipulate the protein structure to favour one of the two reactions because there is no 'formal' binding site for the second substrate that could be modified. However, the partitioning of ribulose-P_2 between the two reactions can be changed; e.g., substituting Mn^{2+} for Mg^{2+} alters the reaction ratio dramatically, suggesting that the metal plays an important role in catalysis, possibly by 'activating' the CO_2 or O_2 substrate. Furthermore, the reaction ratio of different species of the carboxylase varies significantly [18] particularly between those forms that contain or lack a small subunit. Thus, although the carboxylase can generate the bisphosphate enediol, the second substrate may still need to bind, e.g., by coordination to the metal ion, before either reaction can proceed. Clearly, a detailed comparative study of the three-dimensional active site structures of carboxylases with very different carboxylase/oxygenase ratios is necessary.

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