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Review

Determinants of Substrate Specificity and the Role of Metal in the Reactions of Ribulosebisphosphate Carboxylase/Oxygenase

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ABSTRACT

Recent studies have provided a fairly detailed view of the various intermediates involved in the reactions of ribulosebisphosphate carboxylase and the manner in which the catalytically essential metal atom might catalyze their interconversions. A better understanding of how the enzyme distinguishes between its alternate substrates, CO_2 and O_2, has also emerged. The results of these studies should prove useful in anticipating possible ways in which the enzyme's substrate specificity might be manipulated. Together, the techniques that are described constitute a powerful methodology for more refined experimentation aimed at understanding the curious reactivities of ribulosebisphosphate carboxylase.

It is by way of its fickle catalysis of both the carboxylation and oxygenation of RuBP[1] that RuBP carboxylase/oxygenase initiates the major, competing metabolic pathways of photosynthesis and photorespiration. This single, quite abundant enzyme modulates the production of the starting metabolites for both photosynthesis (PGA) and photorespiration (phosphoglycolate) as a result of the competition between CO_2 and O_2 for the enzyme's active site. The expectation that modification of the enzyme's reactivity toward its alternate substrates might allow enhancement of plant productivity (via enhancement of the rate of photosynthesis) lends a practical aspect to its study. These observations have quite naturally engendered a continuing interest in the enzyme's mechanism of action. In this article, I will review recent advances in our understanding of (a) the role of the activating metal ion in the events of catalysis and (b) the catalytic steps that determine the enzyme's specificity for its gaseous substrates. Further background information may be found in an earlier review (8).

ON THE ROLE OF METAL IN CATALYSIS

It has been known for some time that RuBP carboxylase requires CO_2 and metal for activity. (The activating CO_2 molecule is distinct from the molecule of CO_2 which will ultimately be condensed with RuBP.) Activation proceeds by the slow reaction of CO_2 with an evolutionarily conserved lysine group on the enzyme to form an enzyme-carbamate species. A subsequent rapid reaction of the enzyme-carbamate species with a divalent metal atom (usually Mg^{2+}, though many other metals suffice) produces the active, ternary complex (Fig. 1) which is capable of catalyzing the carboxylation *and* oxygenation of RuBP. Recent spectroscopic studies have yielded valuable insight into the environment of the activating metal ion, and provide strong, physical evidence for the intimate involvement of the metal in catalysis.

A number of researchers have studied the model quaternary complex formed between enzyme, CO_2, metal, and the reaction intermediate analog, CABP. This complex is most appropriate for spectral studies since the remarkable stability of the complex [K_d(CABP) ~ 1 pM] suggests faithful mimicry of the actual complex formed with *keto*-CABP during catalysis (see Fig. 1). Preparation of this complex with *Rhodospirillum rubrum* enzyme and Mn^{2+} in ^{17}O-enriched H_2O resulted in spectral broadening of the Mn^{2+} resonances (9). This broadening disappeared upon removal of excess ^{17}O by gel filtration, indicating that, in the quaternary complex, there are one or more slowly exchangeable H_2O molecules bound to the Mn^{2+}. No residual spectral broadening that may have derived from any ^{17}O-enriched carbamate oxygens was observed. When CABP was selectively enriched with ^{17}O at either the carboxyl group or the oxygen at C-2, spectral broadening of the Mn^{2+} resonances was once again observed. This important result shows that the activating metal atom coordinates directly to the reaction intermediate analog via its carboxyl and C-2 oxygen atoms. Similar techniques were used in studies of complexes prepared with spinach enzyme and Cu^{2+} as the activating metal atom (15). In addition to confirming the above results, the narrower resonances observed in the Cu^{2+} spectra allowed these authors to determine that there is precisely one H_2O molecule bound to Cu^{2+} in quaternary complexes made with CABP.

^{13}C and ^{31}P NMR studies of quaternary complexes of *R. rubrum* enzyme prepared with various metals support the above conclusions (12). In addition, the metals' enhancements of the relaxation of the phosphorus atoms of CABP indicated that the phosphorus atoms of CABP are not directly coordinated to the metal but are more or less symmetrically positioned about the metal atom at a distance of not more than 6 Å. Attempts to detect direct coordination between carbamate oxygens and $^{113}Cd^{2+}$ by observation of scalar broadening of ^{13}C-enriched carbamate were unsuccessful. However, obliteration of the ^{13}C-carbamate resonance in quaternary complexes prepared with Mn^{2+}, Co^{2+}, or Ni^{2+} indicated that the carbamate residue is in close proximity (<6 Å) to the activating metal atom.

Spectroscopic studies of spinach enzyme complexes made with Cu^{2+} and RuBP or PGA have also proved informative (1, 2, 15).

[1] Abbreviations: RuBP, ribulose 1,5-bisphosphate; XuBP, xylulose 1,5-bisphosphate; CABP, 2-carboxy-arabinitol 1,5-bisphosphate; *keto*-CABP, 2-carboxy-3-*keto*-arabinitol 1,5-bisphosphate; PGA, glyceric acid 3-phosphate.

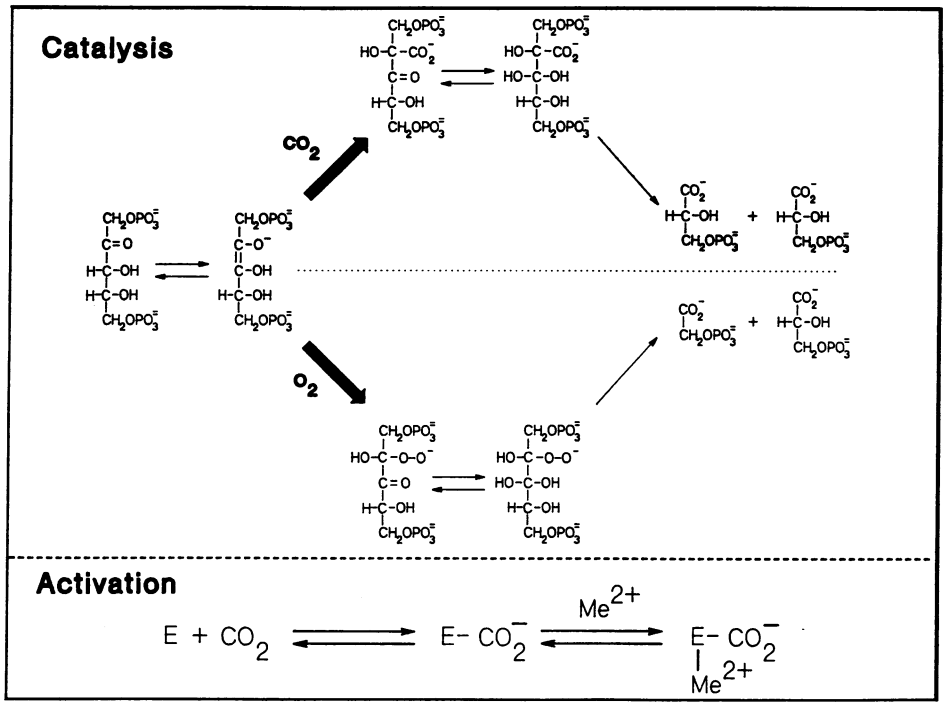

FIG. 1. The ordered reaction mechanisms of RuBP carboxylase/oxygenase.

A nitrogen ligand from the enzyme appears to be displaced from the Cu^{2+} atom upon binding of RuBP, PGA, or CABP. In the PGA complexes, two PGA molecules are bound in the active site, but only one of them is coordinated (via its C-2 oxygen and one carboxyl oxygen) to the metal. Again, no evidence for direct coordination between the activating metal ion and carbamate oxygen was obtained. The spectra of the PGA complexes also indicates a more highly symmetric metal center than in the CABP complexes. No metal bound H_2O molecule could be observed in the PGA complexes, though the authors suggested that an axial H_2O molecule would be difficult to detect due to the more tetragonal symmetry of the metal complex.

It would appear, then, that the activating metal ion is positioned so as to effect many of the steps of catalysis (Fig. 1). Indeed, since O-2 of the reaction intermediate *keto*-CABP derives from the carbonyl oxygen of RuBP, it is most likely that the metal acts as an electron sink to enhance the initial enolization of RuBP. Upon addition of CO_2, the resulting intermediate is stabilized by a two point attachment to the metal atom. The presence of metal bound H_2O might allow facile hydration of the newly formed *keto*-CABP. Subsequent bond scission of the intermediate to form PGA and the aci-acid form of PGA (*i.e.* the stable resonance form of PGA lacking the C-2 proton) would be likewise facilitated by the metal atom. Similar arguments can be adduced for the oxygenase reaction. Studies in which ^{17}O-enriched RuBP was added to Cu^{2+} activated enzyme have provided suggestive evidence for formation of and metal coordination to the five-carbon hydroperoxy intermediate proposed for the oxygenase reaction (2), although in these experiments spectral interpretation is made more difficult by the multiple enzyme forms which exist under steady state conditions.

Despite clear thermodynamic and kinetic evidence that carbamate formation is required for metal binding to RuBP carboxylase, it is important to note that there is still no evidence for direct coordination of the activating metal to the carbamate group. To be sure, the metal atom is close to the activating carbamate, and the acidic nature of the amino acids surrounding the activator lysine make this region an attractive candidate for being the metal binding site. However, it may be that carbamate formation facilitates metal binding merely by masking a positively charged group (lysine) in the vicinity of the metal binding site. In this way, carbamate formation would promote metal binding without direct coordination of the carbamate with the metal.

KINETIC DETERMINANTS OF ENZYME SPECIFICITY

The just discussed physical evidence for the role of metal in the events of catalysis provides a useful framework for constructing a mental picture of the enzyme's reaction mechanism. However, the language which connects these various structures in time (*i.e.* during the course of catalysis) necessarily requires a kinetic component. Too, it is with a kinetic description that one of the most intriguing aspects of the enzymology of RuBP carboxylase emerges—namely, the curious competition between CO_2 and O_2 that defines the relative fluxes of carbon through the major metabolic pathways of photosynthesis and photorespiration.

In a series of elegant studies, Jordan and Ogren (6, 7) showed how the enzyme's relative substrate specificity (τ) is related to the kinetic constants of the enzyme, *i.e.*

$$v_c/v_o = \frac{V_c/K_c}{V_o/K_o} \cdot \frac{[CO_2]}{[O_2]} = \tau \cdot \frac{[CO_2]}{[O_2]}.$$

That is to say, the ratio of carboxylations to oxygenations (v_c/v_o) at equal $[CO_2]$ and $[O_2]$ is constant for a given enzyme. The constant τ, which is simply the ratio of the V_{max}/K_m values for the two reactions, is highly dependent on both the nature of the activating metal ion and the source of the enzyme. For instance, with Mg^{2+} as the activating metal, the spinach enzyme has $\tau \sim 80$, whereas the enzyme from *R. rubrum* has $\tau \sim 15$. When Mn^{2+} is used, the corresponding values change to 3 and 1.5, respectively (7). Obviously, these changes must occur by alteration of the kinetic parameters which τ comprises. What then are the kinetically significant intermediates in the reactions of RuBP carboxylase, and how might the enzyme vary the interplay between them?

A number of the intermediates depicted in Figure 1 have been identified by denaturing the enzyme with acid during steady state

turnover. In this manner, *keto*-CABP was found to exist to the extent of 4 to 12% of spinach enzyme sites in the steady state (10, 14). This intermediate was also detected using *R. rubrum* enzyme (4). In addition, the higher K_m (CO_2) of the *R. rubrum* enzyme allowed Jaworowski *et al.* (5) to observe a substantial fraction of *enol*-RuBP during steady state turnover at low [CO_2]. A subsequent report demonstrated the methodology for obtaining *keto*-CABP via steady state quenching. When this compound was added back to fresh enzyme, it was converted substantially to PGA. These experiments lend a reality to speculations concerning the existence and processing of the various reaction intermediates, and the temporal manner in which the enzyme catalyzes their formation has been recently addressed.

The kinetic order of the RuBP carboxylase reactions has been the subject of much controversy, with most studies favoring a random mechanism for substrate addition. Two recent studies, however, provide strong evidence for an ordered addition of substrates. ^{13}C NMR relaxation studies of enzyme-carbamate-Mn^{2+} complexes demonstrated that, in the absence of RuBP, the HCO_3^- anion, but *not* CO_2 binds within ~4.5 Å of the metal (13). Further, addition of the substrate analog XuBP did not promote CO_2 binding. Attempts at trapping the putative enzyme-substrate CO_2 complex by fast reaction techniques were also unsuccessful. Likewise, equilibrium binding experiments could provide no evidence for O_2 binding in the presence or absence of XuBP at O_2 concentrations far in excess of $K_m(O_2)$ (13). Thus it appears that RuBP must bind to the activated enzyme prior to the addition of the gaseous substrates.

A novel NMR approach has been used to further define the sequence of catalytic events. At very low CO_2 concentrations, the wheat enzyme catalyzed the exchange of H-3 of RuBP with solvent protons. At saturating CO_2 concentrations the exchange reaction was not observed (3). This approach was also used in a more detailed study with the enzyme from *R. rubrum* (13) which demonstrated that as the CO_2 concentration was progressively lowered, the observed rate of the exchange reaction progressively increased. At the lowest CO_2 concentration used, the exchange rate (and by inference, the rate of enolization of RuBP) was larger than the maximum catalytic rate of the enzyme. The clear implication is that enolization of RuBP does not require the presence of substrate CO_2. That is, the enzymic reaction appears to proceed by the ordered binding and enolization of RuBP, followed by reaction with the gaseous substrates.

The methodology for obtaining *keto*-CABP demonstrated by Jaworowski and Rose (5) has been used in a study of the partitioning of this intermediate by various carboxylases (11). *keto*-CABP was shown to exist in solution primarily as the unhydrated, free ketone. In the absence of RuBP carboxylase, it slowly decomposed by decarboxylation. Curiously, CO_2 and metal free (*i.e.* nonactivated) RuBP carboxylase catalyzed the decarboxylation of *keto*-CABP. RuBP was not produced as a result of this decarboxylation. Rather, the decarboxylation produced *enol*-RuBP which suffered β-elimination of its C-1 phosphate group. When activated RuBP carboxylase was used, *keto*-CABP was converted predominantly to PGA. Activated enzymes from spinach, a cyanobacterium, and *R. rubrum*, despite having widely different relative substrate specificities for CO_2 and O_2 (*i.e.* different values for τ), partitioned almost all of the intermediate to PGA. Indeed, even when Mn^{2+} activated enzymes were used, the large majority of *keto*-CABP was hydrolyzed to PGA with very little decarboxylation observed. The implication is that, once formed, *keto*-CABP is committed to product formation. That is, it does not appreciably revert to CO_2 and *enol-RuBP*. It was also suggested by analogy to known chemical reactions, that the corresponding hydroperoxy intermediate of the oxygenase reaction is similarly committed to product formation (11).

We can relate the above kinetic descriptions to provide a clearer understanding of the manner in which the various RuBP carboxylases differentially distinguish between O_2 and CO_2. Since enolization of RuBP appears to precede interaction of the enzyme with the gaseous substrates, the formation of the *enol*-RuBP-enzyme complex is common to both the carboxylase and oxygenase reactions and control of reaction specificity must occur after this step. And again, if *keto*-CABP and the hydroperoxy intermediate are committed to product formation, the enzyme must exert its influence on substrate specificity prior to their formation. Taken together, these kinetic results suggest that the various relative specificities of different RuBP carboxylase enzymes are determined precisely at the step of gaseous substrate addition to the *enol*-form of RuBP.

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