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Evidence that Ribulose 1,5-Bisphosphate (RuBP) Binds to Inactive Sites of RuBP Carboxylase *in Vivo* and an Estimate of the Rate Constant for Dissociation[1]

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ABSTRACT

The binding of ribulose 1,5-bisphosphate (RuBP) to inactive (noncarbamylated) sites of the enzyme RuBP carboxylase *in vivo* was investigated in *Spinacia oleracea* and *Helianthus annuus*. The concentrations of RuBP and inactive sites were determined in leaf tissue as a function of time after a change to darkness. RuBP concentrations fell rapidly after the change to darkness and were approximately equal to the concentration of inactive sites after 60 s. Variations in the concentration of inactive sites, which were induced by differences in the light intensity before the light-dark transition, correlated with the concentration of RuBP between 60 and 120 s after the change to darkness. These data are discussed as evidence that RuBP binds to inactive sites of RuBP carboxylase *in vivo*. After the concentration of RuBP fell below that of inactive sites (at times longer than 60 s of darkness), the decline in RuBP was logarithmic with time. This would be expected if the dissociation of RuBP from inactive sites controlled the decline in RuBP concentration. These data were used to estimate the rate constant for dissociation of RuBP from inactive sites *in vivo*.

Ribulose 1,5-bisphosphate carboxylase catalyzes the fixation of atmospheric CO_2 in the photosynthetic carbon reduction cycle. It is now well established that this enzyme has eight active sites per holoenzyme and that each site is not catalytically competent unless complexed with CO_2 and Mg^{2+}. The addition of CO_2 and Mg^{2+} to a site, termed activation, involves the carbamylation of a lysine residue, and the percentage of activated sites (activation state) can be manipulated *in vitro* by varying the concentrations of CO_2 and Mg^{2+} (9). The mechanism of control for activation state *in vivo* is, however, still controversial; it is unlikely that changes in stromal CO_2 and Mg^{2+} concentrations are large enough to cause the variation in activation state observed. In many plants, the activity of RuBP[2] carboxylase is regulated by the state of activation. However, a tight-binding inhibitor of RuBP carboxylase, carboxyarabinatol 1-phosphate, has been implicated in the control of RuBP carboxylase activity in some plants, independent of changes in activation state (16).

In vitro studies with RuBP carboxylase have demonstrated that the substrate RuBP binds tightly to inactive (noncarbamylated) sites on the enzyme, excluding the activator CO_2 molecule, and thus preventing activation of the site (8). RuBP carboxylase, therefore, activates very slowly in the presence of RuBP, even at high concentrations of CO_2 and Mg^{2+}. The tight binding of RuBP to inactive sites also stabilizes the inactive form of the enzyme and shifts the activation equilibrium toward deactivation (7). Thus, RuBP substantially inhibits the rate of activation and will deactivate previously activated enzyme *in vitro*.

The binding of RuBP to inactive RuBP carboxylase poses questions concerning the control of activation state *in vivo*. In contrast to *in vitro* studies, activation proceeds relatively rapidly *in vivo* in response to an increase in light intensity (13), and is essentially complete in 5 min. Furthermore, the enzyme has been shown to achieve high activation states (approaching 100%) at high light intensities when RuBP concentrations are typically 4 mM or higher.

Several hypotheses have been advanced to explain the activation and maintenance of high activation states of RuBP carboxylase *in vivo*. Mott and Berry (11) found that the apparent k_d for the binding of RuBP to inactive sites *in vitro* increased at high pH values. Activation proceeded rapidly in the presence of high concentrations of RuBP, and high activation states were attainable at air-level CO_2 concentration at pH values of 8.6 or higher. However, there is no evidence that steady state pH values of the stroma reach this value, even at saturating light intensity. Portis *et al.* (15) showed that activation *in vitro* occurred at high RuBP concentrations in the presence of an enzyme extracted from chloroplasts, which they called RuBP carboxylase activase. Activity of RuBP carboxylase activase requires ATP (17), but its mechanism and role in regulating activation state *in vivo* have not been defined. Both of these hypotheses assume that RuBP binds to inactive sites of RuBP carboxylase *in vivo*, but the only data supporting this are those of Brooks and Portis (3), who reported that the amount of protein-bound RuBP varied in parallel with the percentage of inactive RuBP carboxylase sites.

In this study, changes from light to dark were used to investigate the binding of RuBP to inactive sites of RuBP carboxylase in intact leaves. RuBP concentrations and RuBP carboxylase activation state were measured both in the illuminated steady state and as a function of time after a step change to darkness. The photosynthetic carbon reduction

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[2] Abbreviations: RuBP, ribulose 1,5-bisphosphate; CABP, 2-carboxyarabinatol bisphosphate.

cycle should not generate RuBP during the dark period, and active RuBP carboxylase should rapidly consume all remaining free RuBP. RuBP bound to inactive sites of RuBP carboxylase should not be immediately available to the activated sites, but this pool should disappear slowly as RuBP dissociates from the inactive sites and reacts at activated sites. The time-dependent disappearance of RuBP following the light-dark transition is discussed as evidence for binding of RuBP to inactive sites of RuBP carboxylase *in vivo*, and a rate constant for dissociation is estimated.

MATERIALS AND METHODS

Sunflower (*Helianthus annuus*) was grown in sterile potting soil that was watered with nutrient solution, and spinach (*Spinacia oleracea*) was grown either as above or hydroponically. Both species were grown in controlled environment growth chambers in which the light intensity at the top of each plant was maintained at approximately 350 $\mu E(m^2s)^{-1}$. The photoperiods for spinach and sunflower were 10 and 15 h, respectively.

Gas Exchange and Quick-Kill

For experiments that required low CO_2 treatment, leaves were placed in a clamp-on leaf chamber, and gas exchange parameters were measured using a gas mixing and analysis system (10). When the appropriate conditions existed in the chamber, the leaf was quick-killed by firing a stainless steel cutting tube cooled with liquid nitrogen through the chamber with an air driven piston. The top and bottom of the chamber were constructed of parafilm, and they were cut with the leaf tissue and clamped between the bottom of the cutting tube and an aluminum pedestal, also cooled with liquid nitrogen, located under the chamber. The frozen leaf discs were stored in liquid nitrogen before being assayed.

RuBP Assays

Frozen leaf material was ground in a mortar and pestle cooled by liquid nitrogen and then transferred to an ice cold glass homogenizer containing 0.8 mL of 5% perchloric acid. The tissue was further homogenized in the grinder before 200 μL was removed and mixed with 800 μL of acetone for spectrophotometric determination of pheophytin concentration (18). One hundred μL of 1 M Mops was added to the extract before it was neutralized with KOH to a pH of 6.5 to 7.0. The neutralized extract was spun 5 min at 9000 *g* to remove the $KClO_4$ precipitate, and 100 μL of the supernatant was added to 400 μL of a reaction mixture consisting of 100 mM Bicine (pH 8.2), 20 mM $MgCl_2$, 15 mM [^{14}C]$NaHCO_3$ (1 Ci/M), and RuBP carboxylase purified from spinach (5). This carboxylase had no observable ribulose 5-phosphate kinase activity, so other intermediates of the photosynthetic carbon reduction cycle could not be converted to RuBP in the assay mixture. The reaction was allowed to go to completion before acidification with 500 μL of 2 N HCl. The solution was evaporated to dryness, and the remaining (acid stable) radioactivity was determined by liquid scintillation.

RuBP Carboxylase Assays

Leaf tissue and PVP were ground in a mortar and pestle cooled by liquid nitrogen, and then further ground in 1.5 mL 100 mM Bicine (pH 8.2) and 1 mM EDTA using a glass homogenizer. Two-hundred μL were removed to 800 μL of acetone for spectrophotometric Chl determination (1). The remaining extract was spun at 9000 *g* for 30 s, and 50 μL of the supernatant was assayed immediately (as described below) to determine the initial activity (representative of the activity present in the tissue). A portion of the extract was then activated completely with 20 mM $NaHCO_3$ and 20 mM $MgCl_2$. For spinach this activation was at room temperature for 5 min. For sunflower, however, activation was accomplished in 15 min at ice temperature to prevent a rapid loss of activity observed at warmer temperatures.

Each carboxylase assay contained 500 μL of 100 mM Bicine (pH 8.2), 5 mM DTT, 20 mM $MgCl_2$, 15 mM [^{14}C]$NaHCO_3$, and approximately 0.5 mM RuBP (generated from ribose 5-phosphate and ATP with phosphoribuloisomerase and ribulose 5-phosphate kinase). Assays were quenched after 30 s with 500 μL 2 N HCl, evaporated to dryness, and radioactivity was determined using liquid scintillation techniques.

Determination of RuBP Carboxylase Binding Site Concentration

Following complete activation, an aliquot of the carboxylase extract was removed, and a known amount of the transition state analog, CABP, was added. Five or ten min were allowed for complete binding in spinach or sunflower respectively, and the extract was then assayed for carboxylase activity. The difference in activity between this aliquot and the uninhibited total was due to occupation of active sites by CABP and was used to calculate the total number of sites present. This procedure was calibrated, and its accuracy and repeatability were checked using [^{14}C]CABP and subsequent precipitation of the enzyme with antibody (4).

RESULTS AND DISCUSSION

Because CABP binds tightly to activated sites of RuBP carboxylase (14), the degree to which it inhibits the activity of fully activated enzymes should be linearly related to the CABP concentration. This was tested by adding various amounts of CABP to aliquots of an activated leaf extract, allowing 10 min for complete binding RuBP, and then assaying each aliquot for RuBP carboxylase activity. The activity of each aliquot was plotted as a function of the amount of CABP added to that aliquot (Fig. 1). As predicted, there was a linear relationship between enzyme activity and CABP concentration, and other data, not shown, confirm this relationship for the purified enzyme. Although the actual dependence of catalysis rate on CABP concentration should not be linear as CABP concentration approaches that of active sites, the extrapolated linear relationship, shown by the dashed line in Figure 1, should intersect the abscissa at the concentration of active sites.

Because it was difficult to synthesize an exact concentration of CABP, the concentration of the stock CABP solution was

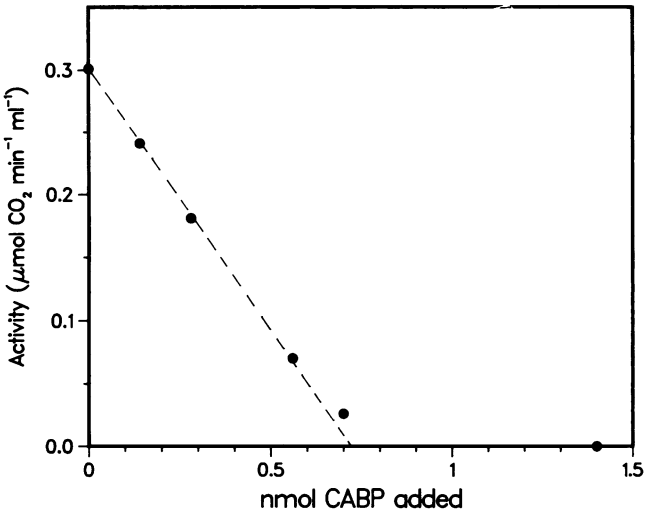

Figure 1. RuBP carboxylase activity of a fully activated spinach leaf extract as affected by CABP. Different amounts of CABP were added to a fully activated leaf extract, and carboxylase activity was measured. The extrapolated linear relationship intersects the abscissa at the binding site concentration of RuBP carboxylase.

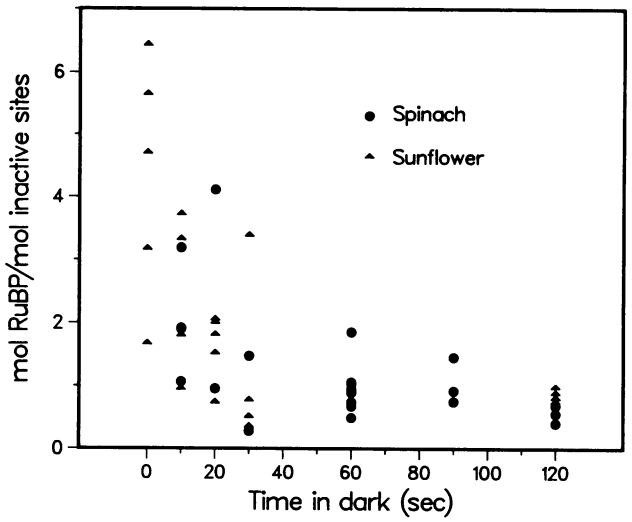

Figure 2. Moles of RuBP per mole of inactive RuBP carboxylase sites in intact leaves of spinach (●) and sunflower (▲) as a function of time after a change from light to dark. Leaf samples were freeze-clamped after various periods of time in the dark. The mol RuBP/mg Chl and mol inactive sites/mg Chl were determined on each sample.

determined indirectly by comparing the results of rate inhibition experiments with the concentration of active sites determined using [^{14}C]CABP (4). The results from several experiments were used to determine the concentration of the stock CABP solution, and all gave similar results. These data indicate that CABP partially inhibits the activity of RuBP carboxylase when CABP concentrations are less than the concentration of binding sites; this is an accurate and repeatable method for determining binding site concentration of RuBP carboxylase.

To determine whether RuBP was bound to inactive sites of RuBP carboxylase *in vivo*, we monitored the decline of RuBP concentrations in intact leaves following a step change from light to dark, and these data are shown in Figure 2. To determine the relationship between RuBP and inactive sites, RuBP concentration was expressed as a function of the concentration of inactive sites in the same tissue.

The rationale behind these experiments was that ATP production by the light reactions, and hence the regeneration of RuBP, should cease within a few seconds after the switch to darkness. However, RuBP should continue to be carboxylated (and oxygenated) at a rate comparable to that existing in the light, so its concentration should decline. If RuBP binds tightly to inactive sites of RuBP carboxylase *in vivo*, as it does *in vitro* (k_d = 0.021 μM) (6), it should remain bound to these sites as free RuBP is consumed by RuBP carboxylase. RuBP bound to inactive sites will become available to activated sites at a rate determined by the rate constant for dissociation of RuBP from inactive sites. Jordan and Chollet (6) reported a k_{off} of 4.9 × $10^{-5}s^{-1}$ at 2°C, which can be used to calculate a half-time for dissociation of 3.9 h. Assuming a Q_{10} of 2, at 22 C the $t_{½}$ for dissociation of RuBP from inactive sites would be approximately 1 h. Therefore, RuBP concentrations should decrease rapidly in darkness until all free RuBP is consumed, but RuBP levels corresponding to the concentration of inactive sites should persist for much longer, and a RuBP concentration of zero should occur only after an extended period of time in the dark.

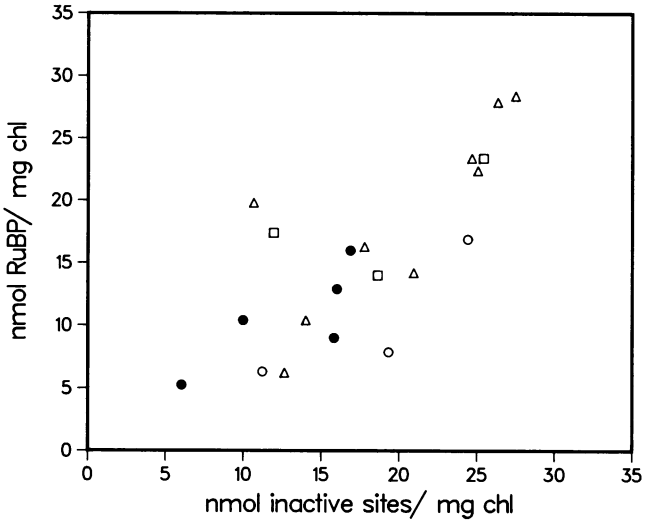

Figure 3. Relationship between RuBP concentration and the concentration of inactive RuBP carboxylase sites. Leaf samples were freeze-clamped after 60 (triangles), 90 (squares), and 120 s (circles) of darkness in spinach (open symbols) and sunflower (closed symbols). The mol RuBP/mg Chl and mol inactive sites/mg Chl were determined on each sample.

Experiments involving light to dark transitions were conducted at an intracellular CO_2 concentration of 100 ± 5 μL/L to ensure high RuBP concentrations at time zero (2, 12, 13). Under these conditions there should be a pronounced decline in RuBP concentration relative to the concentration of inactive sites following the change to darkness. Data shown in Figure 2 confirm this; values for mol RuBP/mol inactive sites were as high as 6 at time zero and declined rapidly to approximately 1 following the change to darkness. Because

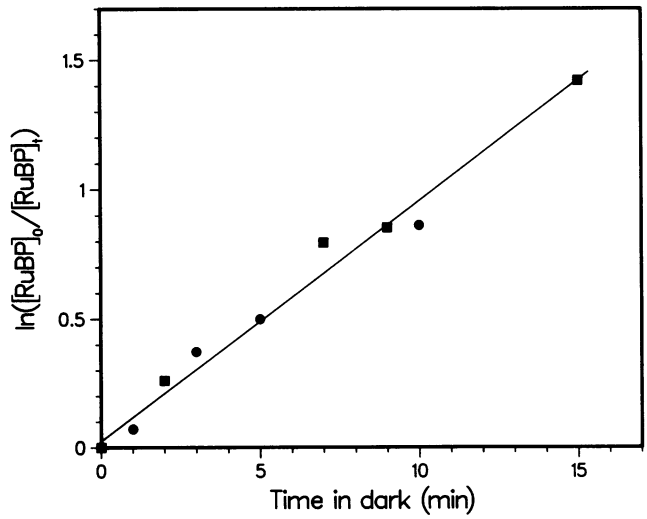

Figure 4. Decline in RuBP concentration with time in darkness for spinach leaves when RuBP concentrations were less than the concentration of inactive sites. For this experiment many freeze-clamped samples were taken from a single spinach leaf at different times after a change to darkness. The mol RuBP/mg Chl was determined for each, and the value at any time ($[RuBP]_t$) was expressed relative to the value at time zero ($[RuBP]_0$) for that leaf. Time zero in these experiments was 60 s in darkness. The different symbols represent data from different leaves. The slope of the line equals the rate constant for dissociation and was determined by linear regression to be $1.54 \times 10^{-3} s^{-1}$.

the CO_2 concentration was low and the RuBP concentration was fairly high, the consumption of free RuBP took up to 60 s, depending on the prevailing rate of carboxylation and the initial concentration of RuBP. Between 60 and 120 s after the change to darkness the RuBP concentration was approximately equal to that of inactive sites.

To demonstrate that mol RuBP/mg Chl and mol inactive sites/mg Chl were varying in parallel when all free RuBP had been consumed, we plotted the values for these two parameters after 60, 90, and 120 s of darkness (Fig. 3). Differences in mol inactive sites/mg Chl were produced by using different light intensities prior to the change to darkness. If the remaining RuBP was bound to and slowly dissociating from inactive sites of RuBP carboxylase, then the RuBP concentration for these times should have been equal to or slightly lower than the concentration of inactive sites. Therefore, some scatter below the theoretical 1:1 relationship would be expected. The data shown in Figure 3 indicate that mol inactive sites/mg Chl varied significantly among plants, consistent with the differences in light intensity prior to the change to darkness. The mol RuBP/mg Chl varied approximately in parallel with this number, and most of the scatter in the data was below the 1:1 relationship, as predicted. The results therefore support the hypothesis that RuBP binds to inactive sites *in vivo*. This agrees with the conclusions of Brooks and Portis (3), which were based on a correlation between the amount of the protein-bound RuBP and the activation state of RuBP carboxylase.

Perchorowicz *et al.* (13) demonstrated that deactivation of RuBP carboxylase is a slow process, requiring up to 1 h to reach a new steady state value following a decrease in light intensity. Activation state should, therefore, remain relatively constant over the first several minutes of darkness, and changes in mol RuBP/mol inactive sites should reflect changes in RuBP concentration rather than in the concentration of inactive sites. For time periods longer than 3 min, however, this assumption becomes increasingly suspect, and changes in mol RuBP/mol inactive sites are probably not a good measure of changes in RuBP concentration. Interplant variability, which was corrected for at times less than 3 min by relating mol RuBP to mol inactive sites, was too large to accurately determine the kinetics of RuBP disappearance for times longer than 3 min in the dark. To solve this problem many samples from one large spinach leaf were taken at different times after a change from light to dark. Two samples were taken after 60 s in the dark to ensure that RuBP concentration at that time was equal to or less than that of inactive sites, and these provided the baseline value for the other points in the time sequence. If the decline in RuBP concentration was exponential, as would be expected if it was a function of RuBP dissociation from inactive sites, then the exact starting point of the time course is irrelevant (as long as the concentration of RuBP is less than the concentration of inactive sites).

To determine k_{off} we expressed these data as the ln $([RuBP]_0/[RuBP]_t)$ where $[RuBP]_0$ and $[RuBP]_t$ are the RuBP concentrations at time zero (in this case time zero is after 60 s of darkness) and at time t, respectively (Fig. 4). Considering the data supporting the binding of RuBP to inactive sites presented above and by Brooks and Portis (3), it seems probable that this decline is a function of the rate at which RuBP is released from the inactive sites. The slope of the straight line in this type of plot is equal to the k_{off}, and by linear regression we calculate this value to be $1.54 \times 10^{-3} s^{-1}$. For the data of Jordan and Chollet (6) for the k_{off} *in vitro* with a correction for temperature ($Q_{10} = 2$), we obtained a k_{off} value of 1.96×10^{-4} at 22°C. The discrepancy between these two values may be attributed to many factors. For example, the value for k_{off} is probably pH dependent because the k_d for RuBP binding to inactive sites is pH dependent (11). The *in vitro* value of k_{off} was determined at a pH value of 8.0, while stromal pH has been measured to be approximately 7.0 in darkened isolated chloroplasts (19). In addition, RuBP carboxylase activase may affect the k_{off} value *in vivo*, although this enzyme requires ATP for activity (17) and would presumably have only low activity in the dark. If the affinity of inactive sites for RuBP is comparable to that of activated sites, there may be some rebinding of RuBP to inactive sites following dissociation, but rebinding would cause an underestimate of k_{off}, thus further increasing the discrepancy between the *in vivo* and *in vitro* values. Because of these problems, the k_{off} value determined in this study may not be a true rate constant for dissociation. It does, however, provide insight into the rate at which inactive sites are freed of RuBP and become available for activation *in vivo*.

In summary, the correlation between RuBP and inactive sites between 60 and 120 s after a switch to darkness supports the hypothesis that RuBP binds to inactive sites *in vivo*. The slow logarithmic decline in RuBP at concentrations below

that of inactive sites is consistent with a dissociation RuBP from inactive sites. The k_{off} value estimated from these data is not strictly comparable to that obtained with the isolated enzyme, but it may have relevance to the functioning of the enzyme *in vivo*. We are currently investigating this possibility.

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