Structural Characterization and the Determination of Negative Cooperativity in the Tight Binding of 2-Carboxyarabinitol Bisphosphate to Higher Plant Ribulose bisphosphate Carboxylase*

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When CO₂/Mg²⁺-activated spinach leaf ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) is incubated with the transition-state analog 2-carboxyarabinitol 1,5-bisphosphate, an essentially irreversible complex is formed. The extreme stability of this quaternary complex has allowed the use of native analytical isoelectric focusing, ion-exchange chromatography, and nondenaturing polyacrylamide gel electrophoresis to probe the mechanism of the binding process and the effects of ligand tight-binding on the structure of the protein molecule. Changes in the chromatographic and electrophoretic properties of the enzyme upon tight binding of the inhibitor reveal that the ligand induces a conformational reorganization which extends to the surface of the protein molecule and, at saturation, results in a 16% decrease in apparent molecular weight. Analysis of ligand binding by isoelectric focusing shows that (i) incubating the protein with a stoichiometric molar concentration of ligand (site basis) results in an apparently charge homogeneous enzyme population with an isoelectric point of 4.9, and (ii) substoichiometric levels of ligand produce differential effects on each of the charge microheterogeneous native enzyme forms. These stoichiometry-dependent changes in electrofocusing band patterns were employed as a probe of cooperativity in the ligand tight-binding process. The tight-binding reaction was shown to be negatively cooperative.

2-Carboxyarabinitol 1,5-bisphosphate is a potent inhibitor of higher plant ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)(1)(EC 4.1.1.39), the large, heteromultimeric enzyme that catalyzes the carboxylation and oxygenation of Ru-P₂. The interaction of 2-carboxyarabinitol-P₃ with CO₂/Mg²⁺-activated Rubisco is characterized by a slow process which results in very tight binding (K₀ = 10 pm) (1). It is postulated that tight binding occurs as a result of structural changes in or around the catalytic site which are promoted by similarities between 2-carboxyarabinitol-P₃ and 2-carboxy-3-keto-arabinitol 1,5-bisphosphate (2), the 6-carbon transition-state intermediate of the carboxylase reaction (3). Studies on the mechanism of 2-carboxyarabinitol-P₃ binding to the active spinach enzyme have demonstrated that this interaction takes place in at least two steps (1).

Step 1: Rubisco + CABP → Rubisco-CABP

Step 2: Rubisco-CABP → Rubisco*CABP

According to this mechanism, 2-carboxyarabinitol-P₃ (CABP) equilibrates rapidly with CO₂/Mg²⁺-activated Rubisco, forming a relatively loose complex (Rubisco-CABP, K₀ = 0.4 µM), which may then undergo a slow, irreversible isomerization to the inactive, exchange-inert quaternary complex of enzyme-CO₂-Mg²⁺-CABP (Rubisco*CABP). Support for an ordered binding mechanism is found in the observation of initially competitive kinetics between Ru-P₃ and 2-carboxyarabinitol-P₃ and the ensuing irreversible, noncompetitive inhibition of enzymic activity (1). Furthermore, the decreased rate of irreversible inhibition of catalysis observed in the presence of Ru-P₃ (2) and the essentially irreversible trapping of activator CO₂ and Mg²⁺ by 2-carboxyarabinitol-P₃ (4) suggest that this phosphorylated ligand binds within the catalytic center. While the mechanism of the slow, irreversible inhibition is not clearly understood, qualitative similarities in the UV difference spectra of enzyme-CABP and non-CO₂/Mg²⁺-activated enzyme-Ru-P₃ complexes are consistent with a mechanism in which the initial binding of the inhibitor promotes conformational changes similar to those occurring during catalysis and which, owing to the catalytic incompetence of the ligand, subsequently form an inactive, exchange-inert complex (2).

The extent to which 2-carboxyarabinitol-P₃ binding distorts the native Rubisco molecule is as yet unanswered, as is the question of whether homotropic effects occur upon subsequent ligand binding. Previous analyses have shown that the CO₂/Mg²⁺-activated carboxylase does not display sigmoidal kinetics with respect to Ru-P₃ and hence is not positively cooperative (5). However, given the complex activation and catalytic mechanisms of Rubisco (6), as well as the sensitivity of the enzyme and gaseous substrate to variations in ionic strength, kinetic analyses, alone, are not likely to elucidate the allosteric effects of ligand binding. As such, determination of the equilibrium binding distribution of a slow, tight-binding inhibitor within a population of enzyme...
molecules seems a more fruitful approach for determining the presence or absence of cooperative effects with respect to ligand binding.

In the research described herein, a variety of techniques were employed to analyze the gross structural effects of tight binding of 2-carboxyarabinitol-P₂ on the CO₂/Mg²⁺-activated spinach carboxylase. These findings were then used to demonstrate that the tight binding of this phosphorylated ligand to CO₂/Mg²⁺-activated spinach leaf Rubisco displays negative cooperativity. The proof is provided by experiments in which the relative preference of 2-carboxyarabinitol-P₂ for binding sites on unigated and partially 2-carboxyarabinitol-P₂-saturated Rubisco was determined by native isoelectric focusing (IEF).

EXPERIMENTAL PROCEDURES

Synthesis and Purification of Ru-P₂ and 2-Carboxyarabinitol-P₂—Ru-P₂ was prepared from ribose 5-phosphate as previously described (7) and purified according to Kuehn and Hsu (8), with the exception that a step gradient (100/200 mM LiCl in 1 mM HCl) was used in the ion-exchange procedure. The final product contained no detectable ADP as determined by absorbance at 260 and 280 nm. 2-Carboxyarabinitol-P₂ and [2-C-14C]2-carboxyarabinitol-P₂ (20 Ci/mmol) were prepared by the method of Brown et al. (10). The epimeric product 2-carboxyribitol 1,5-bisphosphate according to Ref. 1. 2-Carboxyarabinitol-P₂ concentrations were determined by inorganic phosphate analysis (9) of acid phosphatase-treated samples.

Spinach Rubisco Purification and Assay—Homogeneous Rubisco, as determined by denaturing and non-denaturing polyacrylamide gel electrophoresis, was isolated from market spinach leaves according to the method of Brown et al. (10). The protein was stored at 4 °C as an ammonium sulfate precipitate. Prior to use, the enzyme was dissolved in 25 mM Bicine/NaOH, pH 8.0, and desalted by two passes through centrifugal columns, and resolved by native isoelectric focusing in a 5% polyacrylamide horizontal slab gel containing 1.6% LKB, pH 5-8, and 0.4% LKB, pH 3.5-10, carrier ampholytes. The final product contained no detectable ADP as determined by absorbance at 260 and 280 nm. 2-Carboxyarabinitol-P₂ was added to the enzyme solution and allowed to react for 15 min at 30 °C with 20 mM MgCl₂/20 mM NaHCO₃ (0.1 Ci/mmol) in an assay medium containing 25 mM Bicine/NaOH, pH 8.0, 0.2 mg/ml bovine serum albumin, and 0.1 mM Na₂ EDTA. Specific activity was typically 2.3 μmol/min/mg protein for a 1-min assay at 0.3 mM Ru-P₂ and 30 °C. The 0.5-ml assays were stopped and the enzyme was harvested by centrifugation, 2 min at 1840 × g. Protein concentrations (mg/ml) were determined by multiplying A₂₈₀ nm × g with the extinction coefficient of 0.61 (11). Calculations of active-site concentrations were employed to analyze the gross structural effects of tight binding for enzyme species with m ligands irreversibly bound. Simultaneous calculations were performed using the method of Brown et al. (10) for a 1:2 CoM:enzyme ratio, a plot of log F versus separating gel concentration, a generalization of this to the case of n:1 CoM:enzyme, and the number of free and bound CoM:enzyme. The migration of the bromphenol blue dye and protein bands from the bottom of the 3% spacer gel was calculated by a binomial distribution (14-16). Differential Binding Experiments—Binding distributions of any noncooperative system with an equilibrium constant can be calculated as above except that the desalting step centrifugation time was reduced to 15 s in order to concentrate the protein. IEF was performed according to the protocol outlined above. In order to eliminate phase separation, aliquots (100 μl) for the determination of binding stoichiometry were dried at 90 °C in the presence of 100 μl of 6 M CH₃COOH prior to liquid scintillation counting.

Electrophoretic Analysis—Activated spinach Rubisco, 2-carboxyarabinitol-P₂-treated as described above, was subjected to non-denaturing polyacrylamide gel electrophoresis. Slabs (12.2 mm thick) of 4.5, 5.6, or 7% separating gel were prepared using the buffer system described by Laemmli (12), but without sodium dodecyl sulfate, and run at 4 °C until the tracking dye was within approximately 1 cm of the bottom. The position of the dye front was marked, and the gels were stained and destained as previously described. The migration of the bromphenol blue dye and protein bands from the bottom of the 3% separations gel was measured immediately, and the results were expressed as the ratio of protein to dye migration (Rₓ). Since Rₓ varies exponentially with gel concentration, a plot of log Rₓ versus separating gel concentration was used to analyze changes in apparent molecular weight (13). Differential 2-Carboxyarabinitol-P₂-binding Experiments—Phase 1 (see Fig. 1): spinach Rubisco (2.6 mg/ml; 4.6 μM holoenzyme) was activated at 30 °C for at least 20 min with 20 mM NaHCO₃ and 10 mM MgCl₂ in 25 mM Bicine/NaOH, pH 8.0. Aliquots were then incubated for 30 min at 30 °C with 2-carboxyarabinitol-P₂ or [14C]2-carboxyarabinitol-P₂ (20 Ci/mmol) at 2-carboxyarabinitol-P₂ holoenzyme molar ratios of 0, 2, 4, and 6. Phase 2 (see Fig. 1): aliquots of unigated Rubisco from the same stock of activated enzyme used in Phase 1 were added to the Phase 1 enzyme so as to double either the amount of protein (mg/ml) (Fig. 1, Protocol A) or the number of free binding sites (Fig. 1, Protocol B). Nonradioactive 2-carboxyarabinitol-P₂, at a concentration sufficient to bring the second ligand of enzyme to the same partial saturation as the first, was then added. Following another 30-min incubation at 30 °C and centrifugal desalting, samples from both phases were subjected to native IEF on 5% polyacrylamide horizontal slab gels. In order to reduce systematic error in experiments involving both unlabeled and [14C]2-carboxyarabinitol-P₂, the concentrations of these two reagents were kept as matched empirically by determining the aliquot size of each required to produce an identical change in the IEF pattern of native spinach Rubisco (see Fig. 2).

Calculation of Ligand Distribution for Differential Binding Experiments—Binding distributions of any noncooperative system with an initial uniform number of binding sites/protein molecule can be calculated by a binomial distribution (14–16). A generalization of the following steps is derived: (1) was applied to arbitrary initial distributions and two-phase irreversible binding. In Equation 1, F₁ is the fraction of protein with n moles of ligand (L) bound, n is the total number of binding sites/enzyme molecule, and kₘ is the intrinsic probability of tight binding for enzyme species with m ligands reversibly bound. Simultaneous integration was performed using a fourth-order Runge-Kutta procedure. Agreement to six significant figures with the binomial distribution for an 8-site noncooperative case was obtained (starting with F₁ = 1) using 100 steps.
2-Carboxyarabinitol-P₂ Binding to Rubisco

### Results

Effects of 2-Carboxyarabinitol-P₂ Tight-binding on the Activity and Native Isoelectric Focusing Pattern of Spinach Rubisco—A Coomassie Brilliant Blue-stained polyacrylamide slab gel illustrates the native and 2-carboxyarabinitol-P₂-treated spinach carboxylase IEF patterns (Fig. 2). The untreated enzyme routinely focuses as several bands within a diffuse region of protein (11) between pH 5.5 and 5.8, while the 2-carboxyarabinitol-P₂-saturated enzyme (8 2-carboxyarabinitol-P₂/holoenzyme) focuses as a single tight band at a pH of 4.9. These distinct focusing characteristics of the native and ligand-saturated enzymes are independent of protein load and essentially invariant with enzyme preparation or IEF system (including LKB pH 3.5–10, 5–8, or 4–6 gradients; polyacrylamide tube or vertical slab gels or a Bio-Lyte granulated bed). Fractionation, extraction, and assay of focused spinach Rubisco reveal that all portions of the native pattern possess RuP₂-dependent carboxylase activity. Incubation of the activated enzyme with 2-carboxyarabinitol-P₂ at ligand to holoenzyme molar ratios of between 1 and 8 results in an essentially linear decrease in enzymic activity (data not shown).

The effect of binding substoichiometric quantities of 2-carboxyarabinitol-P₂ on the native IEF pattern reveals a complex interaction between ligand tight-binding and the physical properties of the native enzyme forms (Fig. 2). Upon addition of 1 2-carboxyarabinitol-P₂/holoenzyme, the diffuse native carboxylase pattern begins to tighten into a series of discrete bands. Concomitant with this change, some protein migrates to a more acidic region of the pH gradient, although the majority of the sample still remains within the boundaries of the native pattern. Two prominent bands, designated α and β, appear at isoelectric points of 5.45 and 5.30, respectively. α remains a visually distinct band at up to 6 2-carboxyarabinitol-P₂/holoenzyme, while β is detectable at up to 6 2-carboxyarabinitol-P₂/holoenzyme. α is not sufficiently resolved to allow densitometric determination of its relative protein content; scans of β, however, show that it contains 14–19% of the total stained protein at between 1 and 5 2-carboxyarabinitol-P₂/holoenzyme. α remains a visually distinct band at 2-carboxyarabinitol-P₂ levels of between 1 and 5/holoenzyme, while β is detectable at up to 6 2-carboxyarabinitol-P₂/holoenzyme. α is not sufficiently resolved to allow densitometric determination of its relative protein content; scans of β, however, show that it contains 14–19% of the total stained protein at between 1 and 5 2-carboxyarabinitol-P₂/holoenzyme. A new series of closely spaced bands accumulates between pH 5.3 and 4.9 as 2-carboxyarabinitol-P₂ is increased from 1 to 5/holoenzyme, while protein in the original native region is virtually depleted at 2-carboxyarabinitol-P₂ to holoenzyme ratio of 5 to 1. Between 6 and 8 2-carboxyarabinitol-P₂/holoenzyme, the protein pattern tightens markedly, ultimately giving a single tight band at a 2-carboxyarabinitol-P₂ to enzyme ratio of 8 to 1. No effect is observed on the pH 4.9 band when the protein is treated with a molar excess of 2-carboxyarabinitol-P₂ indicat-
ing that, as expected, no more than eight ligands bind to the holoenzyme.

Fluorography of Enzyme-[14C]CABP Complexes—In order to examine the relationship between binding stoichiometry, isoelectric point and banding pattern, fluorography and protein staining were performed on identical halves of gels containing [14C]2-carboxyarabinitol-P2-treated Rubisco. The fluorographs and stained protein patterns on the gels are qualitatively similar at all ratios of ligand to holoenzyme examined (Fig. 3). Comparison of the untreated protein pattern with the fluorographs at 1 and 2 2-carboxyarabinitol-P2/ holoenzyme reveals that 2-carboxyarabinitol-P2 is bound to bands which have pI values identical to those of the unligated protein. When densitometric tracings of the stained and fluorographic patterns are compared at each of the 2-carboxyarabinitol-P2 stoichiometries examined, no marked differences are observed in the amount of ligand bound per unit of stainable protein as a function of pI. However, limitations in the sensitivity and resolution of fluorography and densitometry preclude a more quantitative analysis of the 2-carboxyarabinitol-P2/protein stoichiometry in each of the bands.

Resolution of Enzyme-CABP Complexes by Anion-exchange Chromatography—In light of the magnitude of change observed in the charge microheterogeneity of native Rubisco upon tight binding of 2-carboxyarabinitol-P2 (Figs. 2 and 3), anion-exchange chromatography was employed in an attempt to separate the treated enzyme on the basis of binding stoichiometry, thus permitting quantitation of the binding distribution. Ion-exchange chromatography has been previously used to resolve chemically modified proteins differing by as little as two net surface charges (17). Similarly, this technique is very sensitive to the distribution of charge on the surface of proteins (18).

The bottom panel of Fig. 4 shows the protein desorption

![Fig. 3. Direct comparison of fluorographs and protein-stained gels of focused native and 2-carboxyarabinitol-P2-treated enzymes. For methods, see “Experimental Procedures.” The left side is stained for protein, while the right side is a fluorograph of the complementary gel half. The top and bottom halves of the figure represent separate gels. The 2-carboxyarabinitol-P2/holoenzyme stoichiometry is indicated below each lane. Protein load was 30 μg/lane.](image-url)
profiles of both native and \[^{14}C\]2-carboxyarabinitol-P\(_2\)-treated spinach Rubisco as eluted from a DE53 anion-exchange column with a linear, 150-350 mM, NaCl gradient at pH 8.2. Free ligand is released from the resin at a NaCl concentration of less than 200 mM. \[^{14}C\]2-Carboxyarabinitol-P\(_2\)-saturated Rubisco elutes at 250 mM NaCl, which is approximately 25 mM lower in the gradient than the untreated protein. Enzyme incubated with intermediate molar ratios of 2-carboxyarabinitol-P\(_2\) is eluted between these two extremes. A number of other gradient and buffer systems were also employed, but this system provided the best resolution of the enzyme-CABP complexes.

Measurement of \(^{14}C\) ligand bound to the eluted protein (Fig. 4, top panel) shows a constant stoichiometry of 7.7 2-carboxyarabinitol-P\(_2\)/holoenzyme across the elution peak of the 2-carboxyarabinitol-P\(_2\)-saturated enzyme, while the profiles of enzyme incubated at 1, 2, and 4 2-carboxyarabinitol-P\(_2\)/holoenzyme show the majority of the eluted protein to have a stoichiometry of within ±1 of that present during preincubation. When a mixture of native enzyme and that treated with eight \[^{14}C\]2-carboxyarabinitol-P\(_2\)/holoenzyme is chromatographed, a single, broad \(A_{280}\) nm elution peak results, suggesting that protein-protein interactions may be affecting the resolution of the system. However, determination of \(^{14}C\) radioactivity across the peak indicates that only the front half of the protein profile is radioactive. When eluant samples of enzyme treated at various substoichiometric levels of 2-carboxyara-

Fig. 4. DE53 anion-exchange column chromatography of spinach Rubisco pretreated with 2-carboxyarabinitol-P\(_2\). The lower panel shows the elution profiles \((A_{280}\text{nm})\) of enzyme treated with 0 (\(\bullet\)), 1 (■), 2 (△), 4 (○), and 8 (□) 2-carboxyarabinitol-P\(_2\)/holoenzyme and chromatographed as described under "Experimental Procedures." NaCl concentrations for the elution of the individual protein samples were calculated based on the gradient volume, giving 275, 260, 260, 255, and 250 mM for the peak fractions at 0, 1, 2, 4, and 8 2-carboxyarabinitol-P\(_2\)/holoenzyme, respectively. The top panel shows the 2-carboxyarabinitol-P\(_2\)-binding stoichiometry across each peak as determined by the ratio of \[^{14}C\]2-carboxyarabinitol-P\(_2\)/holoenzyme. When applied to a single protein, this analysis allows a distinction to be made between the effects of ligand binding on the net charge and/or shape of the macromolecule.

Activated spinach Rubisco pretreated with 0, 2, 4, 6, or 10 2-carboxyarabinitol-P\(_2\)/holoenzyme was electrophoresed on nondenaturing vertical slab gels of 4.5, 5.6, and 7% separating gel monomer concentrations. The effect of ligand binding at these various molar ratios on electrophoretic mobility is illustrated in Fig. 5A. The stained gels at each of the different polyacrylamide concentrations demonstrate that each addition of 2-carboxyarabinitol-P\(_2\) increases protein mobility. A plot of log protein mobility relative to the dye front \((R_m)\) versus polyacrylamide gel concentration at the five 2-carboxyarabinitol-P\(_2\) stoichiometries examined yields five nonparallel lines which, when extrapolated, intersect at a 2% separation gel concentration (Fig. 5B). At this hypothetical gel concentration, the free protein and the CABP-enzyme complexes would migrate identically since sieving and charge effects would cancel. Since only sieving effects vary with gel concentration, the finding of nonparallel lines extrapolating to a common point near 0% gel concentration strongly suggests that the various enzyme-CABP complexes differ primarily in hydrodynamic shape (13). That is, under the electrophoretic conditions employed, the various protein samples have essentially identical net charge, but differ in molecular size. The magnitude of these conformational differences is highlighted in Table I, where the effect of 2-carboxyarabinitol-P\(_2\)-binding on the apparent molecular weight of native Rubisco is shown. Since the binding of eight 2-carboxyarabinitol-P\(_2\) and 8 CO\(_2\)/Mg\(^{2+}\) molecules adds a mass of less than 1% of the ~560-kDa Rubisco molecule, the observed differences in hydrodynamic molecular weight must reflect ligand-induced alterations in the shape of the enzyme molecule. The decrease in apparent molecular weight of about 90,000 for the 2-carboxyarabinitol-P\(_2\)-saturated enzyme (Table I) documents the magnitude of this structural reorganization, while the finding of changes in electrophoretic mobility with each change in 2-carboxyarabinitol-P\(_2\) stoichiometry (Fig. 5) shows that sequential ligand binding results in sequential changes in protein structure. In addition, it can be seen that the decrease observed in apparent molecular weight is not linear.
Effect of 2-carboxyarabinitol-P₂ tight-binding on the apparent molecular weight of spinach Rubisco

The molar ratio of 2-carboxyarabinitol-P₂ (CABP) to holoenzyme was that present during the 40 min preincubation with activated enzyme (see Fig. 5). Apparent masses were calculated using the linear slope-molecular weight relationship given by Hedrick and Smith (13).

### TABLE I

**Predicted final average CABP/holoenzyme molar ratio**

| Protocol | Phase 1 protein | Phase 2 protein |
|----------|----------------|----------------|
| A (protein basis) | 2.9 (50%) | 1.1 (50%) |
| B (free site basis) | 2.8 (67%) | 1.0 (43%) |

Differential binding experiments were simulated for a noncooperative, 8-site enzyme using the binding equation described under “Experimental Procedures” (see Equation 1). As indicated, the average binding stoichiometry was made constant at the end of each experimental phase (see Fig. 1). The numbers in each column represent the final mean binding stoichiometry of protein from each phase at the end of the simulated two-phase experiment. The range of stoichiometries about each mean is given by the appropriate binomial distribution since no site-site interaction occurs. The numbers in parentheses represent the per cent of total protein from each phase at the end of the experiment. CABP, 2-carboxyarabinitol-P₂.

**Final mean stoichiometries of 2-carboxyarabinitol-P₂ binding for differential binding experiments with a noncooperative, 8-site enzyme**

### TABLE II

Differential binding experiments were simulated for a noncooperative, 8-site enzyme using the binding equation described under “Experimental Procedures” (see Equation 1). As indicated, the average binding stoichiometry was made constant at the end of each experimental phase (see Fig. 1). The numbers in each column represent the final mean binding stoichiometry of protein from each phase at the end of the simulated two-phase experiment. The range of stoichiometries about each mean is given by the appropriate binomial distribution since no site-site interaction occurs. The numbers in parentheses represent the per cent of total protein from each phase at the end of the experiment. CABP, 2-carboxyarabinitol-P₂.

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with respect to 2-carboxyarabinitol-P₂ stoichiometry (Table I). Since each 2-carboxyarabinitol-P₂ molecule contributes the same overall formal charge and mass to the enzyme, this nonlinearity is suggestive of subunit interactions which vary as a function of the number of ligands bound.

**Differential Binding Experiments**—In view of the above findings, a protocol was devised to investigate possible allosteric effects of the 2-carboxyarabinitol-P₂-induced conformational changes. An outline of the experimental design is presented in Fig. 1. Two variations of the basic experimental protocol (A, B) were employed. In Protocol A equal quantities of protein (i.e., total sites), with and without tightly bound 2-carboxyarabinitol-P₂, competed for the binding of additional 2-carboxyarabinitol-P₂ during Phase 2, while in the second (Protocol B) equal numbers of unligated sites (i.e., sites without ligand irreversibly bound) competed for additional 2-carboxyarabinitol-P₂. In both designs, the amount of 2-carboxyarabinitol-P₂ added in Phase 2 was sufficient to bring 2-carboxyarabinitol-P₂ partial saturation back to the same level obtained at the end of Phase 1. It should be noted that the choice of protocol affects the sensitivity of IEF since with Protocol A protein from Phase 1 comprises 50% of the final protein regardless of 2-carboxyarabinitol-P₂ stoichiometry, while the fraction of protein from Phase 1 in the final mixture varies with 2-carboxyarabinitol-P₂ stoichiometry in Protocol B (see Table II). Although Protocol A has the obvious advantage of maintaining a constant protein ratio in each binding phase, it is also subject to random errors as 2-carboxyarabinitol-P₂ stoichiometry is increased. Consequently, the number of binding sites and the quantity of 2-carboxyarabinitol-P₂...
(relative to sites from Phase 1) increase as a function of 2-carboxyarabinitol-P₂ stoichiometry. For example, if the protein in Phase 1 has 4 2-carboxyarabinitol-P₂/holoenzyme and an equimolar concentration of activated Rubisco is added during Phase 2, the unligated enzyme will have twice as many free catalytic sites in solution as the partially bound enzyme or 67% of the total unoccupied sites. In contrast, Protocol B is less subject to such errors since the amount of both enzyme and 2-carboxyarabinitol-P₂ added in Phase 2 decreases with increasing 2-carboxyarabinitol-P₂ stoichiometry.

Since both an irreversible and a reversible process are involved in 2-carboxyarabinitol-P₂ binding (1), the validity of experiments in which ligand is added in more than one aliquot must be established by showing that, under the conditions employed (i.e. protein and ligand concentrations), the final distribution of ligand within the enzyme population is independent of the path by which it is added. This was verified experimentally by bringing activated Rubisco (4.6 mM, as described under “Experimental Procedures”) to a final 2-carboxyarabinitol-P₂/holoenzyme stoichiometry of 1.5, 3, or 6 in either one or several successive steps. After each aliquot, the solution was allowed to incubate for 30 min at 30 °C prior to the addition of more 2-carboxyarabinitol-P₂. IEF analysis and carboxylase activity measurements showed that this interval was sufficient for complete binding. Both the final activity and native IEF pattern were found to be independent of the number of aliquots by which ligand was added.

Molar ratios of 2, 4, and 6 2-carboxyarabinitol-P₂/holoenzyme were chosen for the differential binding experiments so that distinct components of the IEF profile could be monitored at each 2-carboxyarabinitol-P₂ stoichiometry examined (see Fig. 2). Calculations of the final distribution of ligand among the protein molecules from Phase 1 and Phase 2 (Fig. 1) for an 8-site noncooperative binding process (Table II) allow direct comparison of the actual results from the differential binding experiments to those predicted for a noncooperative process.

The results of an experiment using Protocol A (protein basis) are presented in Fig. 6. The IEF patterns obtained in Phase 1 at ligand ratios of 2, 4, and 6 2-carboxyarabinitol-P₂/holoenzyme (lanes d, e, and f, respectively) are in agreement with those in Fig. 2. The absence of 2-carboxyarabinitol-P₂ exchange between Phase 1 and Phase 2 protein is evidenced by the appearance of native Rubisco in the pH 5.5-5.8 region of the gel when buffer instead of 2-carboxyarabinitol-P₂ was added in Phase 2 (data not shown). By comparison of the protein patterns obtained after each phase at 2, 4, and 6 2-carboxyarabinitol-P₂/holoenzyme (lanes b/c, d/e, and f/g, respectively), it is evident that 2-carboxyarabinitol-P₂ preferentially binds to sites on protein with low levels of ligand bound. The ligand distribution calculation predicts that for an experiment using Protocol A with a noncooperative enzyme at 2 2-carboxyarabinitol-P₂/holoenzyme, protein added in Phase 2 should have a final average stoichiometry of 1 2-carboxyarabinitol-P₂/holoenzyme (Table II). At this stoichiometry, all of the major native enzyme bands (PI 5.5-5.8; lane a) should be readily visible. However, these bands are clearly not evident even though Phase 2 protein makes up 50% of the total protein loaded onto the gel (Table II). Similar results were obtained for the experiments at molar ratios of 4 and 6 2-carboxyarabinitol-P₂/holoenzyme (Fig. 6). The minor banding differences between Phase 1 and Phase 2 proteins (lanes b/c, d/e, and f/g of Fig. 6) can be accounted for by variations in the addition of enzyme and ligand between the two phases.

Fig. 7 illustrates the results of a differential 2-carboxyarabinitol-P₂ binding experiment performed on a free site basis (Protocol B of Fig. 1). At 2 2-carboxyarabinitol-P₂/holoenzyme, the gel patterns of equal amounts of protein after Phase 1 and Phase 2 (lanes a and b) are identical, while the calculations presented in Table II predict that protein added in Phase 1 and 2 should have final average 2-carboxyarabinitol-P₂ stoichiometries of 2.8 and 1.0, respectively, for a noncooperative system. Since the IEF profiles of equal amounts of protein at 1 and 3 2-carboxyarabinitol-P₂/holoenzyme differ markedly (see Fig. 2), these findings clearly demonstrate that at these levels of partial saturation there are changes in the intrinsic (site) rate for the 2-carboxyarabinitol-P₂ tight-binding process. At 4 2-carboxyarabinitol-P₂/holoenzyme (lanes c and d), protein added in Phase 2 should have a final average 2-carboxyarabinitol-P₂ stoichiometry of 2.0 for a noncooperative system (Table II). Direct comparison of the more acidic end of the IEF pattern after Phase 2 (lane d) with protein at 2 2-carboxyarabinitol-P₂/holoenzyme (lane a) shows the stoichiometry to be greater than predicted. At 6 2-carboxyarabinitol-P₂/holoenzyme (lanes e and f), this trend appears to continue, although the errors associated with determination of protein (site) and ligand concentrations, as well as the small fraction of total protein added in Phase 2 (Table II), preclude detailed analysis at this 2-carboxyarabinitol-P₂/holoenzyme level.

In order to examine the effects of competition for 2-carboxyarabinitol-P₂ between unligated and partially saturated Rubisco on the distribution of the partially ligated enzyme species after Phase 2, differential binding experiments were performed using [14C]2-carboxyarabinitol-P₂ in the first phase (see Fig. 1). A single polyacrylamide gel was cut in half after electrofocusing and employed for both protein staining and fluorography. Fig. 8 shows the protein staining (lanes a-f) and fluorography (lanes g-l) patterns from an experiment performed using Protocol B (free site basis) at 2 and 4 2-carboxyarabinitol-P₂/holoenzyme. Lanes a-f show the typical protein patterns obtained with this protocol (see Fig. 7). From the corresponding fluorograph, it can be seen that some migration of protein from the first phase does occur during the second phase. This is particularly evident in the more basic end of the pattern (e.g. lanes h, i, and j, k). However, there is little net shift of the fluorographic patterns to more acidic PI values, indicating that only minor changes occur in binding stoichiometry. These findings are consistent with the presence of negative cooperativity, since the binding of unlabeled 2-carboxyarabinitol-P₂ during the second phase is apparently restricted to Rubisco molecules which have low ligand stoichiometries at the end of Phase 1.

**DISCUSSION**

2-Carboxyarabinitol-P₂ binding to higher plant Rubisco has been investigated previously by kinetic and spectral analyses (1, 2, 4, 19). These studies have shown that the activated enzyme and this phosphorylated ligand interact by a two-step mechanism, which presumably involves a change in protein conformation (2, 19, 20). The extent and effects of this proposed structural reorganization have not been elaborated, however. The experiments reported herein extend these earlier studies by demonstrating that the decrease in carboxylase specific activity is a linear function of the amount of 2-carboxyarabinitol-P₂ bound per holoenzyme and that tight binding is correlated with specific, reproducible changes in the native IEF pattern, as well as in the chromatographic and electrophoretic behavior of the protein. Specifically, the effects of 2-carboxyarabinitol-P₂ on the hydrodynamic shape, charge, and surface properties of the native protein demonstrate the occurrence of a significant, ligand-induced confor-
2-Carboxyarabinitol-P$_2$ Binding to Rubisco

Fig. 6. Results of a differential 2-carboxyarabinitol-P$_2$-binding experiment using Protocol A. Coomassie Brilliant Blue-stained horizontal IEF gel, pH 5–8, of equal amounts of protein (30 μg/lane) following Phase 1 and Phase 2 2-carboxyarabinitol-P$_2$-binding (see Fig. 1, Protocol A). Lane a, native, unligated spinach Rubisco; b, Phase 1 binding at 2 2-carboxyarabinitol-P$_2$/holoenzyme; c, Phase 2 binding at 2 2-carboxyarabinitol-P$_2$/holoenzyme; d, Phase 1 binding at 4 2-carboxyarabinitol-P$_2$/holoenzyme; e, Phase 2 binding at 4 2-carboxyarabinitol-P$_2$/holoenzyme; f, Phase 1 binding at 6 2-carboxyarabinitol-P$_2$/holoenzyme; g, Phase 2 binding at 6 2-carboxyarabinitol-P$_2$/holoenzyme; h, fully ligated Rubisco.

Fig. 7. Results of a differential 2-carboxyarabinitol-P$_2$-binding experiment employing Protocol B. Stained IEF gel (30 μg of protein/lane) from a differential binding experiment in which an equal number of free (i.e. unoccupied) sites on unligated and partially ligated Rubisco compete for 2-carboxyarabinitol-P$_2$ in Phase 2 (see Fig. 1, Protocol B). Lanes a and b, 2 2-carboxyarabinitol-P$_2$/holoenzyme upon completion of Phase 1 and Phase 2, respectively; lanes c and d, 4 2-carboxyarabinitol-P$_2$/holoenzyme after the first binding reaction and upon completion of the second binding phase, respectively; lanes e and f, 6 2-carboxyarabinitol-P$_2$/holoenzyme following Phase 1 and Phase 2 binding, respectively; lane g, fully ligated Rubisco; lane h, native, unligated enzyme.

mational change in the Rubisco molecule. The hypothesis that the changes observed in the properties of the Rubisco molecule upon 2-carboxyarabinitol-P$_2$ tight binding are the result of intersubunit forces exerted by the ligand is supported by the IEF and gel electrophoresis data.

2-Carboxyarabinitol-P$_2$ Binding to the Native Spinach Rubisco Forms—Each of the three independent techniques used in the present study separate, to some extent, on the basis of 2-carboxyarabinitol-P$_2$ stoichiometry. In no case does the observed heterogeneity in binding stoichiometry markedly
2-Carboxyarabinitol-\(P_2\) Binding to Rubisco

**Fig. 8.** Direct comparison of fluorographs and protein-stained gels of a differential 2-carboxyarabinitol-\(P_2\)-binding experiment in which ligated and unligated Rubisco contributed an equal number of free sites to the second phase (Protocol B). Phase 1 and Phase 2 binding employed \(^{14}C\)-2-carboxyarabinitol-\(P_2\) and unlabeled 2-carboxyarabinitol-\(P_2\), respectively. For methods, see “Experimental Procedures” and Fig. 1, Protocol B. The left side of the gel is stained for protein, while the right side is a fluorograph of the complementary gel half. Lanes a and g, native, unligated enzyme; lanes b and l, fully 2-carboxyarabinitol-\(P_2\)-saturated Rubisco; lanes c and h, the Phase 1 reaction at 2 2-carboxyarabinitol-\(P_2\)/holoenzyme; lanes d and i, 2 2-carboxyarabinitol-\(P_2\)/holoenzyme following both phases; lanes e and j, 4 2-carboxyarabinitol-\(P_2\)/holoenzyme after Phase 1; lanes f and k, the binding distribution at 4 2-carboxyarabinitol-\(P_2\)/holoenzyme following the second phase.

Beyond that predicted by a binomial distribution. In particular, the native IEF gels show little or no protein in the pH 4.9 region, where all protein migrates when it is 2-carboxyarabinitol-\(P_2\)-saturated, prior to the addition of 5–6 2-carboxyarabinitol-\(P_2\) ligands to the protein. The binomial distribution model of ligand binding for an 8-site enzyme predicts that only 10% of the protein should have eight ligands bound when the mean 2-carboxyarabinitol-\(P_2\) stoichiometry is 6/holoenzyme. Anion-exchange chromatography resolves only a limited range of 2-carboxyarabinitol-\(P_2\) stoichiometries, and gel electrophoresis, while clearly able to separate enzymes with differences in 2–4 in 2-carboxyarabinitol-\(P_2\) stoichiometries, and gel electrophoresis, while clearly able to separate enzymes with differences in 2–4 in 2-carboxyarabinitol-\(P_2\) stoichiometries, shows fairly tight bands. If the probability of a tight-binding event varied markedly in any subset of the native enzyme population, it would be reflected in the final binding distribution. Thus, each of the native forms of the enzyme appear to have similar rates of ligand tight-binding at each level of 2-carboxyarabinitol-\(P_2\) saturation examined. The possibility of positive cooperativity in the 2-carboxyarabinitol-\(P_2\) tight-binding process is also precluded by these experiments, since this, too, would result in a broad (i.e. nonbinomial) distribution of 2-carboxyarabinitol-\(P_2\) stoichiometries.

**Gross Conformational Effects of 2-Carboxyarabinitol-\(P_2\)**

Tight-binding—Although the tightening of the native IEF titration pattern upon 2-carboxyarabinitol-\(P_2\) saturation is highly suggestive of a ligand-induced conformational change, the possible effects of ligand charge-addition and protein buffering on the native heterogeneity cannot be excluded as the cause of this phenomenon. However, these possibilities are not obviously compatible with two aspects of the IEF titration patterns. Regarding the addition of ligand charge, if the charge of this phosphorylated 6-carbon molecule was the sole determinant of the observed pI shifts, enzyme treated with substoichiometric quantities of 2-carboxyarabinitol-\(P_2\) would be expected to have a more uniform distribution of protein among the isoelectric bands than the native enzyme. This follows directly from the thermodynamics of binding since the random addition of charge to a population of molecules distributed among several discrete charge states must result in a new distribution in which the occupancy of any state approaches one over the number of molecules which can reach that state by charge addition. Thus, the intense protein bands observed at intermediate ligand concentrations, e.g. 3, 4, and 5 2-carboxyarabinitol-\(P_2\)/holoenzyme, are irrefutable with ligand charge addition effects alone. Another possible explanation for the native protein’s microheterogeneity, protein buffering effects, appears equally unlikely. Given the diffuse, multicomponent 0.3 pH-unit range in which the native enzyme focuses, it is not apparent how a change in pI on going from the unbound to the fully ligated state (which yields a single, visually tight band covering less than 0.05 pH-unit) could mask the native surface charge heterogeneity by simple protein buffering, unless it is assumed that the native charge microheterogeneity is a product of amino acid residues at the surface of the protein which all have pK values in the 5.5–6.0 region; this is an unlikely assumption. Consequently, changes in the buffering capacity of multiple, reproducible protein forms does not seem adequate to explain band tightening.

The less sensitive, but complementary techniques of anion-exchange chromatography and native gel electrophoresis yield more specific insight into the extent and nature of the conformational changes which occur upon 2-carboxyarabinitol-\(P_2\) tight binding. A variety of published observations suggest that the active sites of Rubisco are deeply buried within the enzyme molecule (see Discussion in Ref. 20). Thus, the finding that enzyme with 2-carboxyarabinitol-\(P_2\) bound, and hence possessing a higher formal negative charge, elutes at a lower ionic strength than the untreated enzyme (Fig. 4) suggests...
that a structural change occurs in Rubisco upon tight binding of 2-carboxyarabinitol-P₂. This alteration is not limited to the catalytic region of the enzyme, but affects the anion-exchange accessible surface groups of the protein. Native electrophoretic analysis of the untreated enzyme and the enzyme-CABP complexes illustrates the magnitude of the conformational change brought about by tight binding of 2-carboxyarabinitol-

Differential Effects of 2-Carboxyarabinitol P₂ on the Isoelectric Forms of the Native Enzyme—A number of observations from the native IEF protein and fluorography data suggest differential effects of 2-carboxyarabinitol-P₂ on the microheterogeneous charge forms of the native enzyme. First, upon treatment with substoichiometric amounts of 2-carboxyarabinitol-P₂, the diffuse native pattern tightens into a series of discrete bands. Obviously, for this to occur, enzyme forms which, in the unbound state, focus at slightly different pl values must upon addition of 2-carboxyarabinitol-P₂ focus at the same pl. This is best exemplified at 2-carboxyarabinitol-

Implications of the Binding Mechanism—In order to address these possibilities experimentally, we have examined the implications of 2-carboxyarabinitol-P₂ binding to the partially ligated protein. Although there are several questions to be considered, perhaps the most basic of these concerns the elucidation of the mechanism of cooperativity in the tight-binding process. In the case of β, applying the binomial distribution to the protein band intensity as a function of the 2-carboxyarabinitol-P₂ molar ratio leads to the conclusion that 50-70% of the total protein must at some point focus at the pl 5.30 region. Since no bands within the native pattern contain this much protein, either differential conformational changes or negative cooperativity with respect to 2-carboxyarabinitol-P₂ tight binding must be invoked.

The experimental results (Figs. 6-8) demonstrate clearly that the added ligand preferentially binds to the unligated enzyme in a solution of native and partially ligated Rubisco, or, in other words, to the least saturated of two partially complexed CABP-Rubisco species in a solution consisting of two substratated enzyme populations. This was evident even when there were fewer total unligated Rubisco molecules present than ligated enzymes (Fig. 7). Multiple (>2) additions of 2-carboxyarabinitol-P₂ to attain the appropriate ligand concentration also did not alter these conclusions. Consequently, these findings are in accord with the interpretation that this tight binding transition-state analog binds preferentially to the unligated or least ligated form of the enzyme. The analyses presented in this paper have important implications for cooperativity in spinach Rubisco since there is ample precedent in the hemoglobin literature (21, 22) to suggest that the quaternary structure, not the state of ligation per se, controls the ligand affinity and chemical kinetics of an enzyme. More specifically, this literature suggests that with each addition of ligand to a multimeric enzyme, changes in subunit-subunit interactions take place (23); for Rubisco and the purposes of our analysis, these effects are manifested as surface-charge perturbations which are visualized by native IEF. More important, however, these alterations may be responsible for modulating the kinetic properties of the enzyme (24). Consequently, the collective binding distribution results presented herein imply that activated spinach Rubisco is negatively cooperative.

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