Protein Liganding to the Activator Cation of Ribulosebisphosphate Carboxylase†

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ABSTRACT: Spinach leaf ribulosebisphosphate carboxylase forms a quaternary complex with CO_2, carboxyarabinitol bisphosphate, and Cr^{2+} or Co^{2+}. Oxidation of the cation in these complexes produces a protein–cation adduct which is sufficiently stable to be chromatographically isolated after enzyme denaturation. While stoichiometric levels of slowly exchanging cation can be specifically trapped after addition of protein denaturants as well as a vast molar excess of Mg^{2+}, neither CO_2 nor carboxyarabinitol bisphosphate remains bound to denatured protein under the conditions employed in these experiments. These observations demonstrate direct inner-sphere liganding of protein to the exchange-inert cation, which appears to bind at the site normally occupied by the physiologically active cation. Dimeric ribulosebisphosphate carboxylase from *Rhodospirillum rubrum* also forms a quaternary complex containing stoichiometric amounts of enzyme protomer, CO_2, Co^{2+}, and carboxyarabinitol bisphosphate. Lack of a small subunit in the *R. rubrum* enzyme does not impair binding of the components of the quaternary complex in a nonexchangeable mode. Substantial amounts of protein–cation adduct are recovered upon oxidation and denaturation of the *R. rubrum* complex, supporting the prediction that the large subunits of the octameric plant enzyme should be the sites of cation binding. The first direct proof for such a hypothesis has been generated by separation of protein subunits derived from a spinach quaternary complex and by the demonstration that the bound cation is associated with the large subunit.

Ribulosebisphosphate (RuBP)[1] carboxylase accounts for the primary event in photosynthetic carbon fixation, catalyzing the formation of 3-phosphoglycerate in a reaction which requires the participation of a metal ion (Weissbach et al., 1956; Siegel et al., 1972). In the presence of oxygen, the enzyme catalyzes a cation-dependent conversion of the sugar phosphate substrate into phosphoglycolate and phosphoglycerate (Bowes et al., 1971; Andrews et al., 1973). For the generation of a catalytically functional protein, the purified enzyme must be preincubated with CO_2 and a cation (Lorimer et al., 1976). This activation process appears to involve ordered binding, since metal ion does not bind specifically to enzyme in the absence of CO_2 (Miziorko & Mildvan, 1974). The observation that the CO_2 activator combines with an ϵ-amino group of a lysine to form a negatively charged carbamate (Lorimer & Miziorko, 1980) has prompted speculation that activator CO_2 is directly involved in binding divalent cation (Lorimer & Miziorko, 1981). The strong evidence for cation participation in enzyme activation has prompted some investigators to discount the possibility that the cation functions directly in catalysis (Laing & Christeller, 1976). There is, however, evidence that the cation is bound in close proximity to substrate CO_2 (Miziorko & Mildvan, 1974). It has also been observed that partitioning of substrate between carboxylation and oxygenation, processes which both require enzyme activation, depends on which cation is used to support the reactions (Christeller, 1981; Robison et al., 1979). These findings have prompted hypotheses (Lorimer & Miziorko, 1981; Lorimer, 1981b) which invoke a direct role for the cation in catalysis.

Interest in determining whether activator cation interacts directly with activator CO_2 and/or with substrates may explain the relative lack of data concerning any possible direct cation–protein interaction. In ESR studies on a stable quaternary complex which contains stoichiometric amounts of enzyme sites, activator CO_2, activator cation, and the transition-state analogue carboxyarabinitol bisphosphate (CABP), the reported spectra (Miziorko & Sealy, 1980) indicated a marked distortion of the symmetry of Mn^{2+} used in forming the complex. Such distortion was explained by an unusual inner-sphere liganding of the cation. There is only one bound cation per active site in the model quaternary complex; ligands could be donated to the cation by activator CO_2, substrates, or protein. A marked conformational change in the protein occurs upon CABP binding to enzyme–CO_2–cation prior to formation of a stable quaternary complex (Siegel & Lane, 1972; Lane & Miziorko, 1978). Mn^{2+} bound to protein in an enzyme–CO_2–cation complex could experience distortion of symmetry if protein ligands drastically change orientation upon formation of the quaternary CABP-containing complex. Unfortunately, the RuBP carboxylase–activator CO_2–Mn^{2+}–CABP quaternary complex is unsuitable for an investigation of cation–protein liganding by the usual protein chemistry techniques. Upon denaturation of the enzyme, bound cation is readily released (Miziorko & Sealy, 1980). If the model quaternary complex, which has proven so useful in identifying the nature of the protein–activator CO_2 adduct, was to be of any value in investigating protein–activator cation interactions, a cation which exchanged slowly from denatured enzyme would have to be used as a probe.

Exchange-inert cations, e.g., Co^{3+} and Cr^{3+}, have been widely used to form cation–nucleotide complexes that proved to be valuable tools in mechanistic studies on a variety of enzymes (Cleland & Mildvan, 1979; Dunaway-Mariano & Cleland, 1980). Much less interest has been indicated in using these cations to study direct cation–protein interactions, although several reports in the literature (Ryzewski & Takahashi, 1975; Balakrishnan & Villafranca, 1979) suggested that this approach could be fruitful. This report presents data

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[1] Abbreviations: RuBP, ribulose 1,5-bisphosphate; CABP, 2-carboxyarabinitol bisphosphate; $NaDodSO_4$, sodium dodecyl sulfate; ESR, electron spin resonance; Tris, tris(hydroxymethyl)aminomethane.

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indicating that the RuBP carboxylase quaternary complex can incorporate exchange-inert cations and uses the model complexes to demonstrate direct cation–protein liganding. Experiments using the dimeric *Rhodospirillum rubrum* enzyme reinforce this conclusion and, in addition, provide new information on stoichiometries of activator and substrate binding. A preliminary account of parts of this work has appeared (Miziorko & Houkom, 1982).

Experimental Procedures

Materials. RuBP carboxylase was prepared from spinach leaves as described by Paulsen & Lane (1966). *R. rubrum* RuBP carboxylase was the generous gift of Dr. F. C. Hartman. Enzyme activity was assayed by standard radioisotopic (Lorimer et al., 1977) or spectrophotometric (Andrews et al., 1973) procedures. Protein concentration was estimated spectrophotometrically, using extinction coefficients for spinach (Paulsen & Lane, 1966) and *R. rubrum* (Stringer et al., 1981) enzymes that have been previously reported.

RuBP was purchased from Sigma. CABP was synthesized as described by Pierce et al. (1980). Sephadex was supplied by Pharmacia. $NaH^{14}CO_3$, $K^{14}CN$, $^{51}CrCl_3$ and $^{57}CoCl_2$ were purchased from New England Nuclear. Chelex 100 resin was obtained from Bio-Rad Laboratories and was used to prepare metal-free reagents and buffers. Ultrapure Tris base and urea were purchased from Schwarz/Mann. All other reagents were of the highest purity commercially available.

Methods. Enzyme was desalted before use by Sephadex G-25 chromatography in 50 mM Tris-HCl, pH 7.8. Activation of enzyme with CO_2 and cation, as well as formation of the quaternary complex upon incubation with CAPB, was performed as described earlier (Miziorko, 1979). Anaerobic samples were generated by alternately degassing the solution in vacuo and then reintroducing oxygen-free nitrogen. The procedure was carried out by using a Firestone valve (Ace Glass Co.) and repeated for eight cycles. Anaerobic enzyme solutions were brought to 1 mM in dithionite. Solutions which were added to form the quaternary complex were degassed by using the Firestone valve and injected through a serum stopper with a gas-tight syringe. Cr^{2+} was prepared by reduction of $CrCl_3$ (Lingane & Pecsok, 1948).

Quantitation of radioactive components of the isolated quaternary enzyme–CO_2–M–CABP complex was performed by using a Beckman LS-6800 liquid scintillation counter. ^{51}Cr or ^{57}Co is detected in the low-energy channels (Kobayashi & Maudsley, 1974). In double-label experiments employing ^{51}Cr or ^{57}Co as well as ^{14}C-labeled CO_2 or CABP, quantitation of bound material was made by using standard procedures for isotope overlap corrections (Segel, 1976). Independent measurement of ^{51}Cr or ^{57}Co by γ counting produced estimates equivalent to those obtained by liquid scintillation counting.

High-pressure gel permeation chromatography was performed by using a 10-cm precolumn and a 60-cm analytical column packed with TSK 3000-SW resin. Isocratic elution of the column was performed by using 50 mM sodium phosphate, pH 7, containing 0.1% $NaDodSO_4$.

Results

Chromium Supports Formation of an Enzyme–CO_2–Cation–Transition-State Analogue Complex. Previous work (Miziorko & Sealy, 1980; Miziorko, 1979) established that a variety of exchangeable cations would support formation of a stable complex containing spinach RuBP carboxylase and stoichiometric amounts of CO_2, cation, and the transition-state analogue, CABP. Formation of the model complex with a cation that could be converted to an exchange-inert species provides, in principle, a mechanism for isolating components of the denatured complex which are liganded directly to metal. Therefore, methodology was developed to determine whether the quaternary complex could be formed by using chromium as a substitute for the physiological cation activator. A desalted enzyme sample was made anaerobic prior to the addition of 1 mM dithionite. Potassium bicarbonate (20 mM) was used to activate the enzyme before addition of a 2–3-fold excess of $CrCl_2$ (200 μM). The anaerobic sample of activated enzyme was incubated with a 5-fold excess of carboxyarabinitol bisphosphate to permit formation of a quaternary complex. After complex formation was complete, excess Mg^{2+} was added (50 mM) to minimize any nonspecific chromium binding. Isolation of the enzyme complex from unbound small molecules was accomplished by Sephadex G-75 chromatography as previously described (Miziorko, 1979) with the modification that the column buffer contained 1 mM dithionite (Mortenson, 1972). A double-labeling approach, following both the levels of ^{51}Cr and either ^{14}C-labeled CO_2 or CABP that were bound in any particular experiment, was required to ensure that a quaternary complex containing stoichiometric amounts of CO_2, cation activator, and the transition-state analogue was being generated. Failure to maintain anaerobic conditions led to deviation from stoichiometric binding. Oxygen was bubbled through the sample of isolated quaternary complex to ensure complete conversion of the bound cation to the slowly exchanging Cr^{3+} species (Balakrishnan & Villafranca, 1979). Preparation of the sample under strictly anaerobic conditions resulted in isolation of a complex which contained chromium at levels slightly above those observed for other cations (Table I). However, unlike the cations previously examined, substantial amounts of exchange-inert chromium remain bound to the enzyme under denaturing conditions. In contrast, the CO_2 and transition-state analogue components of the isolated chromium-containing quaternary complex do not remain bound (<0.1 per enzyme protomer) under these conditions. The simplest explanation for the chromium binding data is that one or more amino acid residues are directly liganded to the activator cation. Initially, it seemed that the relatively stable chromium-containing complex would be useful for detailed studies leading to identification of the amino acids involved in binding of the cation activator. However, the problem of

Table I: Cation Binding in the RuBP Carboxylase–CO_2–Cation–CABP Complex[a]

expt	sample	cation/ enzyme site
A	enz–CO_2–Cr^{3+}–CABP	1.4
B	enz–CO_2–Mn^{2+}–CABP	1.2[b]
C	enz–CO_2–Co^{2+}–CABP	1.1[b]
D	preformed enz–CO_2–Mg^{2+}–CABP, incubated with Cr^{2+} as in expt A	0.2
E	enz–CO_2–Cr^{3+}–CABP, guanidine denatured and reisolated	0.7

[a] The enzyme (enz) complexes used in the chromium experiments were prepared by degassing solutions of spinach leaf RuBP carboxylase (50–100 μM sites) in 50 mM Tris-HCl, pH 7.8, containing 0.2 M KCl. The sample was brought to 1 mM dithionite and 20 mM $KHCO_3$ prior to addition of $CrCl_2$ (2–3-fold excess over enzyme sites). After 5 min at 30 °C, a 5-fold excess of CABP was added to the anaerobic sample and allowed to incubate for 1 h before Mg^{2+} (50 mM) was added. In experiment D, 50 mM Mg^{2+} was incubated with the enzyme–CO_2 complex prior to subsequent additions of CABP and Cr^{2+}. In experiment E, an isolated sample of enzyme–CO_2–Cr^{3+}–CABP was denatured in 4 M guanidine for 2 h at 30 °C and rechromatographed on Sephadex G-75 equilibrated in 50 mM potassium acetate, pH 5, containing 4 M urea. [b] Data taken from Miziorko & Sealy (1980).

potential nonspecific binding had to be addressed. Since chromium does not appreciably stimulate the RuBP carboxylase reaction, it was necessary to generate some evidence suggesting that this cation is suitable as a specific probe for the activator cation site. Formation of a quaternary complex was performed under anaerobic conditions with Mg^{2+} as the activating cation. Cr^{2+} was added to the enzyme–CO_2–Mg^{2+} sample after activated enzyme had been incubated with CABP. Isolation of the complex by the Sephadex G-75 procedure described above demonstrates a substantial diminution in chromium binding (Table I, experiments A and D), indicating specificity in the chromium binding initially observed. Nonetheless, the background level of nonspecifically bound chromium could complicate any attempt at identification of the amino acid residues involved in cation binding. Moreover, the technical problems encountered in generating Cr^{2+} and in preparing and maintaining anaerobic enzyme samples are not trivial. For these reasons, the suitability of cobalt as an exchange-inert probe of the cation activator site was explored.

Studies on the RuBP Carboxylase–CO_2–Cobalt–Transition-State Analogue Complex. The ability of Co^{2+} to support formation of the spinach RuBP carboxylase quaternary complex (Miziorko & Sealy, 1980) and to support the RuBP oxygenase reaction (Christeller, 1981; Robison et al., 1979) has been well established. Reports of conversion of protein-bound Co^{2+} to slowly exchanging Co^{3+} are available (Ryzewski & Takahashi, 1975; Balakrishnan & Villafranca, 1979), including a study on the ternary complex of *R. rubrum* RuBP carboxylase with CO_2 and cobalt (Robison et al., 1979). However, Co^{3+} has been reported to require several nitrogen ligands in the inner coordination sphere in order to form a slow-exchanging species (Cleland & Mildvan, 1979). It has been suggested that activator CO_2 (Lorimer & Miziorko, 1981) as well as the substrate RuBP (Pierce et al., 1980) may be directly bound to the activator cation on RuBP carboxylase. If such speculations are correct, it seems unlikely that a bound Co^{3+} species could be directly coordinated to multiple nitrogen ligands derived from the protein. Such a rationale prompted our initial investigation of the suitability of Cr^{3+} as a probe. The observation that chromium remained associated with denatured enzyme suggested that direct metal liganding to protein did, in fact, occur and prompted further study on the cobalt-containing quaternary complex.

Formation of an enzyme–CO_2–$^{57}Co^{2+}$ complex prior to inhibition of enzyme by CABP resulted in formation of a quaternary complex which, after isolation free of unbound components, was demonstrated by double-labeling techniques to contain stoichiometrically bound CO_2, Co^{2+}, and CABP. Attempts to convert bound Co^{2+} to exchange-inert Co^{3+} by H_2O_2 oxidation under conditions similar to those described for generating nonstoichiometric enzyme–CO_2–Co^{2+} mixtures (Robison et al., 1979) were not successful in producing from the extremely stable quaternary complex a species which exhibited substantial metal binding upon subsequent denaturation of enzyme. However, simultaneous exposure of the quaternary complex to H_2O_2 and to a protein-denaturing agent produced bound Co^{3+} in good yield (Table II). In order to further test whether the cobalt is specifically trapped at the activator site, we incubated the isolated quaternary complex with a 1000-fold excess of Mg^{2+} prior to and during the oxidation/denaturation step. The stoichiometry of bound cobalt was undiminished. Exposure of a Co^{2+}-containing complex to H_2O_2, followed by depletion of the oxidizing agent with an excess of catalase prior to protein denaturation, led to diminished stable cobalt incorporation (Table II).

Table II: Cobalt Binding in Native and Denatured Spinach RuBP Carboxylase–CO_2–Cobalt–CABP Complexes[a]

expt	sample	cobalt/ enzyme site
A	enz–CO_2–Co–CABP; native complex	1.1
B	enz–CO_2–Co–CABP; guanidine denatured	0.1
C	enz–CO_2–Co–CABP; sample treated with H_2O_2 and guanidine	1.0[b]
D	enz–CO_2–Co–CABP; sample treated with H_2O_2 prior to sequential incubns with excess catalase and guanidine	0.5

[a] Samples were prepared by incubating desalted spinach leaf RuBP carboxylase (in 50 mM Tris-HCl, pH 7.8, and 0.2 M KCl) with 20 mM $KHCO_3$ and $^{57}CoCl_2$ (2-fold excess over enzyme site; specific activity ~1000 cpm/nmol) for 5 min at 30 °C prior to addition of a 5-fold excess of CABP. After 1 h, the quaternary enzyme–CO_2–Co^{3+}–CABP complex was isolated by chromatography on Sephadex G-75 (Miziorko, 1979). In experiment B, an isolated quaternary complex was denatured in 4 M guanidine for 1 h at 30 °C prior to reisolation. In experiment C, the isolated complex was incubated with 3 mM H_2O_2 for 5 min prior to denaturation with 4 M guanidine. In experiment D, the isolated complex was incubated with 3 mM H_2O_2 for 15 min at 30 °C prior to addition of excess catalase (450 units). After 1 h, the sample was brought to 4 M guanidine and incubated at 30 °C for 1 h. Denatured samples were reisolated on Sephadex G-75 and equilibrated with 50 mM Tris-HCl, pH 7, containing 4 M urea. [b] Incubation of the isolated cobalt-containing sample with a 1000-fold excess of Mg^{2+} prior to and during the oxidation and denaturation did not alter the observed stoichiometry.

Table III: Recovery of Enzyme-Bound Activators and Substrate Analogue after Protein Denaturation[a]

	stoichiometry of binding					
	native enzyme			denatured enzyme		
expt	^{57}Co	$KH^{14}CO_3$	[^{14}C]-CABP	^{57}Co	$KH^{14}CO_3$	[^{14}C]-CABP
A	1.0		1.3	0.9		0.2
B	0.9	1.1		0.8	0.1	

[a] Spinach leaf RuBP carboxylase was used to prepare the native quaternary complex as described in Table II. Concentrations of bound components were measured by liquid scintillation counting. Specific activities of ^{57}Co, $KH^{14}CO_3$, and [^{14}C]CABP (2-[^{14}C]-carboxyarabinitol bisphosphate) were approximately 1000 cpm/nmol. Cobalt binding measured by γ counting was identical with that measured by liquid scintillation counting. Isolated native complexes were oxidized with 3 mM H_2O_2 and denatured with 4 M guanidine prior to reisolation by using Sephadex G-75 in 50 mM Tris-HCl, pH 7.0, with 4 M urea. Stoichiometry of binding is calculated per 70 000-dalton enzyme protomer.

The isolation of a stable cobalt–protein complex prompted investigation of whether any of the other components of the complex might be directly coordinated to metal and remain associated with denatured RuBP carboxylase. In contrast to the good recovery of oxidized cobalt bound to denatured enzyme, virtually no CO_2 or CABP remains associated with protein under these conditions (Table III). These findings reinforce the observation described above for the chromium-containing quaternary complexes.

Formation of Cobalt-Labeled Quaternary Complexes by Using R. rubrum RuBP Carboxylase. The CO_2 and divalent cation activation of *R. rubrum* RuBP carboxylase has been well established (Christeller & Laing, 1978), but there has been some controversy over the stoichiometry of binding for the activators in the ternary complex. Preformation of the ternary complex in the presence of excess CO_2 and Co^{2+} followed by incubation with 1 mM CABP results in irreversible inhibition of the enzyme activity and formation of a quaternary

Table IV: Formation and Stability of Enzyme–CO_2–Co–CABP Complexes of *R. rubrum* RuBP Carboxylase[a]

	stoichiometry of binding					
	native enzyme			denatured enzyme		
expt	^{57}Co	$KH^{14}CO_3$	[^{14}C]CABP	^{57}Co	$KH^{14}CO_3$	[^{14}C]CABP
A	1.3		1.3 (1.0)[b]	0.6		0.1
B	1.3	1.1 (1.1)[b]		0.5	0.0	

[a] *R. rubrum* RuBP carboxylase was desalted in 50 mM Tris-HCl, pH 7.8. Enzyme (50–200 μM protomer) was incubated with $KHCO_3$, Co^{2+}, and CABP described for the spinach enzyme (Table II) except that the final CABP concentration was approximately 1 mM in all experiments. Specific activity of radioactive compounds was approximately 1000 cpm/nmol. After a 1-h incubation with CABP, the inhibited enzyme was isolated as described earlier (Table II). The isolated quaternary complex was oxidized with H_2O_2 (3 mM) and incubated with guanidine (4 M) for 1 h at 30 °C prior to rechromatography of the sample (Sephadex G-75; 50 mM Tris-HCl, pH 7, containing 4 M urea) and estimation of the stoichiometry of labeled component bound per enzyme protomer. [b] Stoichiometry measured after incubation of the isolated quaternary complex with an excess of unlabeled CABP (50-fold) or CO_2 (800-fold) for 3 h at 30 °C. Binding levels were calculated after reisolation of the complex, eliminating any contribution from unbound components.

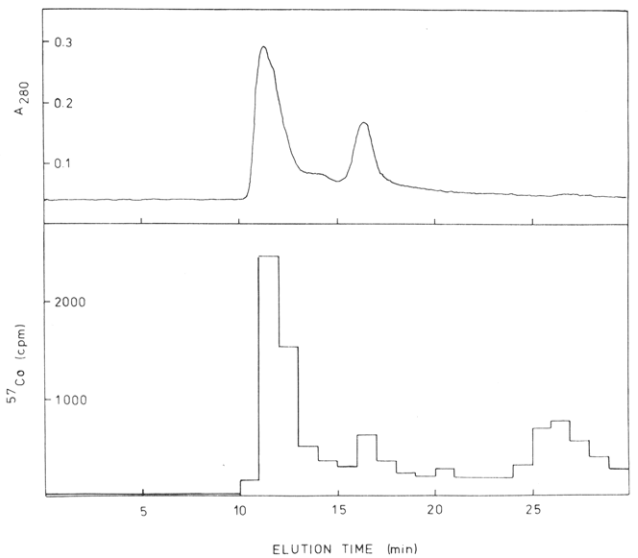

FIGURE 1: High-pressure gel permeation chromatography of ^{57}Co-labeled RuBP carboxylase. 8 nmol of an isolated complex containing stoichiometric amounts of spinach leaf RuBP carboxylase, CO_2, $^{57}Co^{2+}$, and CABP was H_2O_2 treated and guanidine denatured, as described in Table II. The sample (200 μL) was injected onto a TSK 3000-SW column (10-cm precolumn, 60-cm analytical column) equilibrated with 50 mM sodium phosphate, pH 7, containing 0.1% $NaDodSO_4$. Elution was performed at a flow rate of 1.0 mL/min. Fractions (1 mL) of the eluant were collected, and ^{57}Co was detected by γ counting. Protein was detected by monitoring A_{280} with a Hitachi UV–vis spectrometer equipped with an Altex HPLC flow cell. Under the conditions specified, RuBP carboxylase large subunit elutes at t = 12.2 min; the small subunit elutes at t = 16.1 min. Recovery of injected ^{57}Co radioactivity in the eluted fractions is 85%, including free cobalt eluting at t = 27 min.

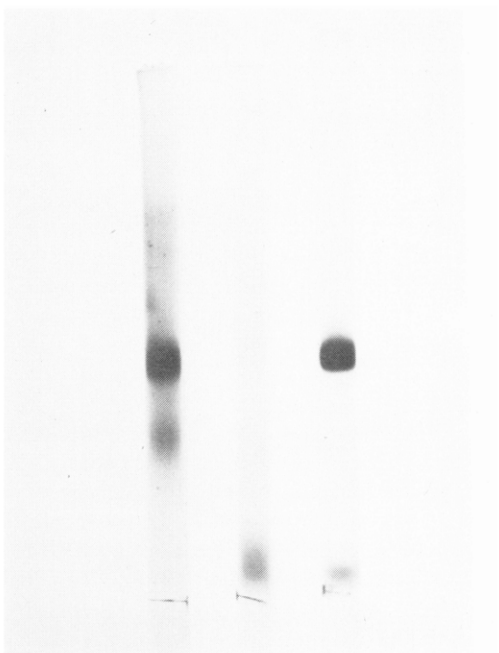

FIGURE 2: Polyacrylamide gel electrophoresis of spinach leaf RuBP carboxylase and its isolated subunits in an $NaDodSO_4$–urea system. Gels were loaded with protein eluting from the TSK 3000-SW column (cf. Figure 1) in fraction 12 (left), fraction 17 (center), or with the native enzyme (right). Samples were prepared for electrophoresis by incubation for 5 min at 100 °C in 8 M urea, 1% $NaDodSO_4$, and 5% mercaptoethanol. 6% polyacrylamide gels contained 4 M urea and 0.1% $NaDodSO_4$. Electrophoresis buffer was 50 mM sodium phosphate (pH 7.0) with 0.1% $NaDodSO_4$. Coomassie blue was used to stain protein in the gels.

complex which is stable upon isolation by Sephadex G-75 chromatography, as described earlier for the spinach enzyme model complex (Miziorko, 1979). It is noteworthy that the *R. rubrum* quaternary complex contains approximately equivalent amounts of CO_2, cation, CABP, and enzyme protomer (Table IV).

Enzyme from *R. rubrum* does not contain the small subunit found associated with the plant enzymes. In order to assess whether the small subunit might be crucial for stabilizing cation binding to protein in the quaternary complex, we oxidized isolated samples of *R. rubrum* RuBP carboxylase–CO_2–Co^{2+}–CABP with H_2O_2 and denatured the samples as described for the spinach enzyme complex. Analysis of the reisolated protein indicated that substantial cobalt remained bound, although the measured stoichiometry was lower than that observed for the spinach enzyme (Table IV). Virtually no bound CO_2 or CABP was detected in the experiments on the oxidized, denatured *R. rubrum* complex (Table IV). Thus, the quaternary complexes formed from bacterial or plant enzyme are similar in this respect.

Localization of the Cation Binding Site. Isolation of a relatively stable cobalt–protein adduct after oxidation and denaturation of the quaternary complex prepared by using spinach leaf RuBP carboxylase prompted further investigation aimed at characterizing the cation binding site. Gel permeation chromatography of an oxidized, denatured quaternary complex on a TSK 3000-SW column equilibrated with 50 mM sodium phosphate (pH 7) containing 0.1% $NaDodSO_4$ produced two protein peaks (Figure 1). The first broad high molecular weight peak corresponds to the unresolved large subunit of the spinach enzyme and its dimer (no reducing agent is employed in the Co^{3+} experiments). The identity of the protein was verified by $NaDodSO_4$ gel electrophoresis of the eluted fractions (Figure 2). The major peak of recovered ^{57}Co radioactivity is associated with this material, accounting for

35% of the total radioactivity injected. Lower levels of radioactivity are observed in the fractions which trail the major peak. Most of this radioactivity can be attributed to unbound cobalt which has slowly exchanged from the protein sample during denaturation and chromatography, since Co^{3+} is not exchange inert in any absolute fashion. Small and variable levels of ^{57}Co appear to remain associated with the second protein peak, which $NaDodSO_4$ gel electrophoresis identifies as the small subunit of RuBP carboxylase (Figure 2). However, the level of small subunit associated radioactivity measured above the trailing background does not account for more than 2% of injected ^{57}Co.

The recovery of ^{57}Co in the fractions containing the large subunit is comparable to that associated with the only ^{57}Co-labeled peptide peak produced upon reverse-phase high-pressure liquid chromatography (HPLC) of a trypsin digest of oxidized, denatured spinach quaternary complex.[2] These observations suggest that the exchange-inert cation probe labels a site on the protein with considerable specificity. However, final evaluation of the utility of this approach must await a more detailed analysis of the putative cation binding peptide.

Discussion

Previous reports of stable cation–protein adducts (Ryzewski & Takahashi, 1975; Balakrishnan & Villafranca, 1979) suggested the utility of cobalt and chromium cations as probes of the RuBP carboxylase metal activator site. If any labeling experiment is to generate useful data, the problem of nonspecific binding must be minimized. The RuBP carboxylase quaternary model complex facilitated the specific trapping of the CO_2 activator (Lorimer & Miziorko, 1980), and, therefore, it promised to be useful in cation site labeling. This expectation proved to be correct. The cation probes are stoichiometrically bound, even when enzyme is denatured in the presence of excess Mg^{2+} in order to minimize rebinding of any cation which dissociates from the activator site. Additional evidence for the specificity of trapping is provided by the demonstration that the exchange-inert cation is localized on the large subunit of the enzyme. If the assumption that the liganding of the exchange-inert cations is similar to that of the physiological activator cation is correct (and the data presented in this report suggest that such extrapolation is not unreasonable), then the direct demonstration of protein–cation liganding has been accomplished, and effort can now be focused on the elucidation of the identity of the amino acid residues which are involved in forming the stable adduct.

The recovery of stoichiometric amounts of protein-bound cation in denatured samples of quaternary complex contrasts with the very low levels of activator CO_2 and CABP which remain associated with enzyme. It should be emphasized that such results do not rule out direct metal activator coordination to activator CO_2 or CABP. Both the number and the chemical nature of the groups involved in liganding will affect whether any cation-coordinated moiety is sufficiently stable to be isolated under the denaturing conditions employed in this study. On the basis of studies on nucleotide complexes of cobalt and chromium (Cleland & Mildvan, 1979), the coordination of cation to several carboxyl- or carbamate-derived oxygens would decrease stability and diminish the likelihood of trapping the molecules donating these ligands upon denaturation of the protein complex. The experiments reported in this study were intended to test whether activator cation binds directly to protein, as opposed to bridging via the carbamate formed at the activator CO_2 site. The experimental conditions employed allowed a clear discrimination between those two possibilities. Future work is aimed at testing whether substrates or activator CO_2 also ligand directly to cation.

A variety of chemical arguments make the hypothesis of cation coordination to CO_2 or CABP an attractive one. A carbamate is formed upon binding of activator CO_2, introducing another negative charge in a peptide possessing strongly anionic character (Lorimer, 1981a). Such a polyanionic region would make a good cation binding site and would explain why cation binds only in the presence of CO_2 (Miziorko & Mildvan, 1974). Carbamate cleavage has been studied in aliphatic amine model systems and involves N-protonation prior to expulsion of CO_2 (Ewing et al., 1980). Cation coordination would retard protonation and stabilize the carbamate. These observations may well explain the need for cation in activation of RuBP carboxylase. The interaction between cation and substrate has also been invoked on chemical grounds (Pierce et al., 1980) as a means to control the stereochemistry of the reaction. However, while a strong chemical rationale can be offered, evidence for cation–activator CO_2 or direct cation–substrate interaction remains to be reported.

Cation binding on the large subunit of plant RuBP carboxylase seems to be a logical hypothesis, since the enzyme from *R. rubrum* contains no small subunits but responds to activation by CO_2 and cation. The stable cation–large subunit adduct described in this report provides the first direct evidence to support this theory. It is interesting that no stable interaction between the small subunit and cation is detectable with the plant enzyme. Thus, the role of this peptide remains unclear. The quaternary complex formed by the plant enzyme is remarkably stable. Denaturation appears to significantly facilitate H_2O_2 oxidation of bound cobalt, suggesting that bound components in the native complex may be partially occluded. There is ample precedent in the literature to support such an interpretation. CO_2 and cation in the quaternary complex exchange very poorly with unbound ligands (Miziorko, 1979; Miziorko & Sealy, 1980; Lorimer & Miziorko, 1980). The failure of $NaBH_4$ to reduce a 3-ketoarabinitol bisphosphate complex (Schloss & Lorimer, 1982) could be accounted for by postulating limited exchange with solvent. The unusual symmetry of Mn^{2+} bound in the quaternary complex is compatible with a complex network of liganding which may limit contact with other molecules in the aqueous medium (Miziorko & Sealy, 1980; Miziorko & Mildvan, 1974). It is conceivable that, without being in the vicinity of the cation activator, the small subunit assists in promoting the conformational change that results in the unique quaternary complex or contributes by some unknown mechanism to the overall stability of the complex. Quaternary complexes lacking the small subunit might be predicted to exchange CO_2 or CABP more readily than that reported for plant enzymes (Miziorko & Sealy, 1980). However, such behavior is not apparent in our limited experiments with the *R. rubrum* enzyme.

Half of sites reactivity has been invoked in reporting stoichiometries of activator binding and active-site modification using *R. rubrum* enzyme (Robison et al., 1979, 1980). Such behavior was not apparent from our stoichiometry measurements on isolated quaternary complexes involving *R. rubrum* enzyme. Moreover, competition of isolated complex against excess unlabeled components failed to diminish the level of tightly bound ligand (Table IV). A recent report on modification of the catalytic site of the *R. rubrum* enzyme concluded that one site exists per protomer (Herndon et al., 1982).

[2] E. C. Houkom, unpublished results.

Thus, while plant and bacterial enzymes may differ in some respects, there is substantial evidence that one set of activator sites and one catalytic site exist per 56 000-dalton peptide of RuBP carboxylase.

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