2<latex>$'$</latex>-Carboxy-3-Keto-D-Arabinitol 1,5-Bisphosphate, the Six-Carbon Intermediate of the Ribulose Bisphosphate Carboxylase Reaction

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2′-carboxy-3-keto-D-arabinitol 1,5-bisphosphate, the six-carbon intermediate of the ribulose bisphosphate carboxylase reaction

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The six-carbon intermediate of the ribulose 1,5-bisphosphate (RuBP) carboxylase reaction, 2′-carboxy-3-keto-D-arabinitol 1,5-bisphosphate (CKABP), was prepared enzymatically by quenching the reaction with acid after a short time (*ca* 12 ms). Over a wide pH range (4–11), CKABP undergoes a slow ($t_{1/2} = 1$ h), pH-independent decarboxylation. No detectable decomposition of CKABP occurs over a six-week period at $-80\,°C$. The decarboxylation of CKABP is acid-catalysed and is also catalysed by deactivated enzyme lacking the activator carbamate–divalent metal ion complex. Decarboxylation is accompanied by β-elimination of the C-1 phosphate from the 2,3-enediolate. Under alkaline conditions (pH > 11) CKABP undergoes hydrolysis. Non-enzymatic hydrolysis of the intermediate is also accompanied by β-elimination of the C-1 phosphate (presumably from the aci-acid of the upper glycerate 3-phosphate) and the formation of pyruvate. Fully activated enzyme catalyses the complete hydrolysis of CKABP to glycerate 3-phosphate, although enzymic hydrolysis of CKABP is limited by an event not on the direct path of carboxylation.

Carbon-13 NMR analysis of [2′,3-¹³C]CKABP indicates that it exists in solution predominantly (> 85%) as the C-3 ketone. In contrast, borohydride trapping of CKABP formed from [3-¹⁸O]RuBP indicates that the intermediate exists on the enzyme predominantly (> 94%) as the hydrated C-3 gem-diol. In solution, the hydration of the C-3 ketone of CKABP proceeds slowly ($k = 2.5 \times 10^{-3}\ s^{-1}$). The enzymatic hydration of CKABP must proceed at least as fast as k_{cat} (*ca.* 5 s^{-1}) or at least 2000 times faster than the hydration of CKABP in solution.

Introduction

The carboxylation of RuBP is a unique reaction in a number of respects, not the least of which is the number of chemical steps involved in transforming substrates to products. The overall reaction can be dissected into five steps (scheme 1): *Enolization,* involving the deprotonation of C-3, generates a nucleophilic centre at C-2 in the form of the 2,3-enediol(ate). *Carboxylation* of the 2,3-enediol(ate) creates the six-carbon intermediate, 2′-carboxy-3-keto-D-arabinitol 1,5-bisphosphate (CKABP). The C-3 ketone form of CKABP undergoes *hydration* to the gem-diol form. Deprotonation of the O-3 gem-diol initiates *carbon-carbon cleavage* to yield a molecule of lower D-glycerate 3-phosphate (*l*PGA) and the C-2 carbanion or aci-acid form of the upper D-glycerate 3-phosphate (*u*PGA). The stereospecific *protonation* of this C-2 carbanion produces *u*PGA.

Shortly after the discovery of RuBP carboxylase (Quayle *et al.* 1954), Calvin (1956) proposed a mechanism involving the intermediacy of the six-carbon, β-keto acid, 2′-carboxy-3-keto-

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enolization   carboxylation   hydration   C–C cleavage   protonation

SCHEME 1
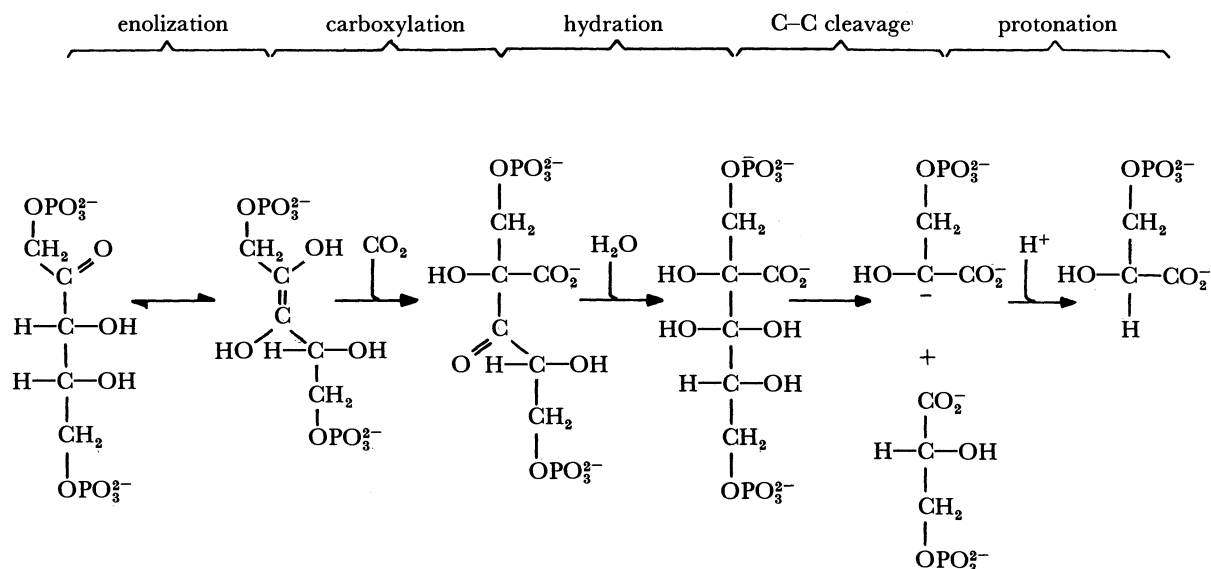
D-pentitol 1,5-bisphosphate. In the ensuing years a number of attempts were made to demonstrate its existence and describe its chemical properties (Moses & Calvin 1958; Sjödin & Vestermark 1973; Siegel & Lane 1973). However, its reputed lability limited efforts to characterize this compound. Nevertheless, the ability of 2′-carboxy-D-pentitol 1,5-bisphosphate, an analogue of the putative intermediate, to act as a potent inhibitor of carboxylation, strengthened the view that the six-carbon, β-keto acid was indeed an intermediate (Siegel & Lane 1972; Wolfenden 1972).

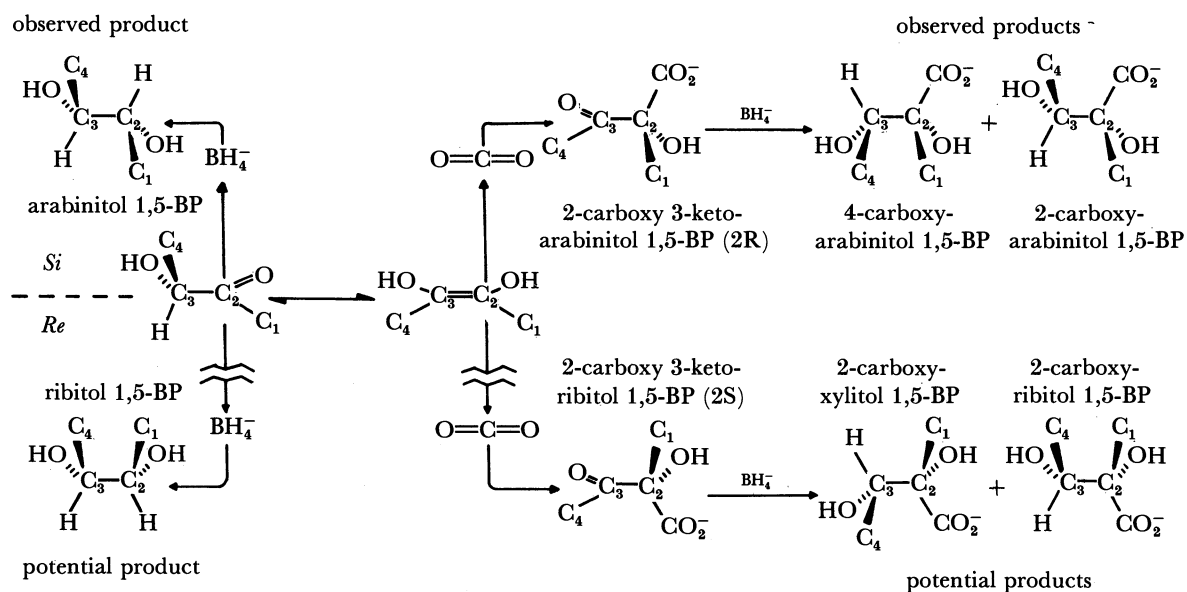### THE STEREOCHEMISTRY OF CARBOXYLATION

In studying the epimeric mixture of 2′-carboxy-D-pentitol 1,5-bisphosphates hitherto used, Pierce *et al.* (1980) chromatographically resolved the mixture and characterized the purified epimers as 2′-carboxy-D-arabinitol 1,5-bisphosphate (2′-CABP) and 2′-carboxy-D-ribitol 1,5-bisphosphate (2′-CRBP). Since 2′-CABP proved to be considerably more potent than 2′-CRBP as an inhibitor, the inference was drawn that the authentic intermediate must also have the same stereoconfiguration about C-2 as 2′-CABP, that is $2R$.

In seeking to define the stereochemical course of the reaction by more direct means, consideration must be given to the planar nature of C-2 both in the substrate and in its 2,3-enediol form. Two questions may be asked: (i) How is the substrate RuBP oriented within the catalytic site? and (ii) Is this orientation related to the direction from which CO_2 attacks the 2,3-enediol(ate)? To answer the first question, use was made of the ability of borohydride to reduce the C-2 carbonyl of RuBP. In solution, the reduction occurred with a slight stereochemical preference for attack on the *Re* face (scheme 2). However, when enzyme-bound RuBP was reduced, the product was exclusively D-arabinitol 1,5-bisphosphate, as if reduction occurred solely on the *Si* face of the C-2 carbonyl (Lorimer & Gutteridge 1986). The *Re* face is presumed to be inaccessible by virtue of steric hindrance by the enzyme.

Since information concerning the stability of the putative six-carbon intermediate was

observed product

observed products

arabinitol 1,5-BP

ribitol 1,5-BP

potential product

2-carboxy 3-keto-arabinitol 1,5-BP (2R)

4-carboxy-arabinitol 1,5-BP

2-carboxy-arabinitol 1,5-BP

2-carboxy 3-keto-ribitol 1,5-BP (2S)

2-carboxy-xylitol 1,5-BP

2-carboxy-ribitol 1,5-BP

potential products

SCHEME 2

lacking, our strategy for (a) demonstrating its existence as an intermediate, and (b) defining the stereochemical course of its formation, was based upon the notion that it could be stabilized by borohydride reduction to the corresponding mixture of carboxypentitol bisphosphates (scheme 2) (Schloss & Lorimer 1982). Depending upon the direction from which CO_2 attacks the 2,3-enediol(ate), either 2′-carboxy-3-keto-D-arabinitol 1,5-bisphosphate (Si attack) or 2′-carboxy-3-keto-D-ribitol 1,5-bisphosphate (Re attack) are to be expected. Reduction of the former by borohydride would be expected to form a mixture of 2′-carboxy-D-arabinitol 1,5-bisphosphate (2′-CABP) and 4′-carboxy-D-arabinitol 1,5-bisphosphate (4′-CABP) (= 2′-carboxy-D-lyxitol 1,5-bisphosphate) while reduction of the latter would be expected to yield a mixture of 2′-carboxy-D-ribitol 1,5-bisphosphate (2′-CRBP) and 2′-carboxy-D-xylitol 1,5-bisphosphate (2′-CXBP). The observed products, 2′-CABP and 4′-CABP, were distinguished from the potential products, 2′-CRBP and 2′-CXBP, both chromatographically and by virtue of their more potent inhibitory properties (Schloss & Lorimer 1982). It may be inferred from this result that the attack of CO_2 on the 2,3-enediol(ate) proceeds on the Si face of C-2, so as to generate 2′-carboxy-3-keto-D-arabinitol 1,5-bisphosphate (CKABP) as an intermediate. This conclusion is thus consistent with the orientation of the substrate within the catalytic site and with the recent evidence that the reaction is ordered with RuBP binding and undergoing enolization prior to reaction with CO_2 (Pierce et $al.$ 1986b).

The ability to trap CKABP with borohydride depends upon first freeing the intermediate from the clutches of the enzyme. This was achieved with a brief acid quench, following the protocol in scheme 3. Attempts to reduce CKABP directly, on the enzyme, with borohydride were unsuccessful. (The orientation of the C-3 carbonyl of CKABP with respect to the enzyme would otherwise have been defined.) In retrospect, this failure can now be attributed to the fact that CKABP exists on the enzyme predominantly, perhaps solely, as the hydrated C-3 gem-diol, a form which is resistance to borohydride reduction.

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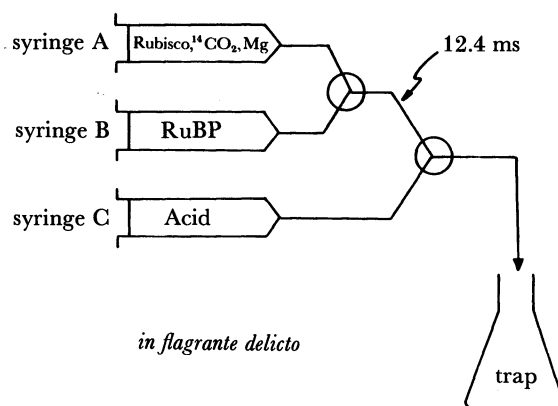
SCHEME 3. Synthesis of the six-carbon intermediate.

SYNTHESIS AND MEASUREMENT OF CKABP

The finding that CKABP is much more stable than previously thought (see below), led to the synthesis of CKABP on a molar scale (Pierce *et al.* 1986 a). The borohydride trap (scheme 3) was replaced with dry ice. After multiple 'pushes' of the rapid-mixing device, a mixture of $[2'\text{-}^{14}C]$CKABP, $[1\text{-}^{14}C]u\text{-PGA}$, lPGA and unreacted RuBP, in a ratio of about $1:1:1:10$, was purified from the accumulated, frozen, acid-quenched reaction mixture. A sensitive but simple method was developed to measure $[2'\text{-}^{14}C]$CKABP and $[1\text{-}^{14}C]u$PGA (scheme 4) (Pierce *et al.* 1986 a). Treatment of the reaction mixture with hot acid brings about the rapid decarboxylation of $[2'\text{-}^{14}C]$CKABP. Only $[1\text{-}^{14}C]$PGA remains to be counted. However, treatment the reaction mixture with borohydride before acidification converts $[2'\text{-}^{14}C]$CKABP into the acid-stable $[^{14}C]$carboxyarabinitol bisphosphate. Both $[1\text{-}^{14}C]$PGA and $[2'\text{-}^{14}C]$CKABP are then counted. The quantity of $[2'\text{-}^{14}C]$CKABP can then be determined as the difference.
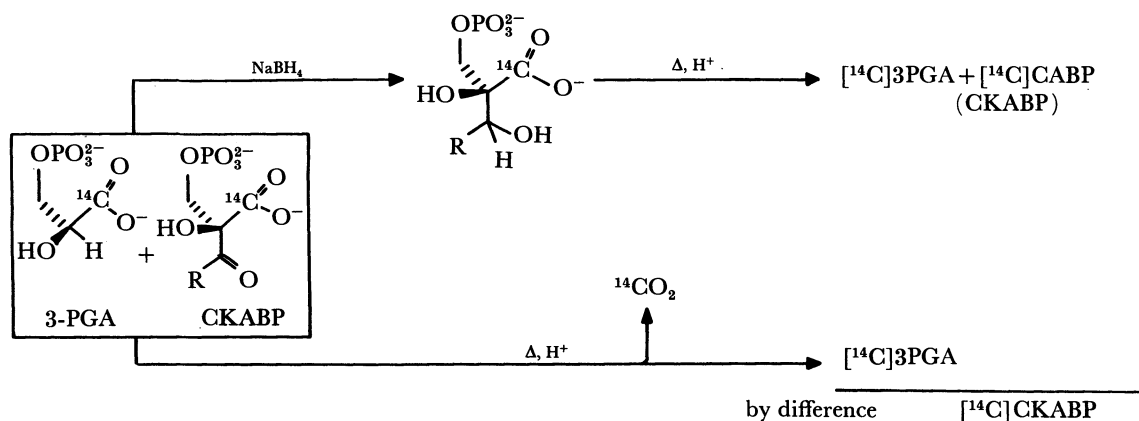
SCHEME 4. Measurement of CKABP.

PROPERTIES OF CKABP

By using such a procedure, together with a more laborious chromatographic method, it was possible to explore the properties of CKABP. The fate of CKABP and its rate of reaction are

pH-dependent (figure 1). Over a wide range (pH 4–11), CKABP undergoes decarboxylation at a constant, pH-independent rate $(2 \times 10^{-4} \text{ s}^{-1})$. This corresponds to a half-life of about an hour at 25 °C. When frozen at -80 °C, no decomposition of CKABP could be detected over a six-week period. However, on decarboxylation CKABP does not revert to pentulose bisphosphates. By using $[1\text{-}^{32}\text{P}]$CKABP, Jaworowski *et al.* (1984) have shown that instead, decarboxylation of CKABP is accompanied by the β-elimination of the C-1 phosphate.
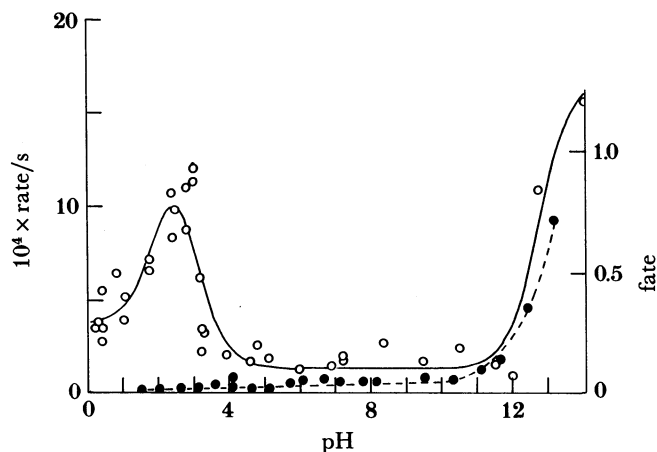

FIGURE 1. The fate and rate of decomposition of CKABP as a function of pH. Fate (closed circles), determined after reaction to completion at the indicated pH, is numerically represented as the partition coefficient, hydrolysis:(decarboxylation plus hydrolysis). Practically this is given by the ratio of change in acid-stable ^{14}C:initial BH_4-stabilizable ^{14}C. Rate (open circles) was determined from first-order plots of the fraction of CKABP remaining after reaction at the indicated pH. CKABP was measured either chromatographically (Schloss & Lorimer 1982), with $[^{3}\text{H}]2\text{-}'$CABP and $[^{3}\text{H}]4'\text{-}$CABP as internal standards, or by the abbreviated ^{14}C method (Pierce *et al.* 1986a).

Additionally, a ^{13}C-NMR study of the decarboxylation of $[2',3\text{-}^{13}\text{C}]$CKABP revealed the formation of a compound with the spectroscopic properties to be expected of 1-deoxy-D-glycero-2,3-pentodiulose 5-phosphate (Pierce *et al.* 1986a). This is the product to be expected from the β-elimination of phosphate from the intermediate 2,3-enediolate (scheme 5). Support for this mechanism comes from the very rapid β-elimination of phosphate from similar enediolates (Richard 1984, 1985) and from the observation that both RuBP and xylulose 1,5-bisphosphate undergo the loss of the C-1 phosphate in basic solution, presumably via the 2,3-enediolate (Paech *et al.* 1978).
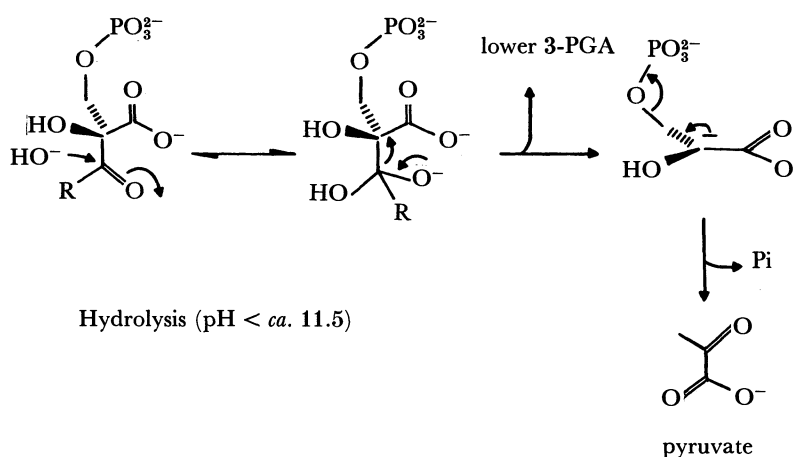

SCHEME 5

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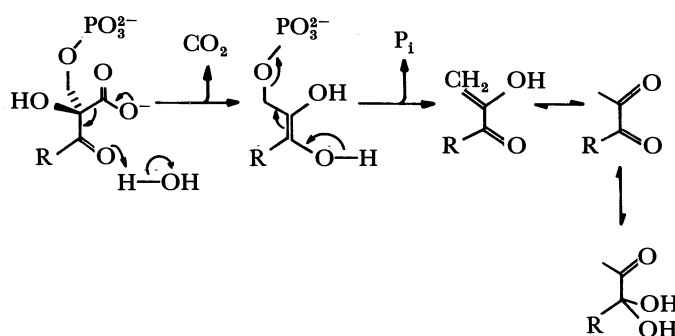
At pH below *ca.* 4, the ultimate fate of CKABP is also decarboxylation. However, two pH-dependent reactions influence the rate of decarboxylation (figure 1). As the pH is lowered below *ca.* 4 the rate of decarboxylation is increased. This is to be expected since the decarboxylation of β-keto acids is typically acid-catalysed. This rate-enhancement is generally attributed to the intramolecular protonation of the carbonyl oxygen by the carboxylic acid (scheme 6). However, below pH *ca.* 2.5, stabilization of CKABP becomes evident (figure 1). This phenomenon remains to be explored. Formation of the γ-lactone is a possible (although unproven) explanation.
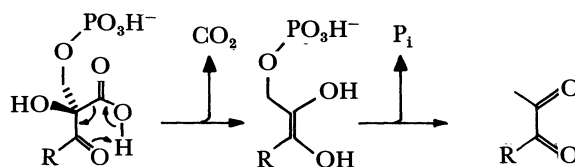
Scheme 6

Under alkaline conditions (above pH *ca.* 11), CKABP increasingly undergoes hydrolysis rather than decarboxylation. Analysis of the hydrolysate with lactate dehydrogenase revealed the presence of substantial (> 80%) quantities of pyruvate. Thus, like the decarboxylation of CKABP, the alkaline hydrolysis of CKABP is also associated with the β-elimination of the C-1 phosphate, rather than with protonation (scheme 7).
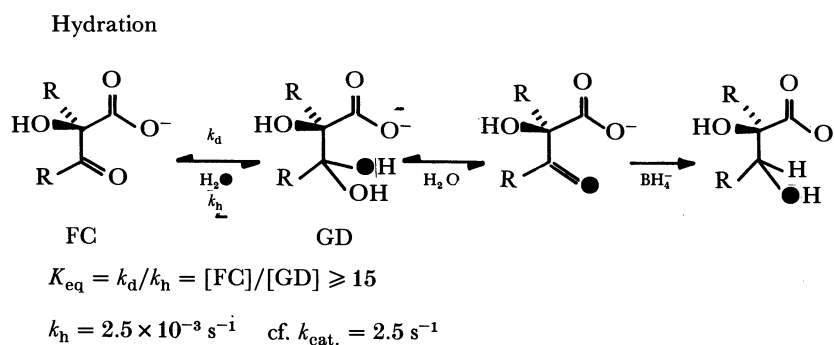

Hydrolysis (pH < *ca.* 11.5)

pyruvate

Scheme 7. Reactions of the six-carbon intermediate.

At pH 14, the rate of hydrolysis of CKABP was 1.5×10^{-3} s^{-1} (figure 1). The rate of phosphate elimination is likely to be many orders of magnitude in excess of this (Richard 1984). At neutral pH, the rate of hydration of CKABP was 2.5×10^{-3} s^{-1} (G. H. Lorimer & J. V. Schloss, unpublished), a rate marginally in excess of the maximum observed rate of hydrolysis. However, since the rate of hydration of carbonyl groups is generally hydroxide-catalysed, it would appear that carbon–carbon cleavage constitutes the rate-determining step in the hydrolysis of CKABP.

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The hydration state of CKABP

The hydration state of CKABP in solution was studied by ^{13}C-NMR with [2'-3-^{13}C]CKABP (Pierce *et al.* 1986 *a*). Although resonances attributable to the 2'-carboxyl carbon and to the C-3 carbonyl carbon (ketone form) were evident, no signal attributable to the hydrated carbonyl (gem-diol form) of CKABP was detected. Thus, taking the signal:noise ratio of the NMR spectrum as the limit, it was concluded that in solution the ketone form of CKABP is favoured over the hydrated gem-diol form by a factor of at least 15:1 (scheme 8).

Hydration

$$K_{eq} = k_d/k_h = [FC]/[GD] \geqslant 15$$

$$k_h = 2.5 \times 10^{-3}\ \mathrm{s}^{-1} \quad \mathrm{cf.}\ k_{cat.} = 2.5\ \mathrm{s}^{-1}$$

SCHEME 8. Reactions of the six-carbon intermediate.

The rate of hydration of CKABP in neutral solution was measured by following the rate at which oxygen-18 was incorporated from labelled water (scheme 8). A value of $2.5 \times 10^{-3}\ \mathrm{s}^{-1}$ was obtained (G. H. Lorimer & J. V. Schloss, unpublished). Since the rate of carboxylation, k_{cat}, is approximately 2.5–$5.0\ \mathrm{s}^{-1}$ under comparable conditions, it follows that the hydration of CKABP by the enzyme is accelerated by a factor of at least three orders of magnitude.

Mention has already been made of the failure to reduce CKABP with borohydride directly on the enzyme. To establish the hydration state of CKABP on the enzyme, an experiment with [3-^{18}O]RuBP, based on the strategy outlined in scheme 9, was devised. Consider the two limiting conditions of scheme 9.

If [3-^{18}O]CKABP exists on the enzyme solely in the ketone form, its release from the enzyme by acid quenching, followed by borohydride reduction in free solution, should yield carboxypentitol bisphosphates with the same isotopic enrichment as the starting [3-^{18}O] RuBP. Conversely, if [3-^{18}O]CKABP exists on the enzyme solely in the hydrated state, its release from the enzyme by acid quenching must be followed by dehydration before reduction can occur. This scenario results in carboxypentitol bisphosphate with only half the isotopic enrichment of the starting [3-^{18}O]RuBP. Table 1 shows the results of such an experiment. The recovered carboxypentitol bisphosphates were very nearly 50 % enriched with oxygen-18 relative to the starting material, regardless of the duration of the acid quench. Thus CKABP is stabilized on the enzyme predominantly (> 94 %) in the hydrated state.

The total PGA formed from [3-^{18}O] RuBP was 49 % enriched (table 1). Allowing for the dilution by *u*PGA, this indicates that the O-3 oxygen of RuBP is completely retained (Lorimer 1978; Sue & Knowles 1978). Although the enzyme clearly catalyses the hydration of CKABP, it does not catalyse the washout of the carbonyl oxygen of CKABP; less than complete retention of the O-3 oxygen of RuBP would otherwise result. Therefore, the hydration of CKABP is either

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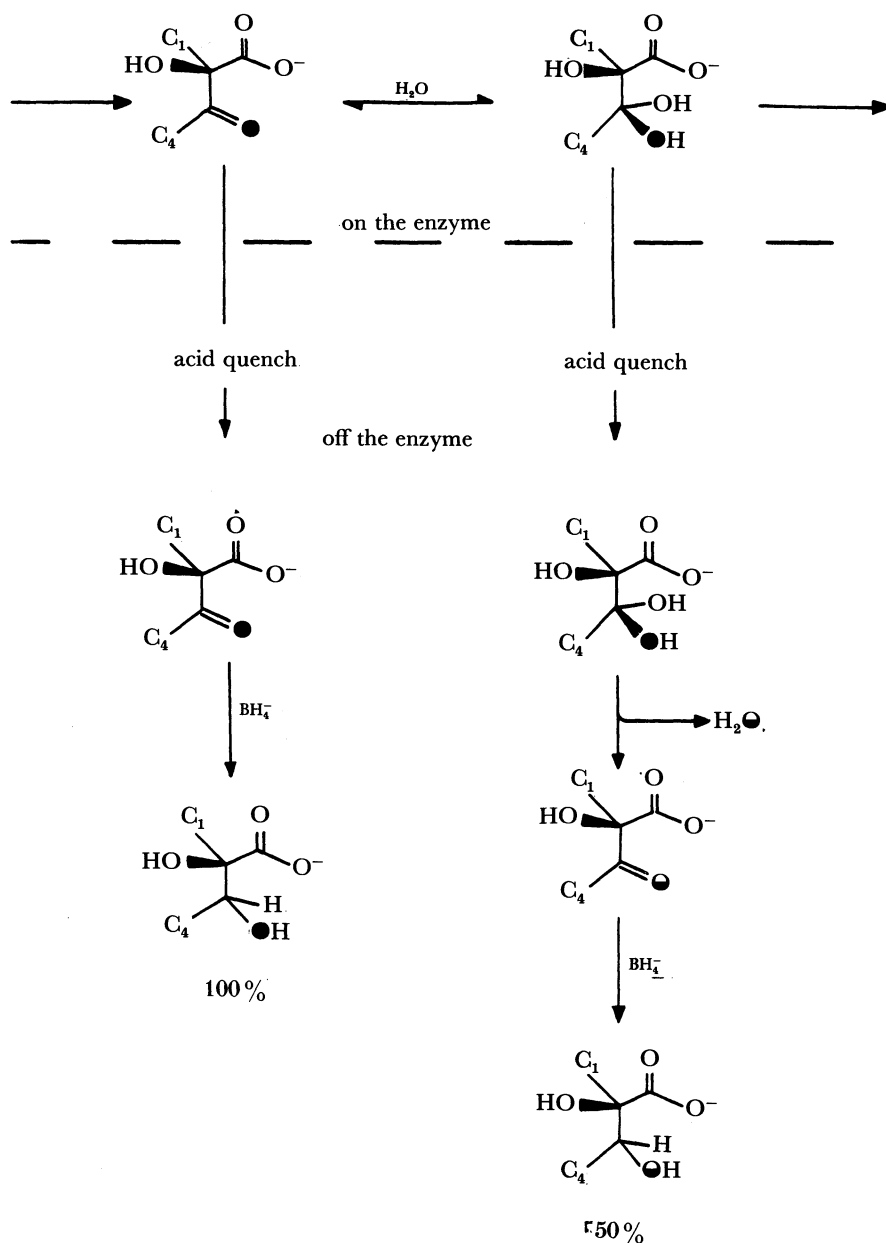
SCHEME 9

TABLE 1. ISOTOPIC ENRICHMENTS OF [3-^{18}O]CARBOXYPENTITOLS AND [1-^{18}O]PGA DERIVED FROM [3-^{18}O]RuBP

duration of acid quench/s	%^{18}O enrichment†	
	carboxypentitols	PGA
0.084	52.8, 49.4	48.7
30	53.6	—
600	45.5	—

† Relative to the isotopic enrichment of substrate [3-^{18}O]RuBP. Rubisco (spinach) was reacted with a slight molar excess of [3-^{18}O]RuBP for 200 ms before being quenched with acid (pH8) for the periods indicated. At the time, a strongly buffered (pH8) solution of NaBH$_4$ was added to reduce CKABP to carboxypentitol bisphosphate.

[100]

kinetically irreversible, or stereochemically directed (i.e. the same oxygen atom is removed as water during dehydration as is added during hydration) or both.

The stabilization of the hydrated form of CKABP on the enzyme, coupled with the presence of Me^{2+} within the catalytic site (Miziorko & Sealy 1984; Styring & Bränden 1985; Pierce & Reddy 1986) is reminiscent of the situation in carbonic anhydrase (Fersht 1985) and suggests that similar mechanisms may be involved. In carbonic anhydrase, water in the inner coordination sphere of the active-site Zn^{2+} is thought to be the source of the hydroxide ion that attacks the substrate CO_2. A recent ^{13}C NMR study of Mn^{2+}-substituted carbonic anhydrase (Led et al. 1982) suggests that the predominant enzyme-bound species is HCO_3^-, the hydrated species. The enzyme-bound HCO_3^- is coordinated directly to the Mn^{2+}, whereas CO_2 is attached only weakly to the enzyme without a direct bond to Mn^{2+}. An analogous mechanism involving the active-site Me^{2+} of Rubisco could account for both the enhanced rate of CKABP hydration and the preferential stabilization of the hydrated gem-diol form.

ENZYME-CATALYSED REACTIONS OF CKABP

Hydrolysis

The availability of CKABP gave us the opportunity to study its interaction with both activated and deactivated enzymes. Activated enzyme catalysed the complete hydrolysis of CKABP to PGA. Over the pH range 7.0–8.9, essentially no decarboxylation occurred provided that precautions were taken to ensure complete activation with CO_2 and Mg^{2+}. We may conclude that, with the formation of CKABP, the enzyme is fully committed to PGA formation (Pierce et al. 1986a).

However, kinetic analyses of the enzyme-catalysed hydrolysis of CKABP revealed that the rates fell considerably short of the rates of overall carboxylation with RuBP as substrate (table 2). Nevertheless, these rates exceed the non-enzymatic rate of hydration of the carbonyl group of CKABP, indicating that the free ketone form of CKABP is the substrate on which the enzyme acts. On the other hand, it would appear that the enzyme-catalysed hydrolysis of CKABP is limited by an event that is not on the direct catalytic pathway. Perhaps processing of CKABP involves a slow isomerization of the enzyme–CKABP complex to the form which normally participates in catalysis. In this regard, the two-step reaction of the activated enzyme with the CKABP analogue 2'-CABP involves a slow isomerization of the enzyme–2'-CABP complex with a rate constant $(4 \times 10^{-2} \text{ s}^{-1})$ of similar magnitude to that defining the hydrolysis of CKABP (Pierce et al. 1986a). Perhaps the two processes, CKABP hydrolysis and 2'-CABP binding, involve a similar change in enzyme conformation.

TABLE 2. RATE CONSTANTS FOR NON-ENZYMATIC AND ENZYME-CATALYSED REACTIONS OF CKABP

	rate constants $/\text{s}^{-1}$			
	hydrolysis of CKABP	hydration of CKABP	decarboxylation of CKABP	k_{cat}
non-enzymatic	1.5×10^{-3} (pH14)	2.5×10^{-3} (pH8)	2.0×10^{-4} (pH8)	0
spinach Rubisco	8.4×10^{-2}	$\geqslant k_{cat}$	2.9×10^{-3} (15)	2.5
Synechococcus Rubisco	2×10^{-1}	$\geqslant k_{cat}$	4.4×10^{-2} (220)	7.8

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Decarboxylation

To date, the deactivated enzyme, lacking the carbamate–Me^{2+} complex on Lys 201, was thought to be devoid of catalytic activity. However, when challenged with CKABP, it was unexpectedly found to catalyse the decarboxylation of CKABP (table 2). To be sure, the enhancement of decarboxylation over the non-enzymatic rate was a modest 200-fold (for the *Synechococcus* enzyme). However, the evidence indicates that the reaction was specific for the catalytic site. No catalysis was observed with boiled enzyme and the analogue of CKABP, 2'-CABP, severely inhibited the enzyme-catalysed decarboxylation (Pierce *et al.* 1986*a*). Additionally, both large and small subunits were required for the enhanced decarboxylation (Andrews *et al.* 1986).

The deactivated enzyme does not catalyse the protonation of C-3 of the 2,3-enediol arising from the decarboxylation of CKABP. That is to be expected, since deactivated enzyme does not catalyse the deprotonation of C-3 of RuBP. Instead, like the non-enzymatic decarboxylation, the enzyme-catalysed decarboxylation of CKABP ultimately leads to the β-elimination of the C-1 phosphate group of the 2,3-enediol(ate). It is not known if the elimination of phosphate occurs within the catalytic site or after dissociation of the 2,3-enediol from the enzyme. Although the β-elimination is likely to be rapid in any event (Richard 1984), a case can be made for the catalytic involvement of the deactivated enzyme (scheme 10). If indeed the decarboxylation of CKABP is catalysed by the protonation of the O-3 carbonyl oxygen by a group within the catalytic site, the product should be the 2,3-enediol. Yet this same group can now function as a general base, withdrawing the proton to yield the 2,3-enediolate. It is from the enediolate that β-elimination of the C-1 phosphate proceeds (Richard 1984).

The non-enzymatic decarboxylation of CKABP is acid-catalysed (figure 1 and scheme 6). This suggests that the specific protonation of the O-3 carboxyl oxygen of CKABP by a group within the catalytic site of the deactivated enzyme at least contributes to the observed rate enhancement (scheme 10). In so far as the decarboxylation catalysed by the deactivated enzyme represents a faithful reversal of the carboxylation of the 2,3-enediol(ate) by the activated enzyme, it seems reasonable to conclude that carboxylation is facilitated by the specific deprotonation of the O-3 of the 2,3-enediol(ate).
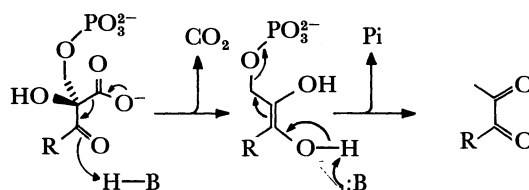
SCHEME 10. Deactivated-enzyme-catalysed decarboxylation and β-elimination.

The ability to dissect the overall reaction into partial reactions (enolization, carboxylation and hydrolysis), which can be independently analysed, promises to be very useful. When coupled with structural analysis (Brändén *et al.*, this symposium) and site-directed mutagenesis, it offers an experimental approach to identifying the various enzyme groups that participate in catalysis.

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