Trapping of a reaction intermediate by tetranitromethane during catalysis by ribulose bisphosphate carboxylase

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Carbanion intermediate *Tetranitromethane* *Ribulose bisphosphate carboxylase*

1. INTRODUCTION

Catalysis by ribulose bisphosphate (RuBP) carboxylase probably proceeds via deprotonation of the 3-hydroxyl of the 2,3-enediol of RuBP and consequent generation of a nucleophilic center at carbon 2 [1]. Carbon dioxide then attacks the carbanion to yield 2-carboxy-3-keto-D-arabinitol 1,5-bisphosphate and subsequently two molecules of 3-phospho D-glycerate [2-4].

Tetranitromethane (TNM) has been used to examine enzymic reactions thought to involve carbanionic intermediates [5,6]. In general, TNM reacts with such intermediates to yield nitroform, which absorbs strongly at 350 nm [7]. The reaction may lead either to oxidation of the carbanion in which case nitronium ion is reduced to nitrite [6,8] or to nitration of the carbanion by nitronium ion formed stoichiometrically with nitroform. Side reactions occur with enzyme leading to nitration of tyrosine residues or oxidation of sulfhydryl groups [9].

We now present evidence for a carbanion intermediate in the reaction catalyzed by spinach RuBP carboxylase using TNM as a probe.

2. MATERIALS AND METHODS

2.1. *Materials*

The tetrasodium salt of RuBP and trisodium salts of fructose 1,6-bisphosphate, and 6-phospho-D-gluconate were from Sigma Chemical Co. $NaH^{14}CO_3$ was purchased from ICN. Ribulose bisphosphate carboxylase was purified to homogeneity from fresh spinach leaves [10].

2.2. *Methods*

The enzyme stored as a suspension in 55% saturated $(NH_4)_2SO_4$ (at 4°C) was centrifuged and dissolved in 50 mM Tris–HCl (pH 8.3, 23°C), which had been freshly prepared using boiled H_2O that was gassed continuously with N_2 during pH adjustment to minimize dissolved CO_2. The dissolved enzyme was then passed through a Biogel P-10 column equilibrated with the same buffer to further reduce the carryover of HCO_3^- and Mg^{2+} and stored at 4°C in a tube sealed with Parafilm. A portion was activated at 30°C with 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ for 10 min. To initiate the reaction at 23°C, active enzyme was added to the sample cuvette containing the same buffer-Mg^{2+} solution, HCO_3^- at a final concentration of 5 mM, 0.4 mM tetranitromethane (TNM), and 1.0 mM ribulose bisphosphate (RuBP) in a volume of 0.5 ml. The reference cuvette contained an equivalent amount of unactivated enzyme and Tris buffer lacking Mg^{2+} and HCO_3^- but containing 0.4 mM TNM and 1.0 mM RuBP. The initial rate of nitroform production was measured at 350 nm and a value of 14 400 $M^{-1} \cdot cm^{-1}$ used as Σ_M [7].

3. RESULTS

Nitroform was produced from TNM in the presence of numerous buffers like Bicine, MOPS and HEPES but Tris was inert. Decomposition of

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Table 1

Effect of competitive inhibitors on nitroform production

Additions	Concn. (mM)	ΔA_{350}/min	% of Initial rate of nitroform production
None	–	0.085	100
6-Phosphogluconate	1	0.052	61
	2	0.040	47
Fructose 1,6-bisphospate	1	0.050	58
	2	0.030	35
	3	0.005	5
2-Carboxymannitol 1,6-bisphosphate	0.85	0.032	38
	1.7	0.022	25
	2.5	0.010	12

The enzyme which had been activated with 10 mM HCO_3^- and 20 mM Mg^{2+} was used to measure nitroform production. The rates are corrected for the non-specific decomposition of tetranitromethane by using an unactivated enzyme preparation, RuBP, and inhibitor in the reference cuvette. The reaction mixture contained 50 mM Tris (pH 8.3), 20 mM Mg^{2+}, 2.5 mM HCO_3^-, 0.4 mM TNM, and 1.0 mM RuBP

TNM to nitroform also occurred in the presence of potential effectors or inhibitors of RuBP carboxylase such as NADPH, xylulose 5-phosphate and sedoheptulose 1,7-bisphosphate. No decomposi-

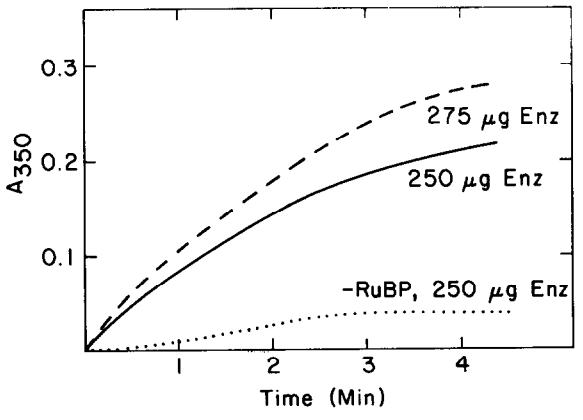

Fig.1. Nitroform production in the presence of the indicated amount of RuBP carboxylase and 1.0 mM RuBP (where present) under conditions described in the text. The reference cuvette lacking Mg^{2+} contained 1.0 mM RuBP, 0.4 mM TNM, and an identical concentration of unactivated RuBP carboxylase in 50 mM Tris, pH 8.3.

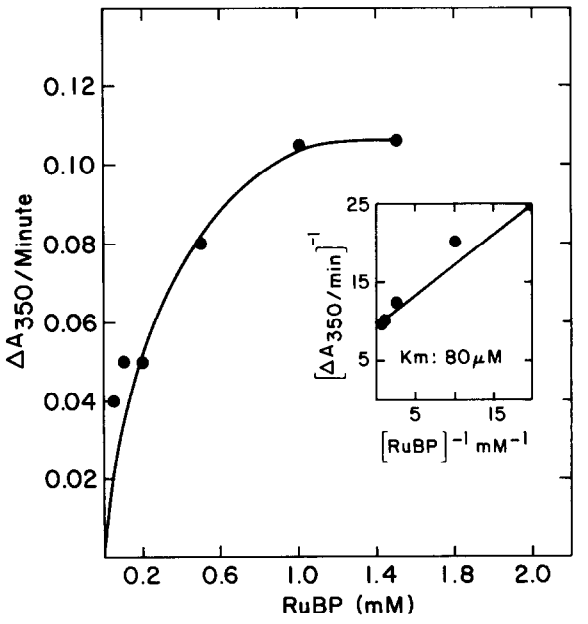

Fig.2. Nitroform production as a function of RuBP concentration using 140 μg of RuBP carboxylase. The reference cuvette contained an identical concentration of RuBP and unactivated RuBP carboxylase. Other conditions were identical to those described in fig.1.

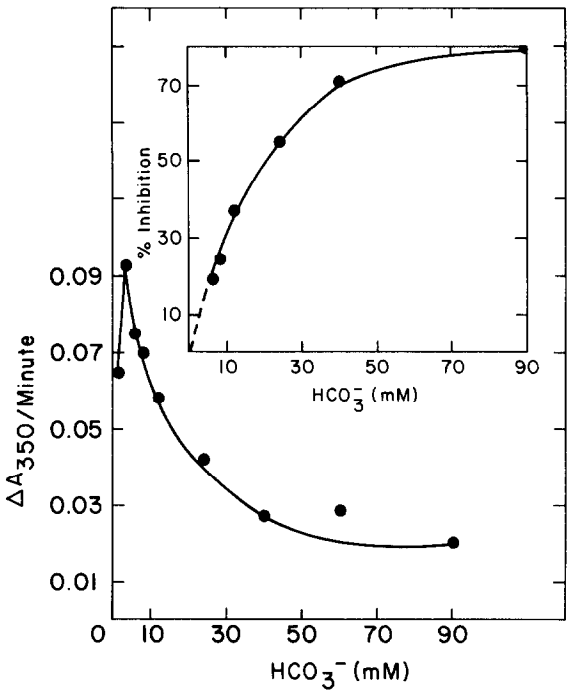

Fig.3. Nitroform production as a function of HCO_3^- concentration using 100 μg of RuBP carboxylase. Bicarbonate concentration was matched during activation and catalysis. The reference cuvette contained no HCO_3^- and no Mg^{2+} but an identical concentration of unactivated enzyme and of RuBP (1.0 mM). Other conditions were as described for fig.1.

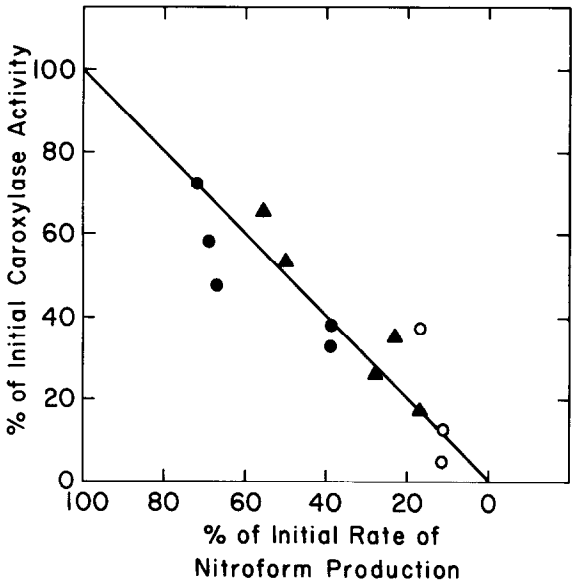

Fig.4. The relationship between RuBP carboxylase activity and nitroform production. The enzyme was modified by diethylpyrocarbonate (DEP) at pH 7.0 [15] for: 0.5, 1.0, 2.0, 5.0 and 10.0 min with 0.25 mM DEP (•—•); 0.5, 1.0, 2.0, 5.0 and 10.0 min with 0.5 mM DEP (▲—▲); and for 0.5, 1.5 and 3.0 min with 1.0 mM DEP (○—○). The reaction was quenched [15], and carboxylase [18] and nitroform production were each assayed independently in aliquot portions. The correlation coefficient in an analysis of linear regression is 91%.

tion of TNM occurred in the presence of 50 mM Tris containing 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ (pH 8.3, 23°C). On addition of RuBP carboxylase to this buffer, there was a low rate of nitroform production (fig.1) that may have been due to oxidation and nitration of the activated enzyme and slow decomposition of TNM catalyzed by basic groups on this protein [11]. However, when the enzyme was added to this buffer containing 1.0 mM RuBP a considerable rate enhancement occurred (fig.1). There was also a slow non-enzymatic reaction of RuBP with TNM. To correct for this in all experiments to be reported, the reference cuvette lacking Mg^{2+} contained unactivated enzyme and RuBP at concentrations equivalent to those present in the sample cuvette.

Nitroform production was dependent upon both RuBP (fig.2) and enzyme concentration (data not shown). It was inhibited by HCO_3^- at a concentration above 5 mM (fig.3) and was dependent upon Mg^{2+} showing an overall $K_{0.5}$ of 2.08 mM in experiments in which the Mg^{2+} concentration was matched during activation and catalysis.

Inhibitors of higher plant RuBP carboxylase such as 6-phospho D-gluconate [12], fructose 1,6-bisphosphate [13] and 2-carboxy D-mannitol 1,6-bisphosphate [14], none of which reacted with TNM, inhibited nitroform production (table 1).

Carbethoxylation of an essential histidine residue by diethylpyrocarbonate [15,16] resulted in concordance between the decay of nitroform production and of RuBP carboxylase activity (fig.4).

4. DISCUSSION

In this paper it is demonstrated that the decomposition of tetranitromethane to nitroform is catalyzed by RuBP carboxylase and is substrate-dependent. Although side reactions of both enzyme and substrate occur, they are quantitatively minor. All characteristics of the major enzyme-catalyzed process suggest that a common substrate-derived intermediate partitions between reactions leading either to nitroform production or carboxylation to yield 3-phosphoglycerate. For example, the production of nitroform requires Mg^{2+} and the K_m for RuBP of 80 μM at 23°C and pH 8.3 is similar to that of 100 μM for the barley carboxylase measured in Tris buffer at pH 8.0 and 30°C [17]. Known inhibitors of higher plant RuBP carboxylase also inhibit nitroform production. Covalent modification of an essential histidine residue affects both carboxylase and nitroform-producing activities equivalently. Especially noteworthy is the observed inhibition of nitroform production by CO_2 (provided as HCO_3^-). This would be expected if a common intermediate such as a carbanion of RuBP was diverted into the carboxylation pathway as a function of CO_2 concentration.

Although details of the chemistry of the process of RuBP-dependent enzyme-catalyzed nitroform production must still be elucidated, the process may be of interest in studies of RuBP carboxylase. Certainly the assay is rapid and reasonably sensitive. It is likely that nitroform production reflects enzyme-catalyzed events prior to the rate-limiting addition of CO_2 to an intermediate derived from RuBP. The production, therefore, may be extremely useful in elucidating aspects of catalysis by RuBP carboxylase.

ACKNOWLEDGEMENT

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