Quo Vadis photorespiration: A tale of two aldolases

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Abstract An O_2-consuming side reaction of D-ribulose 1,5-bisphosphate carboxylase causes photorespiration in plants. This reaction may be an inevitable consequence of the enzyme's inability to protect its ene-diolate reaction intermediate from O_2, a notion that is supported by the failure of persistent efforts to eliminate selectively its oxygenase activity by genetic manipulation. We have examined two aldolases with similar ene-diolate intermediates, L-rhamnulose 1-phosphate aldolase and L-fuculose 1-phosphate aldolase. The former enzyme has an oxygenase activity, while the latter does not, suggesting that the reaction with O_2 is not inevitable.

Key words: D-Ribulose 1,5-bisphosphate carboxylase/oxygenase; Photorespiration; L-Rhamnulose 1-phosphate aldolase; L-Fuculose 1-phosphate aldolase; Ene-diol; Oxygenase

1. Introduction

Discovery of D-ribulose 1,5-bisphosphate carboxylase's (Rubisco) oxygenase activity (Fig. 1) in the early 1970s caused considerable confusion among biochemists [1–4]. Although this activity could account for production of phosphoglycolate [2] in plants exposed to aerobic photosynthetic conditions (photorespiration) and inhibition of photosynthesis by oxygen (the 'Warburg effect') [4], it is an unusual oxygen-consuming reaction [5,6]. Rubisco requires none of the cofactors normally associated with O_2-utilizing enzymes. Biochemists were reluctant to accept that Rubisco catalyzed the reaction shown in Fig. 1 with Mg^{2+} as the only required cofactor, confirmed by the fact that the enzyme was mistakenly thought to be a copper-containing oxygenase [7] or that the oxygen-consuming reaction might be attributed to a separate enzyme [8,9]. Eventually, after it was shown that both activities were expressed by the same gene [10] and rigorous analyses [11] demonstrated that other metals or flavins were not required for activity, the oxygenase activity was accepted to be an intrinsic feature of Rubisco from all known sources [12].

Considerable effort has been devoted to divorcing the oxygenase activity from Rubisco by genetic manipulation [5].

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Abbreviations: Rubisco, D-ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39); RhuA, L-rhamnulose 1-phosphate aldolase (EC 4.1.2.19); FucA, L-fuculose 1-phosphate aldolase (EC 4.1.2.17); FruA, D-fructose 1,6-bisphosphate aldolase (EC 4.1.2.13); Mops, 3-(*N*-morpholino)propanesulfonic acid.

Studies have demonstrated that the oxygen-consuming side reaction of Rubisco reduces the yields of crop plants by as much as 50% [13]. Speculation that the oxygenase activity is a necessary consequence of the ene-diolate chemistry of Rubisco casts some doubt on whether it is even possible to produce an enzyme that can carry out the normal carboxylation reaction free from sensitivity to O_2 [14,15]. According to this premise, it would be expected that other enzymes with similar oxygen-sensitive intermediates should also react with O_2. A number of enzymes, that are commonly thought to have carbanionic reaction intermediates, do have low levels of oxygen-consuming side reactions [16]. However, the similarity of the intermediates for these other enzymes is somewhat remote, making comparisons with the oxygenase chemistry of Rubisco difficult. Considerable evidence exists that the first step of the reaction catalyzed by Rubisco involves the formation of an ene-diolate, shown in Fig. 1, by abstraction of the C-3 proton of D-ribulose 1,5-bisphosphate [5,6]. This ene-diolate (equivalent to the C-3 carbanion) then isomerizes to the ene-diolate that reacts with CO_2 or O_2 (equivalent to the C-2 carbanion) [6]. Several metal-dependent (class II) aldolases exist that utilize a similar ene-diolate intermediate for comparable carbon-carbon ligation chemistry. We have examined three such enzymes: L-rhamnulose 1-phosphate aldolase (RhuA), L-fuculose 1-phosphate aldolase (FucA), and D-fructose 1,6-bisphosphate aldolase (FruA) isolated from *Escherichia coli* [17], for their ability to catalyze oxygen-consuming side reactions.

2. Materials and methods

2.1. Enzymes

RhuA, FucA, and FruA from *E. coli* were purified as previously described [17]. Enzymes were freed of their endogenous metal (Zn^{2+}) by exhaustive dialysis [17]. After reconstitution of apo-RhuA or apo-FucA with Co^{2+}, it was no longer possible to remove this metal from either enzyme by dialysis.

2.2. Enzymatic assays and dihydroxyacetone phosphate

Aldolase activities of RhuA, FucA, and FruA were determined at 25°C in 50 mM Mops-NaOH, pH 7, and 60 mM KCl. Each assay (1 ml) contained various concentrations of the substrates for these enzymes (or 1 mM L-rhamnulose 1-phosphate, L-fuculose 1-phosphate, or D-fructose 1,6-bisphosphate, respectively, for routine assay), 0.2 mM NADH, and a mixture of triosephosphate isomerase and glycerol phosphate dehydrogenase (10 units, Sigma Chemical Co.) [17]. Production of dihydroxyacetone phosphate in these coupled assays was monitored by loss of absorbance at 340 nm ($\varepsilon = 6220\ cm^{-1}\ M^{-1}$) due to oxidation of NADH.

2.3. Oxygen consumption

Oxygen consumption was determined by use of a Hansatech oxygen electrode. All assays were conducted at 25°C in 50 mM Mops-NaOH, pH 7, 60 mM KCl, and various concentrations of dihydroxyacetone phosphate. Assay mixtures were equilibrated with air (0.24 mM O_2) prior to initiation of the reaction by addition of enzyme [6].

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2.4. Hydrogen peroxide production

Substrate-dependent production of hydrogen peroxide by RhuA was determined by use of a peroxidase-coupled spectrophotometric assay at 25°C [18]. Each 1 ml assay contained 50 mM Mops-NaOH, pH 7, 60 mM KCl, 1 mM 2,2′-azinodi(3-ethylbenzthiazoline-6-sulphonic acid) ($\varepsilon = 36000$ and 12000 M^{-1} cm^{-1} at 414 and 730 nm, respectively [18]), 25 μg/ml horse radish peroxidase, and various concentrations of dihydroxyacetone phosphate. Assay mixtures were equilibrated with air prior to initiation of the reaction by addition of enzyme.

2.5. Phosphohydroxypyruvaldehyde production

To 0.99 ml of 1 mM dihydroxyacetone phosphate, 50 mM Mops-NaOH, pH 7, and 60 mM KCl, was added 10 μl of a 5 μM solution of RhuA that had been freed of metal by dialysis [17]. For assays that contained Co^{2+} as the activating metal, 10 μM Co^{2+} was added to the final assay mixture. For assays that contained Ni^{2+} as the activating metal, the apo-RhuA was preincubated with 1 mM Ni^{2+} prior to dilution into the final assay mixture. After 1 h of incubation at 25°C, 10 μl of a solution of catalase (1000 units/ml, 0.2 μg) was added and the sample was allowed to incubate at 25°C for an additional 5 min. An aliquot (0.1 ml) of the reaction mixture was diluted to a final volume of 1 ml to give a solution containing 50 mM Mops-NaOH, pH 7, 60 mM KCl, 0.2 mM NADH, and the residual dihydroxyacetone phosphate was determined as described in Section 2.2. To 0.5 ml of the sample that had been freed of H_2O_2, 6.25 μl of 10 N NaOH was added and the solution was incubated at 30°C for 30 min. 3-Phosphoglycerate, that results from base treatment of phosphohydroxypyruvaldehyde, was determined by a coupled spectrophotometric assay after neutralization of the sample by addition of 6.15 μl of concentrated HCl (37% w/w) [19]. An aliquot (200 μl) of the neutralized sample was diluted to a final volume of 1 ml to give a solution containing 50 mM Mops-NaOH, pH 7, 60 mM KCl, 3 mM ATP, 10 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 10 units of pyruvate kinase, and 10 units of glycerol-3-phosphate dehydrogenase. After establishing a constant absorbance (340 nm) at 25°C, a 20 μl aliquot containing 20 units of 3-phosphoglycerate kinase and 20 units of glyceraldehyde-3-phosphate dehydrogenase was added and the change in absorbance at 340 nm that resulted was determined.

3. Results

3.1. Oxygenase activity of RhuA, FucA, and FruA

As isolated, RhuA, FucA, and FruA, all contain tightly bound Zn^{2+}. In this form, none of the enzymes has substantial oxygenase activities (< 0.001 μmol O_2 consumed min^{-1} mg^{-1}). However, if Co^{2+} is substituted for the naturally occurring Zn^{2+} in these enzymes, RhuA will catalyze an oxygen-consuming reaction in the presence of dihydroxyacetone phosphate (0.4 μmol O_2 consumed min^{-1} mg^{-1}), while FucA and FruA have virtually no oxygenase activity (0.0019 and 0.0015 μmol O_2 consumed min^{-1} mg^{-1} detected, respectively). With respect to their 'normal' aldolase activities, both RhuA and FucA have somewhat higher activity with Co^{2+} as the activating metal. The relative activities in the direction of aldol cleavage of the Zn^{2+} and Co^{2+} forms of the enzymes, respectively, are 10 and 32 μmol min^{-1} mg^{-1} for RhuA and 3.1 and 7.0 μmol min^{-1} mg^{-1} for FucA. Several other metals, Ni^{2+}, Cu^{2+}, and Mn^{2+}, support the oxygenase activity of RhuA (Table 1). The levels of activity for Ca^{2+}-, Mg^{2+}-, Zn^{2+}-, Cd^{2+}-, Ba^{2+}-, and Al^{3+}-substituted RhuA were all less than 0.001 μmol min^{-1} mg^{-1} and, with the exception of Zn^{2+}, these metals did not support aldolase activity. Comparable levels of oxygenase activity were obtained for Ni^{2+}- and Co^{2+}-activated RhuA, while substantially lower levels of activity were obtained for the Cu^{2+}- and Mn^{2+}-activated enzymes (Table 1).

3.2. Products formed in the oxygen-consuming reaction of RhuA

Stoichiometric levels of hydrogen peroxide and phosphohydroxypyruvaldehyde were produced relative to the O_2 consumed (Tables 1 and 2). By contrast to the oxygenase reaction of Rubisco, where no reactive species of oxygen are released [5], the oxygenase reaction of RhuA gives one molecule of hydrogen peroxide for each molecule of oxygen consumed (Table 1).

4. Discussion

Although the specific activity of Ni^{2+}- or Co^{2+}-activated RhuA for oxygen consumption is substantially higher than Rubisco at atmospheric levels of O_2, the fate of the peroxide intermediate of the RhuA oxygenase reaction is quite different from that of the corresponding intermediate for Rubisco (Fig. 1). These results suggest that Rubisco directs the fate of its

Table 1
Kinetics of RhuA

Rates observed at 1 mM dihydroxyacetone phosphate		
Activating metal	O_2 consumption (μmol min^{-1} mg^{-1})	H_2O_2 production (μmol min^{-1} mg^{-1})
Ni^{2+}	0.53	0.47
Co^{2+}	0.42	0.39
Cu^{2+}	0.07	0.09
Mn^{2+}	0.029	0.016

Kinetic constants						
Activating metal	Aldolase		O_2 consumption		H_2O_2 production	
	V_{max} (μmol min^{-1} mg^{-1})	K_m[a] (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_m[b] (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_m[b] (mM)
Zn^{2+}	7.1 ± 0.05	0.35 ± 0.05	< 0.005			
Ni^{2+}	4.5 ± 0.2	3.7 ± 0.3	0.64 ± 0.08	0.31 ± 0.15	0.67 ± 0.02	0.36 ± 0.03
Co^{2+}	48 ± 4	1.6 ± 0.3	1.0 ± 0.06	0.26 ± 0.06	0.57 ± 0.02	0.21 ± 0.02
Cu^{2+}	< 0.01		0.11 ± 0.01	0.09 ± 0.02	0.16 ± 0.01	0.033 ± 0.008
Mn^{2+}	55 ± 1.5	4.2 ± 0.2	0.027 ± 0.002	0.12 ± 0.03	0.021 ± 0.002	0.32 ± 0.07

[a]Michaelis constant for rhamnulose 1-phosphate.
[b]Michaelis constant for dihydroxyacetone phosphate. V_{max} and K_m values were obtained at atmospheric levels of O_2; no attempt was made to vary the concentration of the gaseous substrate.

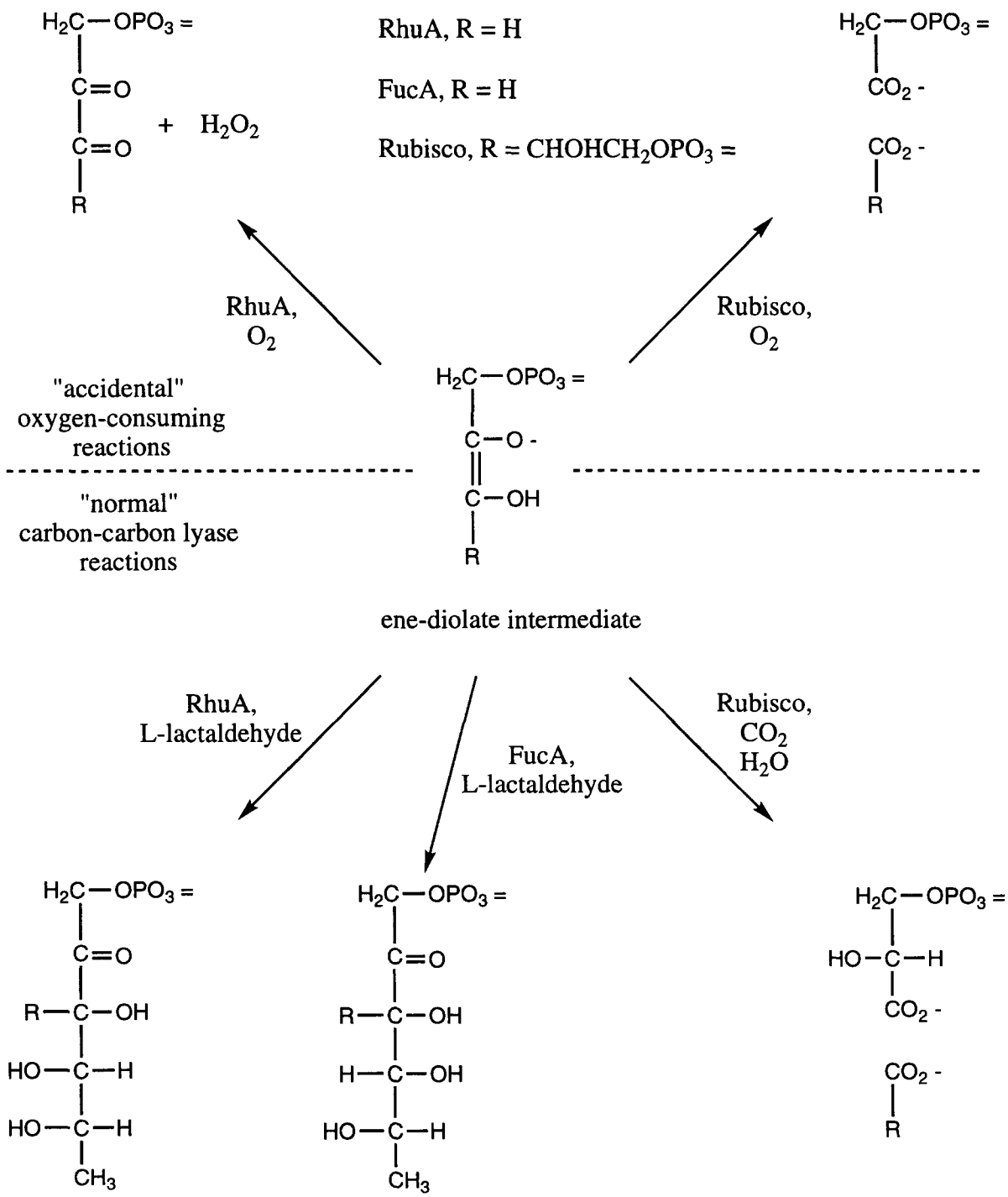

Fig. 1. The fate of putative ene-diolate reaction intermediates for the carbon-carbon ligation reactions or oxygenase reactions of Rubisco, RhuA, and FucA.

corresponding peroxide intermediate down the same path as the carboxylation reaction, towards cleavage of the bond between C-2 and C-3 [5]. Recently, a mutant of the *Rhodospirillum rubrum* Rubisco was described that carries out an oxygenase reaction similar to RhuA, giving a dicarbonyl and hydrogen peroxide as products [20]. This suggests that the difference in the ultimate fate of the oxygenase reactions of RhuA and Rubisco is due to additional catalytic potential of the latter, rather than a more fundamental difference in the chemistry of these two reactions.

Interestingly, the wild-type *R. rubrum* Rubisco is exclusively an oxygenase (devoid of carboxylase activity) when the enzyme is activated with Co^{2+} [21], while the Cu^{2+}-activated RhuA supports only the oxygen-consuming reaction (Table 1). The fact that an absolute bias towards oxygenase activity vs. carbon-carbon ligation occurs with different metals for Rubisco and RhuA (Co^{2+} for the former and Cu^{2+} for the latter) indicates that this is not directed by an intrinsic feature of the metal's chemistry. RhuA actually has a higher level of aldolase activity when activated with Co^{2+} than when acti-

Table 2
Products of the oxygenase reaction of RhuA[a]

Activating metal	Dihydroxyacetone phosphate consumed (μmol)	Phosphohydroxypyruvaldehyde produced (μmol)
Ni^{2+}	0.44 ± 0.02	0.42 ± 0.01
Co^{2+}	0.47 ± 0.02	0.47 ± 0.01

[a]No dihydroxyacetone phosphate was consumed and no phosphohydroxypyruvaldehyde was formed in control samples that lacked either enzyme or metal. The data presented are the average of four replicates.

vated by its physiological metal, Zn^{2+} (32 vs. 10 μmol min^{-1} mg^{-1}, respectively). The highest level of oxygenase activity for Rubisco is for the Mg^{2+}-activated enzyme (0.24 μmol min^{-1} mg^{-1}) and the relative levels of oxygenase activity for the enzyme activated by various metals differs from the metal specificity of RhuA. While the activating metal appears to play an important role in the oxygenase reactions of RhuA and Rubisco, the protein is also a determining factor in regulating this side reaction, as suggested by the absence of such a reaction for FucA.

RhuA and FucA catalyze reactions that differ only in the stereochemistry of their physiological products (epimeric at C-4). Both enzymes will carry out condensations between dihydroxyacetone phosphate and a variety of aldehydes [17]. Despite the fact that the ene-diolate of dihydroxyaldehyde phosphate bound to FucA is resistant to attack by O_2, it is actually more reactive towards formaldehyde than when bound to RhuA (the relative rates for condensation with formaldehyde as acceptor compared to L-lactaldehyde for FucA and RhuA are 44 and 22%, respectively) [17]. It would seem that FucA can exhibit a high degree of selectivity in avoiding reaction with O_2, but has no greater degree of substrate specificity than RhuA for aldehydic substrates. Although there is some sequence homology between RhuA and FucA (18% identity), they are thought to have arisen by convergent, rather than divergent evolution [22]. Crystals and a crystal structure for both RhuA [23] and FucA [24], respectively, have been obtained. Comparison of the structures of these two ene-diolate utilizing enzymes may help to suggest a path forward for engineering an oxygenase-less Rubisco by genetic manipulation. Although such a mutant has not been obtained by design or selection to date, the examples of RhuA and FucA would indicate that such a mutant should, in principle, be possible. For the doubtful, it should be remembered that natural selection only favors the best Rubisco necessary for survival of photosynthetic organisms, not necessarily the best enzyme for human agriculture.

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