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## Oxygenase Side Reactions of Acetolactate Synthase and Other Carbanion-Forming Enzymes

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**ABSTRACT:** Enzymes that mediate carbanion chemistry must protect their reactants from solvent and undesirable electrophiles, such as molecular oxygen. A number of enzymes that utilize carbanionic intermediates were surveyed for O<sub>2</sub>-consuming side reactions. Several of these enzymes, acetolactate synthase, pyruvate decarboxylase, class II aldolase, and glutamate decarboxylase, catalyze previously undetected oxygen-consuming reactions, while others such as class I aldolase, [(phosphoribosyl)amino]imidazole carboxylase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase, and triosephosphate isomerase do not. Prior to this work, only ribulosebiphosphate carboxylase was known to catalyze an oxygenase side reaction. These new examples indicate that while O<sub>2</sub>-consuming side reactions are a more general feature of enzyme-mediated carbanion chemistry than has been previously appreciated, they are not necessarily an inevitable consequence of this chemistry. Expression of an oxygenase activity not only depends on the accessibility of the carbanionic intermediate to molecular oxygen but also may depend on the ability of the enzyme to stabilize the initially formed peroxide anion either through protonation with an appropriate enzymic group or through metal coordination.

The oxygen-consuming reaction of ribulosebiphosphate carboxylase is an undesirable feature of the enzyme and accounts for photorespiration with concomitant inhibition of photosynthesis and plant growth by oxygen (Ogren & Bowes, 1971; Hardy et al. 1978). It has been proposed that the oxygenase activity may be an unavoidable reaction with O<sub>2</sub> of the carbanionic intermediate that normally reacts with CO<sub>2</sub> (Lorimer & Andrews, 1973; Andrews & Lorimer, 1978). If the oxygenase reaction is simply a consequence of carbanion chemistry, then any enzymic reaction that involves oxygen-accessible carbanionic reaction intermediates should have an oxygenase activity. Philipp Christen demonstrated that the carbanionic intermediates of several enzymes (class I and class II fructose-1,6-bisphosphate aldolase, 6-phosphogluconate dehydrogenase, aspartate aminotransferase, and pyruvate decarboxylase) were accessible to various oxidants, such as hexacyanoferrate(III) and tetranitromethane (Healy & Christen, 1973). These results demonstrate that there are a number of enzymes with intermediates capable of reacting with external electrophiles that should in principle also be capable of reacting with molecular oxygen. A number of enzymes that catalyze reactions involving carbanionic intermediates were examined for oxygenase activity in order to determine which

factors might be important for expression of an oxygenase activity. Those factors that contribute to discrimination between O<sub>2</sub> and CO<sub>2</sub> are particularly important in the case of ribulosebiphosphate carboxylase, as there is currently a considerable effort devoted to elimination of the oxygenase activity of this enzyme by genetic manipulation (Hartman, 1991).

### EXPERIMENTAL PROCEDURES

#### Materials

The 96.8% <sup>18</sup>O<sub>2</sub>, 98% H<sub>2</sub><sup>18</sup>O, and [2-<sup>13</sup>C]pyruvate were obtained from Merck, Sharpe and Dohme isotopes. Acetolactate synthase isozyme II (ALS II)<sup>1</sup> from *Salmonella typhimurium* was prepared as previously described (Schloss & Aulabaugh, 1990). The flavin analogue 5-deaza-FAD was a gift from Professor Colin Thorpe of the University of Delaware. [(Phosphoribosyl)amino]imidazole carboxylase from *Escherichia coli* and the substrate [(phosphoribosyl)amino]imidazole were a gift from Professor JoAnn Stubbe and

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<sup>1</sup> Abbreviations: TPP, thiamin pyrophosphate; FAD, flavin adenine dinucleotide; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; Tricine, N-tris(hydroxymethyl)methyl glycine; Mes, 2-(N-morpholino)ethanesulfonic acid, ALS II, acetolactate synthase isozyme II; 3-PGA, 3-phosphoglycerate.

Erik Myer of the Massachusetts Institute of Technology. NADP<sup>+</sup>-dependent isocitrate dehydrogenase from pig heart was obtained from Boehringer Mannheim. All other enzymes were obtained from Sigma.

### Methods

**Measurements of O<sub>2</sub> Consumption.** A Hansatech oxygen electrode thermostated at 25 °C was used to monitor consumption of oxygen. Buffer solutions were equilibrated with air (0.24 mM O<sub>2</sub>) prior to each assay.

**Assay Conditions for Specific Enzymes.** Acetolactate synthase activity was measured in 0.1 M Tricine-NaOH buffer, pH 7.8, containing 0.1 mM TPP, 0.1 mM FAD, 10 mM MgCl<sub>2</sub>, and either 30 mM pyruvate, 30 mM  $\alpha$ -ketobutyrate, or 5.5 mM acetolactate. The specific activity of ALS II was 27  $\mu$ mol of acetolactate produced min<sup>-1</sup> mg<sup>-1</sup>.

Glutamate decarboxylase (*E. coli*) oxygenase activity was measured in 0.1 M ammonium acetate buffer, pH 4.7, containing 0.01 mM pyridoxal phosphate and 30 mM L-glutamate. The maximal rate of glutamate decarboxylation was 20  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup>. Oxygenase activity could not be detected with 4-aminobutyrate as substrate.

Assays for pyruvate decarboxylase (brewers' yeast) consisted of 0.1 M Mes-NaOH buffer, pH 6, containing 0.1 mM TPP, 10 mM MgCl<sub>2</sub>, and either 30 mM pyruvate or 30 mM  $\alpha$ -ketobutyrate. The maximal rates of acetaldehyde formation from pyruvate or of propionaldehyde from  $\alpha$ -ketobutyrate were 6.7 and 4.7  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup>, respectively. Oxygenase activity could not be detected with acetaldehyde as substrate.

Assays for class II aldolase (Zn<sup>2+</sup> dependent) from *Staphylococcus aureus* contained 0.1 M HEPES-NaOH buffer, pH 7.5, with either 3 mM fructose bisphosphate or 8 mM dihydroxyacetone phosphate. The maximal rate of fructose bisphosphate conversion to dihydroxyacetone phosphate and glyceraldehyde phosphate observed for this enzyme was 21  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup>. No oxygen consumption was observed for class I aldolase from rabbit muscle under identical conditions.

Triosephosphate isomerases from bakers' yeast and porcine muscle, which converted D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate at rates of 10 000 and 5000  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup>, respectively, were assayed in the presence of 100 mM HEPES-NaOH buffer, pH 7.5, and 8 mM dihydroxyacetone phosphate. No oxygen consumption was observed.

[(Phosphoribosyl)amino]imidazole carboxylase (*E. coli*) was assayed in 100 mM Tricine buffer at pH 7.8 with 8.8 mM [(phosphoribosyl)amino]imidazole. The presence or absence of 1 mM MgCl<sub>2</sub> and/or 1 mM ATP did not affect the carboxylase activity nor the lack of oxygenase activity. The enzyme carboxylated [(phosphoribosyl)amino]imidazole at a rate of 6.6  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup>.

6-Phosphogluconate dehydrogenase (*Torula* yeast) was examined for oxygenase activity in 100 mM HEPES buffer pH 7.5, starting with either 5.5 mM D-ribulose 5-phosphate and NADPH or 8.3 mM 6-phosphogluconate and NADP<sup>+</sup>. The nucleotide concentration was varied from 200  $\mu$ M to 1 mM in both cases. In the normal reaction, the enzyme produced 28.8  $\mu$ mol of D-ribulose 5-phosphate min<sup>-1</sup> mg<sup>-1</sup> from 6-phosphogluconate. No activity was observed spectrophotometrically starting with D-ribulose 5-phosphate in the presence of NADPH.

Isocitrate dehydrogenase (NADP<sup>+</sup> dependent) from pig heart was examined for oxygenase activity in an assay mixture containing 100 mM HEPES-NaOH buffer, pH 7.5, and either 30 mM isocitrate in the presence of NADP<sup>+</sup> or 30 mM  $\alpha$ -ketoglutarate in the presence of NADPH. The nucleotide concentration was varied from 200  $\mu$ M to 1 mM in both cases.

The enzyme had an activity of 2 units/mg when converting isocitrate to  $\alpha$ -ketoglutarate.

**<sup>18</sup>O Labeling Experiments.** A reaction mixture with a total volume of 0.5 mL was prepared that contained 200 mM Tricine-NaOH buffer, pH 7.8, 10 mM MgCl<sub>2</sub>, 10  $\mu$ M FAD, 10  $\mu$ M TPP, 0.8 mM [2-<sup>13</sup>C]pyruvate, 20% H<sub>2</sub><sup>18</sup>O, and 40  $\mu$ L of 2 mg/mL ALS II. The pyruvate concentration was kept low in order to favor the oxygenase reaction ( $K_m$  pyruvate < 10  $\mu$ M) compared to the normal synthase reaction ( $K_m$  pyruvate = 10 mM). After 1 h at 25 °C, 50  $\mu$ L of 100 mM EDTA, pH 8.0, was added to quench the reaction along with 50  $\mu$ L of dioxane-d<sub>8</sub>, which was used as a reference. The <sup>13</sup>C NMR spectra were obtained with a Bruker AM 600 nuclear magnetic resonance spectrophotometer. Only a single peak at 182.068 ppm was observed for the carboxylate of acetate. However, in the 212 ppm region, a set of four peaks was present for the carbonyl group of acetolactate. The two major peaks at 212.631 and 212.348 ppm were separated by 0.28 ppm, the correct value for a <sup>13</sup>C-<sup>13</sup>C coupling constant. Each of the major peaks was accompanied by a minor peak, at 212.579 and 212.296 ppm, respectively. These minor peaks are upfield-shifted by 0.052 ppm, the expected shift for <sup>18</sup>O incorporation into a carbonyl group (Risley & Van Etten, 1981). While no <sup>18</sup>O incorporation was observed into acetate, <sup>18</sup>O incorporation was observed into the acetolactate produced by the normal reaction of ALS II due to exchange with solvent. This experiment was repeated several times using as much as 50% H<sub>2</sub><sup>18</sup>O, and still no <sup>18</sup>O incorporation was observed into acetate.

A reaction mixture similar to that prepared above was used for the <sup>18</sup>O<sub>2</sub> labeling experiment. All of the reaction components except [2-<sup>13</sup>C]pyruvate and ALS II were combined and the reaction vessel was prepared under anaerobic conditions and equilibrated with an <sup>18</sup>O<sub>2</sub> atmosphere. The reaction vessel was sealed and [2-<sup>13</sup>C]pyruvate was added to a concentration of 0.8 mM, along with 40  $\mu$ L of 2 mg/mL ALS II. The reaction was allowed to proceed for 1.5 h at 25 °C. For the last 5–10 min of the reaction, the sample was opened to the atmosphere to introduce a small amount of <sup>16</sup>O label as an internal reference. The <sup>13</sup>C NMR spectra for this sample showed two peaks in the carboxylate region for acetate at 182.068 and 182.092 ppm with the major peak upfield-shifted by 0.027 ppm from the minor peak. This is the shift expected for <sup>18</sup>O incorporated into the carboxylate group of acetate (Risley & Van Etten, 1981). The upfield-shifted peak was larger, indicating that a majority of the acetate contained 1 equiv of <sup>18</sup>O, as would be expected from the conditions of the experiment.

**Identification of Products from Oxygenase Reactions.** The presence of acetate as a product from the oxygenase reaction of ALS II was verified by NMR (see above) and by derivatization of an ALS II reaction mixture with *tert*-butyldimethylsilane and subsequent analysis by gas chromatography-mass spectrometry. Reaction mixtures containing both ALS II and pyruvate showed the presence of acetate in the mass spectrum, while control experiments that contained either pyruvate or ALS II alone were found to contain no acetate.

The presence of hydroxypyruvaldehyde phosphate in the oxygenase reactions of aldolase (class II) using fructose-1,6-bisphosphate as substrate was confirmed by two methods. The first involved the use of glyoxylase I as previously described by Healy and Christen (1972). The second method involved treating the samples with base (Healy & Christen, 1972) and NaBH<sub>4</sub>, followed by a coupled enzymatic assay for 3-phosphoglycerate (3-PGA) using phosphoglycerate kinase,

Table I: Oxygenase Activities of Various Carbanion-Utilizing Enzymes

enzyme	rate of oxygen consumption [ $\mu\text{mol min}^{-1}$ (mg of enzyme) $^{-1}$ ]
ribulosebiphosphate carboxylase <sup>a</sup>	0.24
acetolactate synthase <sup>b</sup>	
acetolactate	0.20
pyruvate	0.16
$\alpha$ -ketobutyrate	0.020
glutamate decarboxylase	0.027
pyruvate decarboxylase	
$\alpha$ -ketobutyrate	0.021
pyruvate	0.012
class II aldolase	
dihydroxyacetone phosphate	0.003
fructose-1,6-bisphosphate	0.003

<sup>a</sup> Activity for the enzyme from *Glycine max* (soybean) at pH 8.2 and saturating ribulose biphosphate (Christeller, 1981). Similar values for the enzymes from *Spinacea oleracea* (spinach), *Anabaena variabilis* (a cyanobacterium), *Rhodospirillum rubrum* (a purple, non-sulfur photosynthetic bacterium) were 0.2 (Badger & Lorimer, 1976), 0.047 (Badger, 1980), and 0.41 (Christeller, 1981), respectively. The maximal rates observed for the soybean, spinach, *A. variabilis*, and *R. rubrum* enzymes at saturating  $\text{O}_2$  were 0.65, 0.6, 0.26, and 0.89  $\mu\text{mol min}^{-1}$  (mg of enzyme) $^{-1}$ . The maximal rates for  $\text{CO}_2$  fixation were 1.6 (Christeller, 1981), 1.7 (Badger & Lorimer, 1976), 2.3 (Badger, 1980), and 3.4 (Christeller, 1981)  $\mu\text{mol min}^{-1}$  (mg of enzyme) $^{-1}$ , respectively.

<sup>b</sup> The maximal rate obtained with saturating  $\text{O}_2$  and pyruvate was 0.26  $\mu\text{mol min}^{-1}$  (mg of enzyme) $^{-1}$ . The Michaelis constants for  $\text{O}_2$ , acetolactate, pyruvate, and  $\alpha$ -ketobutyrate were 0.2, 1.2, <0.01, and 0.03 mM, respectively. The maximal rates of  $\alpha$ -acetolactate formation from pyruvate,  $\alpha$ -propio- $\alpha$ -hydroxybutyrate formation from  $\alpha$ -ketobutyrate, or pyruvate formation from  $\alpha$ -acetolactate and  $\text{CO}_2$  were 26, 1.7, and 0.010  $\mu\text{mol min}^{-1}$  (mg of enzyme) $^{-1}$ , respectively. The Michaelis constant for  $\text{CO}_2$  in the reverse reaction was 6 mM (0.4 M  $\text{NaHCO}_3$ ).

glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, and glycerolphosphate dehydrogenase. As a control, non-base-treated samples were also assayed enzymatically and were found to contain 3-PGA. The amount of hydroxy-pyruvaldehyde phosphate formed during the reaction was found to be the same whether the assay was done with glyoxylase I or with the coupled assay. Control experiments showed that the glyceraldehyde 3-phosphate formed during the aldolase reaction did not survive the work-up.

## RESULTS AND DISCUSSION

Oxygenase activity was observed for acetolactate synthase (ALS II), pyruvate decarboxylase, glutamate decarboxylase, and class II ( $\text{Zn}^{2+}$ -dependent) fructosebiphosphate aldolase. These results are summarized in Table I. Enzymes surveyed that did not support detectable oxygen consumption (<0.001  $\mu\text{mol min}^{-1}$  mg $^{-1}$ ) included class I (Schiff base dependent) fructosebiphosphate aldolase, triosephosphate isomerase, [(phosphoribosyl)amino]imidazole carboxylase, and a couple of nucleotide-dependent oxidative decarboxylases such as 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase ( $\text{NADP}^+$  dependent). All of the enzymes surveyed, with the exception of triosephosphate isomerase, not only are thought to catalyze reactions utilizing carbanionic intermediates but also contain an access channel to the carbanion for a small gaseous substrate ( $\text{CO}_2$ ) or for a much larger electrophile, such as various aldehydes in the case of aldolase (Rutter et al., 1966).

ALS II is the first common enzyme in branched-chain amino acid biosynthesis and requires thiamin pyrophosphate (TPP),  $\text{Mg}^{2+}$ , and flavin adenine dinucleotide (FAD) for catalysis. Under aerobic conditions with acetolactate as the substrate and in the absence of added bicarbonate, the products

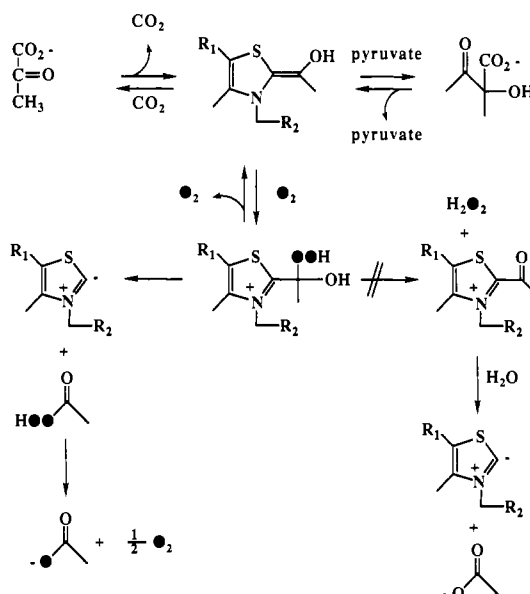


FIGURE 1: Products and isotopic labeling of the oxygenase reaction of acetolactate synthase.

are pyruvate and acetate as confirmed by mass spectral analysis. The maximal rate for this reaction is 0.26  $\mu\text{mol min}^{-1}$  mg $^{-1}$ . The rate of pyruvate production under anaerobic conditions in the presence of bicarbonate is 0.01  $\mu\text{mol min}^{-1}$  mg $^{-1}$ , making this enzyme a better oxygenase than a carboxylase in the reverse physiological direction.

The oxygenase activity of ALS II was examined with  $^{18}\text{O}_2$  and [2- $^{13}\text{C}$ ]pyruvate as substrates. Incorporation of  $^{18}\text{O}$  into the product acetate was observed by an upfield  $^{18}\text{O}$  shift of 0.027 ppm on the  $^{13}\text{C}$  nuclear magnetic resonance signal for the carboxylate of acetate (Risley & Van Etten, 1981). Similar reactions conducted in the presence of  $^{16}\text{O}_2$  and 20–50% mixtures of  $\text{H}_2^{18}\text{O}$  and  $\text{H}_2^{16}\text{O}$  produced no  $^{18}\text{O}$  labeling of acetate, although  $^{18}\text{O}$  incorporation into the carbonyl group of acetolactate produced from the normal condensation reaction was observed as evidenced by an upfield shift of 0.052 ppm (Risley & Van Etten, 1981). This labeling pattern is consistent with the mechanism shown in Figure 1, where the initial (hydroxyethyl)thiamin pyrophosphate hydroperoxide intermediate decomposes to peracetic acid and TPP. Peracetic acid is known to decompose to acetate and  $\text{O}_2$  by nonhydrolytic mechanisms (Swern, 1970). The presence of catalase in the reaction mixtures did not affect the rate of oxygen consumption, which is consistent with the absence of substantial peroxide production and the mechanism proposed in Figure 1.

Substitution of FAD with 5-deaza-FAD had no effect on the observed oxygenase activity, despite the known inability of 5-deaza-FAD to carry out one-electron chemistry or activate other flavin-dependent oxidases (Hersh & Walsh, 1980). Treatment of ALS II and reaction mixtures with the chelating resin Chelex (Bio-Rad) had no effect on the observed level of oxygenase activity. Similarly, the presence of superoxide dismutase in the reaction mixtures did not affect the rate of oxygen consumption. These results indicate that the oxygenase reaction does not depend on the redox properties of FAD or adventitious metal ions for oxygen activation.

Pyruvate decarboxylase utilizes the same (hydroxyethyl)-thiamin pyrophosphate intermediate as acetolactate synthase but lacks a flavin cofactor. Both enzymes have comparable levels of oxygenase activity when  $\alpha$ -ketobutyrate is used as substrate (Table I). The oxygenase activity of pyruvate decarboxylase with pyruvate as the substrate is 1000-fold greater

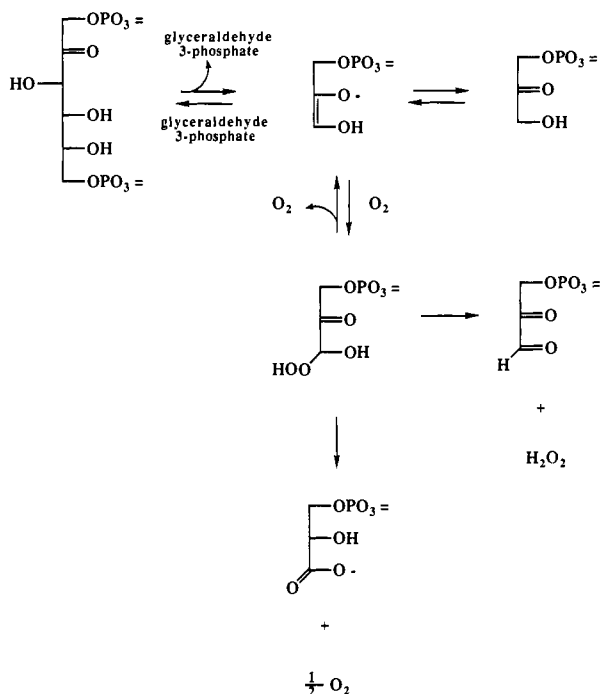


FIGURE 2: Products of the oxygenase reaction of aldolase.

than that recently reported for a flavin-modified form of the enzyme (Annan & Jordan, 1990). These results further support the conclusion that flavin activation of oxygen is not required for the observed oxygenase activity of acetolactate synthase.

Glutamate decarboxylase, which is a pyridoxal 5'-phosphate dependent enzyme, also has an oxygenase side reaction (Table I). Unlike ALS II, however, both pyruvate decarboxylase and glutamate decarboxylase did not exhibit an oxygenase activity in the presence of products. In order for glutamate decarboxylase and pyruvate decarboxylase to exhibit an oxygenase activity with their respective products,  $\gamma$ -amino-butyrate and acetaldehyde, a proton must be abstracted from the adduct formed between cofactor and product. Absence of an enzymic base in the appropriate position to abstract a proton would preclude formation of the carbanionic intermediate, making these reactions effectively irreversible, and thus minimize the consequences of the oxygenase reactions.

Possible products of the oxygenase reaction of class II aldolase are illustrated in Figure 2. Analysis of reaction mixtures, with known extents of oxygen consumption, gave 0.25 equiv of hydroxypyruvaldehyde phosphate (the same product obtained with other oxidants) (Healy & Christen, 1972) and 0.25 equiv of 3-phosphoglycerate. The enzyme underwent inactivation during the course of the reaction with a half-time of 17 min. Substrate-dependent inactivation of aldolase was previously observed during trapping experiments using external oxidants such as hexacyanoferrate (Christen, 1977).

For those enzymes that do not catalyze significant oxygen-consuming side reactions, the results can only be partly rationalized in terms of accessibility of the carbanionic reaction intermediate. Clearly, the carbanion of triosephosphate isomerase need not be accessible to molecular oxygen, and this enzyme was considered unlikely to have an oxygenase side reaction. For the oxidative decarboxylases, rate-limiting release of nucleotide (Dalziel, 1975) would lower the effective concentration of enzyme-bound carbanion in the steady-state distribution of enzyme forms. However, the carbanion of 6-phosphogluconate dehydrogenase can react with tetra-

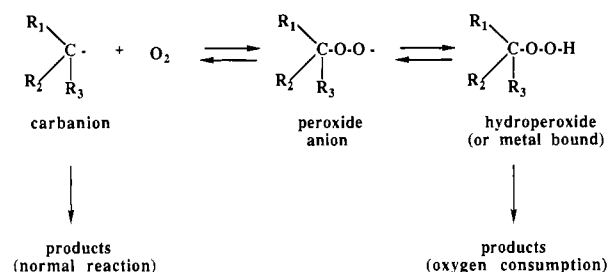


FIGURE 3: A minimal mechanism for an oxygenase reaction.

nitromethane despite its apparent inability to do so with  $O_2$ . Similarly, the carbanionic intermediate of class I aldolase can react with a variety of aldehydes (Rutter et al, 1966) as well as external oxidants (Healy & Christen, 1972), but no reaction was observed with molecular oxygen. [(Phosphoribosyl)-amino]imidazole carboxylase fixes  $CO_2$ , as does ribulosebiphosphate carboxylase, although only the latter enzyme has an oxygenase activity. Further, the substrate [(phosphoribosyl)amino]imidazole consumes oxygen in the absence of the enzyme at a rate of  $0.7 \mu M/min$  with  $8.8 mM$  [(phosphoribosyl)amino]imidazole.  $CO_2$  suppresses the rate of this reaction, indicating the ability of the substrate to react with either  $CO_2$  or  $O_2$  in the absence of enzyme. This enzyme is successful, therefore, in differentially accelerating the rate of substrate carboxylation without enhancing the rate of the oxygenase reaction. These results suggest that some subtlety of enzymic reactions other than accessibility must limit the reaction with oxygen. Although  $O_2$ -consuming reactions are more general than has been previously appreciated, they are not necessarily an inevitable consequence of carbanion chemistry. The lifetime and accessibility of the carbanion is important, but so must also be stabilization of the initially formed peroxide anion.

Although a carboxylation reaction need not have an associated oxygenase activity, as evidenced by its absence in [(phosphoribosyl)amino]imidazole carboxylase, modulation of the polarity of the access route to the carbanion might be expected to alter the specificity for  $CO_2$  and  $O_2$  (Schloss, 1990). A more polar gas "pore" or access channel would be expected to favor  $CO_2$  over  $O_2$ . Acetolactate synthase supports both a carboxylase (reverse direction of its physiological reaction) and an oxygenase reaction. The specificity factor, which is the ratio of the rate constants for reaction of two gaseous substrates, ( $V_c K_o / V_o K_c$ ), for acetolactate synthase, is approximately 0.001 compared with a typical value of 80 (Jordan & Ogren, 1981) for ribulosebiphosphate carboxylase. The active site of acetolactate synthase should be rather hydrophobic to facilitate decarboxylation of its (lactyl)thiamin pyrophosphate reaction intermediate (Kluger & Brandl, 1986). Consistent with this expectation, acetolactate synthase is a much better oxygenase than a carboxylase.

The carbanionic intermediates of both class I and class II aldolases are susceptible to oxidation by external oxidants such as hexacyanoferrate(III) (Christen, 1977), but only class II aldolase has an oxygenase activity. As illustrated in Figure 3, in the absence of stabilization, the initially formed peroxide anion could regenerate oxygen and the carbanion. Stabilization of the peroxide anion could include its protonation and/or metal coordination. Elimination of hydrogen peroxide would be unlikely to occur if doubly ionized peroxide were the product. Similarly, cleavage of the oxygen-oxygen bond, as occurs for the oxygenase reaction of ribulosebiphosphate carboxylase (Lorimer & Andrews, 1973), would be unlikely to proceed if  $O^{2-}$  were produced. As the peroxide anion can

be spatially distant from the initial position of the carbanion, it could extend to accessible enzymic groups, solvent protons, or metal coordination sites inaccessible to the carbanion. It is perhaps relevant that only the metal- ( $\text{Zn}^{2+}$ -) dependent form of aldolase has oxygenase activity and the metal-independent carboxylase [(phosphoribosyl)amino]imidazole carboxylase is devoid of oxygenase activity. If the metal participates in peroxide anion stabilization and product formation, then substitution of different metals might be expected to alter the specificity for  $\text{CO}_2$  and  $\text{O}_2$ . Substitution of  $\text{Mg}^{2+}$  with different metals alters the specificity of ribulosebisphosphate carboxylase with respect to gaseous substrates; the *Rhodospirillum rubrum* enzyme is exclusively an oxygenase in the presence of  $\text{Co}^{2+}$  (Christeller, 1981).

In principle, it should be possible to eliminate the oxygenase activity of ribulosebisphosphate carboxylase on the basis of the absence of such activities in other enzyme-mediated carbanion reactions. In practice, however, the required structural changes may not be accessible by mutagenesis if they must serve to alter metal coordination chemistry.

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