The Oxygenase Reaction of Acetolactate Synthase

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Received May 14, 1993; Revised Manuscript Received July 20, 1993®

ABSTRACT: In addition to the physiological reactions catalyzed by acetolactate synthase, it supports an oxygen-consuming side reaction. Although the synthase and oxygenase activities are activated to somewhat different extents by various metals (Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, Ba^{2+}, Al^{3+}), the modest degree of these differences (at most 6-fold) and the high degree of promiscuity of the enzyme with respect to its metal requirement suggest that the metal is not intimately involved in the chemistry of either reaction. Saturation of the oxygenase reaction occurs at pyruvate concentrations below the limit of sensitivity for the oxygen electrode (<10 μM), at higher concentrations pyruvate inhibits the rate of oxygen consumption. At a noninhibitory concentration of pyruvate (1 mM), inhibition of the reaction is also observed with α-ketobutyrate. Inhibition of the oxygenase reaction by high concentrations of pyruvate or α-ketobutyrate is presumably due to competition between these substrates and molecular oxygen for a common carbanionic reaction intermediate, the conjugate base of (hydroxyethyl)thiamin pyrophosphate. Inhibition of the reaction indicates that the lactylthiamin pyrophosphate intermediate can decarboxylate prior to binding of the second pyruvate or α-ketobutyrate. At high concentrations of pyruvate or α-ketobutyrate, only incomplete inhibition of the oxygenase reaction is achieved (65–89 % or 89–93 % maximal inhibition, respectively). This incomplete inhibition of the oxygenase reaction by α-keto acids indicates that the reaction is not Theorell–Chance with respect to addition of the second α-keto acid and that oxygen has more than one route of access to the carbanionic reaction intermediate.

Acetolactate synthase (EC 4.1.3.18, also commonly referred to as acetohydroxy acid synthase) is the first common enzyme in the biosynthetic pathway for branched-chain amino acids. Considerable interest in this enzyme has resulted from the discovery that it is the site of action of several different classes of commercial herbicides (Schloss et al., 1988). The physiological reactions catalyzed by this enzyme are the condensation of two molecules of pyruvate to form (*S*)-acetolactate and carbon dioxide (Crout, 1990) or the condensation of one molecule of pyruvate and one of α-ketobutyrate to form α-aceto-α-hydroxybutyrate (Figure 1). Partitioning between these two physiological reactions determines the ratio of precursors for valine (and also leucine) or isoleucine biosynthesis (Barak et al., 1990). The partitioning ratio is a constant, although it varies from one form of the enzyme to another, ranging from 2 to 65 for various isozymes of the enzyme from enteric bacteria (Chipman et al., 1990). Although the kinetics associated with this branch point have been elegantly elucidated, several details of the kinetic mechanism remain in question. In particular, whether decarboxylation of the first pyruvate can occur prior to binding of a second molecule of pyruvate or α-ketobutyrate has yet to be determined. Since the enzyme must protect the product of decarboxylation, the conjugate base of (hydroxyethyl)thiamin pyrophosphate, from protonation by solvent, there would seem to be some advantage to binding both α-keto acid substrates before product (CO_2) is released.

Recently it was discovered that acetolactate synthase supports an oxygen-consuming side reaction with either pyruvate, α-ketobutyrate, or acetolactate as substrate (Abell & Schloss, 1991). This reaction is most likely a consequence of the reaction of molecular oxygen with the conjugate base of (hydroxyethyl)thiamin pyrophosphate, the same intermediate that reacts with pyruvate or α-ketobutyrate in the step that determines the partitioning ratio of the enzyme (Chipman et al., 1990). The oxygenase reaction provides a convenient monitor of this carbanionic intermediate of the reaction. Use of the oxygenase reaction to determine the kinetic mechanism of ALSII,[1] its dependence on substrate concentration and various metals, is the subject of the present communication.

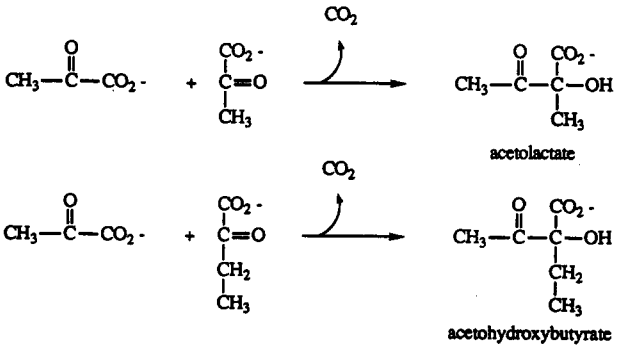

FIGURE 1: Physiological reactions catalyzed by acetolactate synthase.

MATERIALS AND METHODS

Materials. Racemic acetolactate was prepared from ethyl 2-ethoxy-2-methylacetoacetate (Aldrich Chemical Co.) by addition of 1.9 equiv of 10 N NaOH to an aqueous suspension of the diester with vigorous mixing at room temperature. Analysis of a 0.5 M stock solution of racemic acetolactate [0.25 M (*S*)-acetolactate] prepared in this fashion by use of ketol-acid reductoisomerase and NADPH (Aulabaugh & Schloss, 1990) indicated quantitative conversion. Sodium pyruvate, sodium α-ketoglutarate, thiamin pyrophosphate,

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® Abstract published in *Advance ACS Abstracts,* September 15, 1993.

[1] Abbreviations: Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; ALSII, acetolactate synthase isozyme II from *Salmonella typhimurium.*

0006-2960/93/0432-10398$04.00/0

flavin adenine dinucleotide, Tricine, creatine, α-naphthol, β-NADH, β-NADPH, and lactate dehydrogenase were obtained from Sigma Chemical Co. All inorganic chemicals used were of reagent grade. Acetolactate synthase isozyme I from *Escherichia coli*, ALSII, and ketol-acid reductoisomerase were prepared by published procedures (Aulabaugh & Schloss, 1990; Schloss et al., 1985).

Assay of Synthase Activity. At high concentrations of pyruvate, where the rate of pyruvate consumption is predominantly due to the conversion of pyruvate to acetolactate (>99%), the rate of the synthase reaction could be determined by the loss of absorbance at 333 nm (ϵ = 17.5 M^{-1} cm^{-1}; Schloss et al., 1985). These assays were carried out by addition of 10 μL of 24 mg/mL ALSII to 1 mL of 0.1 M Tricine–0.05 N NaOH (pH 8), 1 mM metal, 0.1 mM flavin adenine dinucleotide, 0.1 mM thiamin pyrophosphate, and 50 mM sodium pyruvate at 25 °C.

At lower pyruvate concentrations, due to the lack of sensitivity of the continuous assay and the increasing fraction of pyruvate consumption due to the oxygenase reaction, a modification of the fixed-time colorimetric assay of Westerfeld was employed (Westerfeld, 1945). After incubation of a 0.45-mL solution of 0.1 M Tricine–0.05 N NaOH (pH 8), 0.1 mM thiamin pyrophosphate, 0.1 mM flavin adenine dinucleotide, 1 mM metal, and 5.1 μg of ALSII in a polyethylene tube for 5 min at 25 °C, 0.05 mL of a pyruvate solution was added to initiate the enzymic reaction. After an additional 5-min reaction time, 0.1 mL of 2 N HCl was added to stop the enzymic reaction. Samples were placed in a microwave oven (Litton Generation II) and irradiated at the highest setting for 15 s. In rapid succession, with mixing, were added 0.1 mL of 2 N NaOH, 0.15 mL of 0.5% creatine, and 0.15 mL of 5% α-naphthol in 2.5 N NaOH. The samples were irradiated in a microwave oven for an additional 15 s, cooled in a 25 °C water bath, and centrifuged in a microcentrifuge (Hermle Z229) for 5 min. Acetolactate formation was determined by measuring the absorbance of the supernatant at 530 nm. An absorbance of 0.72 ± 0.05 is obtained for each 0.1 μmol of acetolactate (or acetoin) produced in this assay. With the exception of Co^{2+}, none of the metals examined had any effect on the color yield obtained from acetolactate in this assay. In the presence of 1 mM Co^{2+}, the absorbance obtained from acetolactate is reduced to 44% of that observed in its absence.

Assay of Oxygenase Activity. Oxygen consumption was monitored by use of an oxygen electrode (Hansatech). To 0.95 mL of a solution containing 0.1 mmol of Tricine–0.05 mequiv of NaOH (pH 8), 1 μmol of metal, 0.1 μmol of flavin adenine dinucleotide, 0.1 μmol of thiamin pyrophosphate, 0.23 μmol of O_2 (air equilibrated; Schloss, 1990), and 0.24 mg of ALSII at 25 °C was added 0.05 mL of a solution that contained various concentrations of pyruvate, acetolactate, or mixtures of pyruvate and α-ketobutyrate.

When acetolactate was used as substrate, the oxygenase reaction could also be followed by a coupled spectrophotometric assay. This assay relies on the much greater rate of the oxygenase reaction than the carboxylase reaction (reverse of the synthase reaction) under aerobic conditions, especially in the absence of added bicarbonate (Abell & Schloss, 1991). Similar assay conditions were employed, except for the inclusion of 25 μg/mL lactate dehydrogenase and 0.1 mM NADH and the use of 0.11 mg/mL ALSII. Assays were initiated by the addition of 10 μL of a solution that contained various concentrations of acetolactate and monitored at 340 nm.

Table I: Comparison of Synthase and Oxygenase Specific Activities Obtained with Various Metals

metal	synthase[a] (μmol/min/mg)	%	oxygenase[b] (μmol/min/mg)	%	oxygenase/synthase (×100)
Mn^{2+}	12.9	100	0.22	100	1.7
Mg^{2+}	9.7	75	0.17	77	1.7
Ca^{2+}	7.3	56	0.13	59	1.8
Co^{2+}	6.7	52	0.052	24	0.78
Zn^{2+}	6.5	50	0.026	12	0.40
Ni^{2+}	4.8	37	0.032	14	0.67
Cd^{2+}	6.9	53	0.031	14	0.45
Cu^{2+}	6.4	50	0.026	12	0.41
Ba^{2+}	5.2	40	0.026	12	0.50
Al^{3+}	5.8	45	0.016	7.2	0.28

[a] Rate obtained at 50 mM pyruvate in the continuous assay of pyruvate consumption at 333 nm. [b] Rate of oxygen consumption obtained at 1 mM pyruvate by use of the oxygen electrode.

Data Analyses. The following equations were fit to data by use of the program GraFit (Leatherbarrow, 1992):

$$v = \frac{V_{max}S}{K_m + S} \quad (1)$$

$$v = V_1 - \frac{(V_1 - V_2)S}{K_i + S} \quad (2)$$

where v is the rate of the enzymic reaction, V_{max} is the maximum rate obtained at high substrate, K_m is the Michaelis constant, S is substrate concentration, V_1 is the rate obtained at low concentrations of substrate, V_2 is the rate obtained at high concentrations of substrate, and K_i is the concentration of substrate that gives half-maximal substrate inhibition.

RESULTS

Even after preincubation of ALSII with flavin adenine dinucleotide, thiamin pyrophosphate, and metal, assay time courses of the synthase reaction exhibit a lag at high pyruvate concentrations. This increase in rate over a 5–10-min period is likely due to a change in the enzyme's oligomeric state (conversion of a substantial fraction of the dimeric $\alpha_2\beta_2$ structure into a tetrameric $\alpha_4\beta_4$ one) induced by pyruvate (Grimminger & Umbarger, 1979; D. E. Van Dyk and J. V. Schloss, unpublished observation). This inherent nonlinearity of the assay at high concentrations of pyruvate complicates comparison of kinetic behavior at different pyruvate concentrations. Further, some of the metals, most notably Ba^{2+}, have exceedingly slow rates of activation of ALSII that are affected by the concentration of pyruvate used in the assay. Many of the metals examined (e.g., Cu^{2+}, Co^{2+}, and Cd^{2+}) will irreversibly inactivate ALSII upon extended incubation. At high concentrations of pyruvate, the time course of oxygen consumption will dramatically change at the point where most of the pyruvate has been converted to acetolactate, relieving the inhibition of the oxygenase reaction by pyruvate.

Despite the difficulties in reliably determining a representative steady-state rate under conditions that favor the synthase (50 mM pyruvate) or oxygenase (1 mM pyruvate) activities, comparison of the relative activities listed in Table I reveals that a wide variety of metals will satisfy either reaction. There is surprisingly little difference in the relative levels of activity for either synthase or oxygenase reactions obtained with various metals. Essentially equivalent relative rates are obtained for the two activities with Mn^{2+}, Mg^{2+}, and Ca^{2+}, while the level of oxygenase activity is 17–46% of what would be expected if it were to be activated to the same extent as the synthase

Table II: Kinetic Constants for Synthase Activity Obtained with Various Metals

metal	V_{max} (units/mg)	K_m (mM)
Mn^{2+}	7.1 ± 0.4	1.3 ± 0.3
Mg^{2+}	5.6 ± 0.1	2.2 ± 0.2
Ca^{2+}	3.0 ± 0.1	3.4 ± 0.5
Co^{2+}	1.6 ± 0.1	9 ± 3
Zn^{2+}	1.2 ± 0.1	8 ± 3
Ni^{2+}	1.9 ± 0.3	15 ± 7
Cd^{2+}	2.6 ± 0.2	12 ± 3
Cu^{2+}	0.44 ± 0.05	5 ± 2
Ba^{2+}	0.56 ± 0.03	0.3 ± 0.1
Al^{3+}	1.9 ± 0.4	24 ± 12

Table III: Kinetic Constants for Oxygenase Activity with Various Metals and Pyruvate as Substrate

metal	V_1 (units/mg)	V_2 (units/mg)	K_i (mM)	V_2/V_1 (×100)
Mn^{2+}	0.27 ± 0.01	0.030 ± 0.002	2.2 ± 0.7	11
Mg^{2+}	0.18 ± 0.01	0.045 ± 0.001	3.6 ± 0.9	25
Ca^{2+}	0.123 ± 0.002	0.025 ± 0.001	6 ± 1	20
Co^{2+}	0.022 ± 0.001	0.0046 ± 0.0006	11 ± 7	21
Zn^{2+}	0.022 ± 0.001	0.0078 ± 0.0004	5 ± 2	35
Ni^{2+}	0.019 ± 0.001	0.0043 ± 0.0004	11 ± 4	23

activity with Co^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, Ba^{2+}, and Al^{3+}. The lowest extent of activation for the synthase reaction is 37% for Ni^{2+}, while the least activity in the oxygenase reaction is obtained for Al^{3+}, which gives 7.2% of the activity seen with Mn^{2+}. Although not examined in detail, isozyme I of acetolactate synthase has an oxygenase activity comparable to that seen for ALSII. Isozyme I has the following levels of oxygenase activity in the presence of 1 mM Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+} (0.1 mM; higher concentrations inhibit the enzyme), Ba^{2+}, or Al^{3+} at 1 mM sodium pyruvate: 0.17, 0.18, 0.13, 0.0073, 0.0029, 0.011, 0.0036, 0.006, 0.0082, and 0.0021 μmol/min/mg, respectively. At 50 mM sodium pyruvate, isozyme I has the following levels of synthase activity for these metals: 6.5, 9.2, 6.1, 4.7, 3.2, 8.5, 2.2, 3.2, 5.2, and 4.8 μmol/min/mg, respectively.

Simple hyperbolic saturation of the synthase reaction by pyruvate is observed for ALSII activated by each of the metals listed in Table II. At low pyruvate concentrations the rate is directly proportional to the concentration of pyruvate (rather than sigmoidal, i.e., second order in pyruvate), indicating that addition of the first pyruvate is highly committed. The maximum rates obtained at saturating pyruvate for the fixed-time assay (Table II) are about half of the values obtained with the continuous assay (Table I). This is presumably due to the nonlinearity in the assay noted earlier. The maximum rates obtained when Co^{2+}, Zn^{2+}, Cu^{2+}, and Ba^{2+} are used to activate ALSII are substantially lower than those obtained by use of the continuous assay (7–24%) and are primarily due to loss of enzymic activity during the preincubation. The kinetic parameters calculated from the data obtained by use of the fixed-time assay are summarized in Table II.

Incomplete inhibition of the oxygenase activity by high concentrations of pyruvate is observed for all of the metals listed in Table I. The kinetic constants that define this substrate inhibition were determined for a number of these metals, and those results are listed in Table III. Within experimental error, the inhibition constants for pyruvate in the oxygenase assay (Table III) are equivalent to the Michaelis constants obtained for saturation of the synthase reaction (Table II). The maximum extent of inhibition of the oxygenase reaction at high pyruvate ranges from 65% for Zn^{2+}-activated ALSII to 89% for Mn^{2+}-activated ALSII. At the lowest

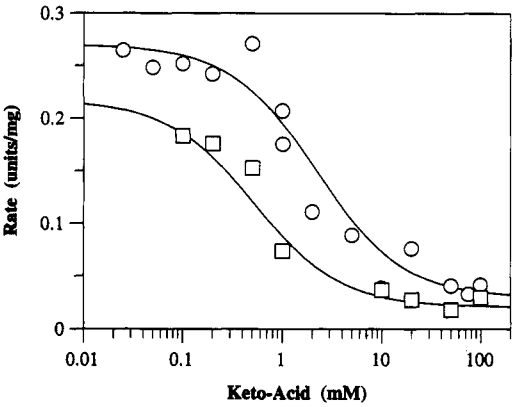


FIGURE 2: Dependence of the oxygenase rate for Mn^{2+}-activated ALSII on pyruvate concentration (○) or on ketobutyrate concentration in the presence of 1 mM pyruvate (□).

Table IV: Kinetic Constants for Oxygenase Activity with Various Metals and a Mixture of Pyruvate and Ketobutyrate[a]

metal	V_1 (units/mg)	V_2 (units/mg)	K_i (mM)	V_2/V_1 (×100)
Mn^{2+}	0.22 ± 0.03	0.021 ± 0.003	0.5 ± 0.2	9.5
Mg^{2+}	0.18 ± 0.04	0.014 ± 0.003	0.4 ± 0.2	7.8
Ca^{2+}	0.12 ± 0.05	0.010 ± 0.002	0.3 ± 0.3	8.3
Co^{2+}	0.052 ± 0.008	0.0048 ± 0.0007	0.3 ± 0.1	9.3
Zn^{2+}	0.06 ± 0.03	0.004 ± 0.001	0.2 ± 0.1	7
Ni^{2+}	0.018 ± 0.002	0.0019 ± 0.0003	1.1 ± 0.4	11

[a] Pyruvate was maintained at 1 mM, and α-ketobutyrate was varied from 0.1 to 100 mM.

concentration of pyruvate that allows assays to be carried out by use of the oxygen electrode (10 μM), the rate of oxygen consumption is saturated with respect to this substrate. Figure 2 illustrates the effect (inhibition) of pyruvate concentration on the rate of oxygen consumption for Mn^{2+}-activated ALSII.

In the presence of a mixture of pyruvate and α-ketobutyrate, formation of α-aceto-α-hydroxybutyrate competes with acetolactate formation. Although ALSII can catalyze the condensation of α-ketobutyrate with a second molecule of α-ketobutyrate, in the presence of pyruvate this reaction does not occur at detectable rates (Chipman et al., 1990). Inhibition of the rate of oxygen consumption by α-ketobutyrate in the presence of 1 mM pyruvate was examined for the same range of metals employed in defining the inhibition constants for pyruvate (Table III). Table IV summarizes the kinetic constants for the inhibition of the oxygenase reaction by α-ketobutyrate. Under these conditions, α-ketobutyrate is substantially more effective in capturing the carbanionic reaction intermediate than pyruvate (4–37-fold, $K_{i\ pyruvate}/K_{i\ ketobutyrate}$). The maximum extent of suppression of the oxygenase reaction is also somewhat greater than is observed under conditions of saturating pyruvate (89–93% inhibition). The inhibition of oxygen consumption by α-ketobutyrate for Mn^{2+}-activated ALSII is illustrated in Figure 2.

Unlike pyruvate or α-ketobutyrate, acetolactate does not inhibit the rate of oxygen consumption at high concentration. The lack of inhibition of the reaction by high concentrations of acetolactate is presumably due to the inability of acetolactate to compete with oxygen for the reactive intermediate. Further, much higher concentrations of acetolactate are required to saturate the oxygenase reaction. The inefficiency of the oxygenase reaction with acetolactate as substrate likely reflects the higher energetic barrier to the formation of the carbanionic intermediate with this substrate. Such a difference between pyruvate and acetolactate is to be expected on the basis of the

Table V: Kinetic Constants for Oxygenase Activity with Various Metals and Acetolactate as Substrate

metal	V_{max} (units/mg)	K_m (mM)
Mn^{2+}	0.222 ± 0.006	0.70 ± 0.03
Mg^{2+}	0.135 ± 0.003	0.55 ± 0.02
Ca^{2+}	0.105 ± 0.006	0.56 ± 0.06

overall equilibrium of the synthase reaction ($K_{eq} = 3 \times 10^{-9}$; Schloss, 1992). Simple hyperbolic saturation of the oxygenase reaction is observed for acetolactate. The kinetic constants for the oxygenase reaction with acetolactate as substrate are summarized in Table V. Due to interference of a number of metals with the lactate dehydrogenase couple, reliable data were only obtained for Mn^{2+}, Mg^{2+}, and Ca^{2+}.

DISCUSSION

The likely mechanism of acetolactate synthase is illustrated in Figure 3. Attack of the thiazole of thiamin pyrophosphate on pyruvate would give the lactylthiamin pyrophosphate intermediate. Decarboxylation of the lactylthiamin pyrophosphate intermediate would give the conjugate base of (hydroxyethyl)thiamin pyrophosphate. Although the conjugate base could be stabilized as its ene–amine form, as illustrated in Figure 3, the enzyme would need to prevent protonation of this intermediate by solvent or its tautomerization to the ketone (Kluger, 1987) so that it could react with a second molecule of pyruvate (or α-ketobutyrate) to give an adduct of acetolactate (or acetohydroxybutyrate) and thiamin pyrophosphate, or with molecular oxygen to give a peroxide intermediate. Release of acetolactate or acetohydroxybutyrate would complete the synthase catalytic cycle, while release of peracetate would complete the oxygenase catalytic cycle. Although acetate is the ultimate product of the oxygenase reaction, peracetate is the initial product of the reaction and undergoes nonhydrolytic conversion to acetate and one-half equivalent of molecular oxygen (Abell & Schloss, 1991). Competition between pyruvate and ketobutyrate for the (hydroxyethyl)thiamin pyrophosphate intermediate defines the partitioning ratio for acetolactate synthase with respect to the two physiological reactions (one specific to isoleucine biosynthesis and the other specific to valine or leucine biosynthesis; Figure 1). Competition between molecular oxygen and pyruvate or ketobutyrate for the (hydroxyethyl)-thiamin pyrophosphate intermediate would account for inhibition of the oxygenase reaction by high concentrations of either of these two α-keto acids. Similarly, competition between oxygen and carbon dioxide is expected in the reverse of the synthase reaction. However, the Michaelis constant for carbon dioxide is so high (6 mM, 0.4 M bicarbonate under assay conditions; Abell & Schloss, 1991) that under aerobic conditions ALSII functions almost exclusively as an oxygenase in the reverse direction.

If the metal ion required for acetolactate synthase were to play a role in stabilizing intermediates of either the synthase or the oxygenase reaction, it would be unlikely that the enzyme would use such a wide variety of metals for both reactions. The rather modest differences in the kinetic parameters for the synthase and oxygenase reactions of ALSII activated with various metals suggest that the metal does not play an intimate role in either reaction. In the crystal structures of the thiamin

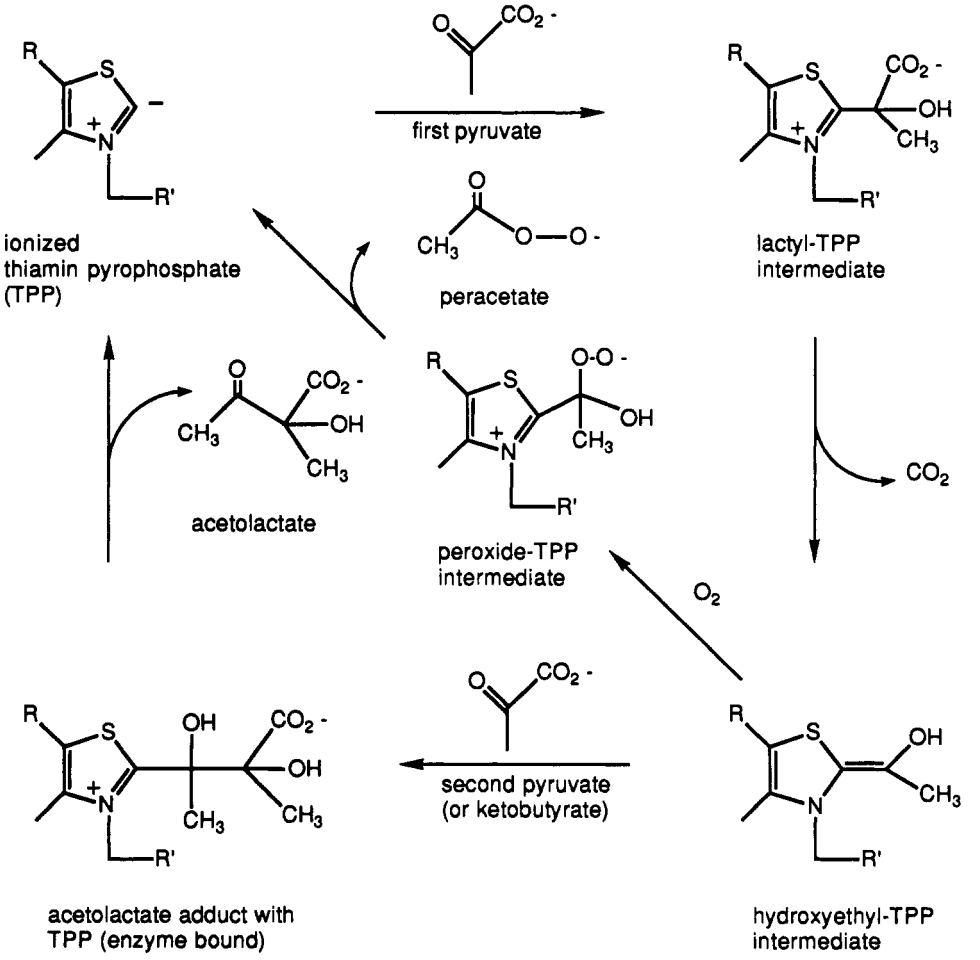
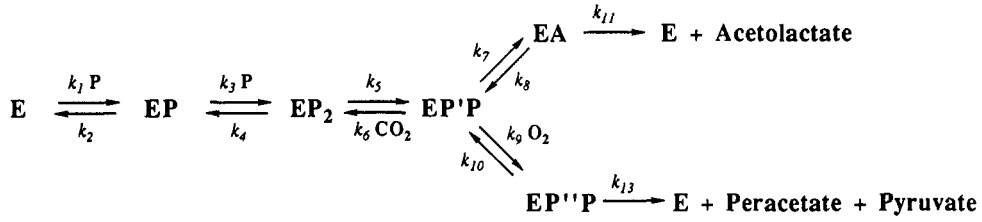
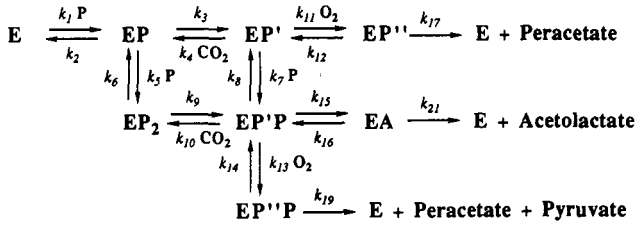


FIGURE 3: Catalytic cycles of the synthase and oxygenase reactions for acetolactate synthase.

Scheme I



Scheme II



pyrophosphate-dependent enzymes transketolase (Lindqvist et al., 1992), pyruvate decarboxylase (Dyda et al., 1993), and pyruvate oxidase (Muller & Schulz, 1993) (pyruvate decarboxylase and pyruvate oxidase are related to ALSII in mechanism and sequence homology), the metal is coordinated to the α and β phosphates of the cofactor, remote from the C-2′ position of the thiazole moiety that adds to pyruvate. If the metal in ALSII is bound in a similar fashion, then it would be unlikely to play any role in the enzymic reaction other than in molecular recognition of the thiamin pyrophosphate cofactor. Thiamin pyrophosphate and various cofactor analogs (tetrahydrothiamin pyrophosphate, thiamin thiazolone pyrophosphate, and (hydroxyethyl)thiamin pyrophosphate) all bind to the enzyme in a metal-dependent fashion (Schloss & Aulabaugh, 1990). By contrast, flavin adenine dinucleotide binds tightly to the enzyme in the absence of metal (Schloss et al., 1985).

The oxygenase reaction of ALSII provides a useful approach to determine the kinetic mechanism of the synthase reaction. Since it is unlikely that molecular oxygen can react with intermediates of the enzymic reaction prior to decarboxylation of lactylthiamin pyrophosphate, the effect of pyruvate and α-ketobutyrate on the rate of the oxygenase reaction determines whether they bind before or in competition with oxygen (concurrently). If binding of the second α-keto acid had to occur before decarboxylation of lactylthiamin pyrophosphate, as illustrated in Scheme I, then the enzyme form that reacts with oxygen would increase at high pyruvate or ketobutyrate. In this kinetic mechanism, the rate of the oxygenase reaction would most likely increase at high pyruvate concentrations, and there should be no inhibition of the rate of oxygen consumption at saturating pyruvate. In Scheme I, E is enzyme, P is pyruvate, P′ is decarboxylated pyruvate [the conjugate base or ene–amine form of (hydroxyethyl)thiamin pyrophosphate], P″ is the peroxide intermediate, A is acetolactate, and EP, EP_2, EP′P, EP″P, and EA are the respective complexes with enzyme. If decarboxylation of lactylthiamin pyrophosphate can occur prior to binding of the second α-keto acid, as illustrated in Scheme II, then the second pyruvate or ketobutyrate will compete for the carbanionic intermediate with molecular oxygen. If the second α-keto acid simply reacts with the carbanionic reaction intermediate, without forming a Michaelis complex, then the rate of this reaction will increase in proportion to the solution concentration of the α-keto acid. At sufficiently high concentrations of α-keto acid in a Theorell–Chance mechanism, the rate of the addition of the α-keto acid would drive the rate of oxygen consumption to zero. Since there is a residual rate of oxygen consumption at high concentrations of pyruvate (11–35%) or ketobutyrate (7–11%), the second step of the synthase reaction cannot be Theorell–Chance and oxygen can still react with the carbanion in the Michaelis complex that contains the second α-keto acid (as illustrated in Scheme II). Since oxygen can still react with the carbanion in the complex with the second pyruvate bound, it must have a different orientation with respect to the carbanion than the second pyruvate. Either this is the primary route of access of oxygen to the carbanion or oxygen may have more than one route of access to the (hydroxyethyl)-thiamin pyrophosphate intermediate. In Scheme II, E, P, P′, P″, EP, EP_2, EP′P, EP″P, and EA have the same definitions as in Scheme I. Scheme II differs from Scheme I only by the inclusion of EP′ and EP″, the complexes of enzyme with the conjugate base or ene–amine form of (hydroxyethyl)thiamin pyrophosphate and the peroxide intermediate, respectively. At sufficiently low pyruvate concentrations (well below the Michaelis constant for pyruvate in the synthase reaction), or in the presence of acetolactate, acetolactate synthase functions predominantly as an oxygenase. This kinetic scheme would predict that the Michaelis constant for the synthase reaction would approximate the K_i for pyruvate observed for substrate inhibition of the oxygenase reaction. The K_m for the synthase reaction is nearly equivalent to the K_i for the oxygenase reaction with ALSII activated by six different metals (Tables II and III).

ALSII is one of several enzymes that have oxygen-consuming side reactions (Abell & Schloss, 1991). Several other such enzymes include ribulosebisphosphate carboxylase (Rubisco), fructose 1,6-bisphosphate aldolase from *Staphylococcus aureus*, glutamate decarboxylase from *E. coli*, and pyruvate decarboxylase from yeast. All of these enzymes catalyze reactions that are thought to involve carbanionic intermediates. Several different cofactors are involved in stabilizing these intermediates. ALSII and pyruvate decarboxylase are both thiamin pyrophosphate-dependent enzymes. Glutamate decarboxylase is a pyridoxal phosphate-dependent enzyme. Aldolase involves a Schiff's base mechanism (Goetz et al., 1980). Ribulosebisphosphate carboxylase is a metal-dependent enzyme, where the metal probably stabilizes the carbanionic intermediate that reacts with oxygen (Schloss, 1990; Schneider et al., 1990), unlike the role for metal in ALSII. In contrast to ALSII, the ribulosebisphosphate carboxylase from *Rhodospirillum rubrum* is an exclusive oxygenase when activated by Co^{2+}, and the relative levels of carboxylase and oxygenase activities are extremely sensitive to the metal used to activate the enzyme (Christeller, 1981). ALSII, pyruvate decarboxylase, and glutamate decarboxylase favor reaction with O_2 over CO_2, whereas the reaction of ribulosebisphosphate carboxylase favors CO_2 over O_2 when activated with Mg^{2+}. Decarboxylation is the favored direction for the physiological reactions of the former three enzymes, while the carboxylation reaction of the latter enzyme is

irreversible. A better understanding of those factors that contribute to the discrimination between CO_2 and O_2 in these analogous oxygenase reactions could lead to more rational strategies for the manipulation of this ratio in photosynthesis. The oxygenase activity of ribulosebisphosphate carboxylase accounts for oxygen inhibition of photosynthesis and reduces the net photosynthetic efficiency in plants (Hartman & Harpel, 1993).

These oxygenase reactions are also potential sources of reactive oxygen species that are presently not widely recognized. Although the oxygenase reaction of ribulosebisphosphate carboxylase is thought to involve efficient cleavage of the oxygen–oxygen bond of the peroxide intermediate to eliminate any reactive species (Lorimer et al., 1973), ALSII and glutamate decarboxylase give peracetate and hydrogen peroxide as products, respectively (Abell & Schloss, 1991; unpublished observation). When activated by Mn^{2+}, the oxygenase reaction of ribulosebisphosphate carboxylase has an associated chemiluminescence that has been attributed to the formation of singlet oxygen (Mogel & McFadden, 1990). The oxygenase reaction of ALSII has a level of an associated chemiluminescence comparable to that reported for ribulosebisphosphate carboxylase (J. V. Schloss, unpublished observation). The reaction of oxygen with these and other carbanion-forming enzymes bears further scrutiny to assess the physiological relevance of these interactions, if any, and the molecular details of those factors that limit the reaction of various types of carbanionic intermediates with molecular oxygen.

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