Reaction of Serine-Glyoxylate Aminotransferase with the Alternative Substrate Ketomalonate Indicates Rate-Limiting Protonation of a Quinonoid Intermediate†

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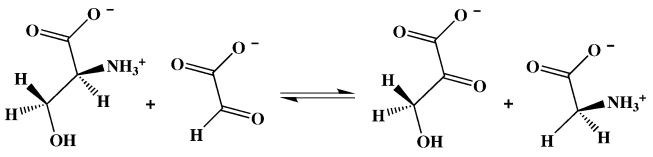
ABSTRACT: Serine-glyoxylate aminotransferase (SGAT) from *Hyphomicrobium methylovorum* is a pyridoxal 5′-phosphate (PLP) enzyme that catalyzes the interconversion of L-serine and glyoxylate to hydroxypyruvate and glycine. The primary deuterium isotope effect using L-serine 2-D is one on $(V/K)_{serine}$ and V in the steady state. Pre-steady-state experiments also indicate that there is no primary deuterium isotope effect with L-serine 2-D. The results suggest there is no rate limitation by abstraction of the α proton of L-serine in the SGAT reaction. In the steady-state a solvent deuterium isotope effect of about 2 was measured on $(V/K)_{L-serine}$ and $(V/K)_{ketomalonate}$ and about 5.5 on V. Similar solvent isotope effects were observed in the pre-steady-state for the natural substrates and the alternative substrate ketomalonate. In the pre-steady-state, no reaction intermediates typical of PLP enzymes were observed with the substrates L-serine, glyoxylate, and hydroxypyruvate. The data suggest that breakdown and formation of the ketimine intermediate is the primary rate-limiting step with the natural substrates. In contrast, using the alternative substrate ketomalonate, pre-steady-state experiments display the transient formation of a 490 nm absorbing species typical of a quinonoid intermediate. The solvent isotope effect results also suggest that with ketomalonate as substrate protonation at C_α is the slowest step in the SGAT reaction. This is the first report of a rate-limiting protonation of a quinonoid at C_α of the external Schiff base in an aminotransferase reaction.

Serine-glyoxylate aminotransferase (SGAT,[1] EC 2.6.1.45) from *Hyphomicrobium methylovorum* GM2 is a pyridoxal 5′-phosphate (PLP) containing enzyme that catalyzes the following interconversion.



H. methylovorum is a methylotrophic bacterium that assimilates C1 compounds via a serine pathway (*1*, *2*). In the serine pathway, glycine is converted to L-serine by the enzyme serine hydroxymethyltransferase with concomitant conversion of $N^{5,10}$-methylene THF to THF. SGAT converts L-serine to hydroxypyruvate, which is then converted to phosphoenolpyruvate by the sequential action of the enzymes hydroxypyruvate reductase, glycerate kinase, and phosphopyruvate hydratase. In *H. methylovorum*, the enzymes of the serine pathway are induced by methanol but decline to a minimum when the cells are transferred to a C2 source (*1*).

SGAT appears to be a homotetramer with a subunit molecular mass of 43 880 Da, and the gene encoding the enzyme has been cloned and sequenced (*3*). On the basis of the initial velocity pattern and dead-end inhibition studies, a ping-pong kinetic mechanism has been proposed for the enzyme (*4*, *5*). The UV−vis spectrum of SGAT reveals an absorbance maximum at 414 nm and a shoulder centered at 330 nm, corresponding respectively to the ketoenamine and enolimine tautomers of the protonated internal aldimine. Incubating the enzyme with L-serine converts the enzyme to the pyridoxamine 5′-phosphate (PMP) form of the cofactor, generating hydroxypyruvate. Enzyme−PMP may be converted back to E−PLP by incubating with hydroxypyruvate to give L-serine or glyoxylate to give glycine. On the basis of pH studies, an acid−base mechanism has been proposed for SGAT (*5*). L-Serine is proposed to bind with its α-amine protonated, and an enzymatic base accepts a proton from the amino group of serine. The resulting unprotonated α-amine nitrogen of serine attacks C4′ of the PLP, leading to a *gem*-diamine intermediate. Collapse of the *gem*-diamine with expulsion of the ϵ-amine of the active-site lysine forms the external aldimine followed by abstraction of the α proton of L-serine by the ϵ-amino group of lysine to form the quinonoid intermediate. Proton transfer from the ϵ-amino group of lysine to C4′ will lead to the

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DGD, dialkylglycine decarboxylase; LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; SGAT, serine-glyoxylate aminotransferase.

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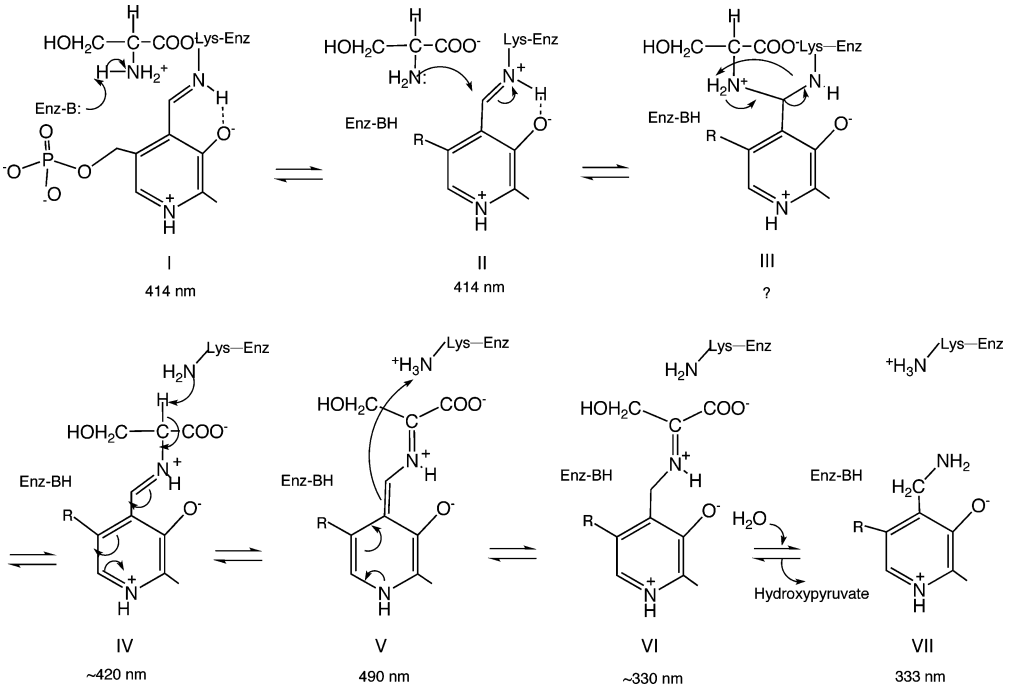
Scheme 1: Proposed Chemical Mechanism for SGAT



ketimine, and finally, hydrolysis of the ketimine forms hydroxypyruvate and the PMP form of SGAT. The proposed acid−base mechanism is shown in Scheme 1. One of the major goals of the current work was to attempt to determine if the quinonoid intermediate could be detected in the SGAT reaction because it has rarely been observed in reactions catalyzed by PLP-dependent enzymes.

The current study reports on the primary and solvent deuterium isotope effects on the SGAT-catalyzed reaction to identify rate-limiting steps in the reaction. We also report pre-steady-state kinetic studies to detect spectral intermediates in the reaction.

MATERIALS AND METHODS

Chemicals and Reagents. Reduced nicotinamide adenine dinucleotide (NADH), L-serine, ketomalonate, hydroxypyruvate, glyoxylate, and lactate dehydrogenase (LDH) were purchased from Sigma Chemical Company and used without further purification. Protein concentrations were determined by the method of Bradford (*6*) with the Bio-Rad protein assay kit and bovine serum albumin as a standard.

Two sources of the *H. methylovorum* SGAT were used. The enzyme was expressed from the SGAT gene housed in the pKK223 plasmid (*3*) or the pQE30 plasmid, which introduces a 6-His tag on the N terminus of the enzyme. Plasmids were contained in the *Escherichia coli* HB101 strain. The enzyme was purified after expression from pKK223 as described in ref *3* and modified in ref *5*. The His-tagged enzyme was purified on a Ni-NTA column (Qiagen) according to the recommendations of the manufacturer. The enzyme purified by either method was ≥90% pure as judged by SDS−PAGE. The His-tagged enzyme was kinetically indistinguishable from the enzyme derived from pKK223.

Isotope Effect Studies. Enzyme assays were carried out using a Beckman DU 640 spectrophotometer. Reactions were carried out in 1-mL volumes using 1-cm path length cuvettes. L-Serine 2-D was prepared using *O*-acetylserine sulfydrylase (*7*). Briefly, L-serine was dissolved in D_2O and lyophilized, and the dried powder was redissolved in D_2O. The L-serine in D_2O was then incubated with *O*-acetylserine sulfydrylase for 48 h. The enzyme was removed by filtration using an Amicon ultrafiltration membrane. The primary deuterium isotope effect was determined by direct comparison of initial rates using either L-serine 2-H or L-serine 2-D (*5*). A typical assay contained 100 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethane-sulfonic acid (Hepes) at pH 8, varied concentrations of L-serine 2-H or L-serine 2-D, 2 mM ketomalonate, 4 units of LDH, 0.2 mM NADH, and about 9 μg of SGAT. The disappearance of NADH was followed at 340 nm (ϵ_{340} = 6220 M^{-1} cm^{-1}) upon reduction of hydroxypyruvate by LDH. For the solvent deuterium isotope effects, each of the reaction components except the enzyme were dissolved in D_2O, lyophilized to dryness, and redissolved in D_2O. The pD of the solutions was adjusted to the desired pD by the addition of either KOD or DCl. A correction factor of 0.4 was added to the pH-meter reading made in D_2O to correct for the isotope effect on the electrode (*8*). The pD dependence of the enzyme activity was determined to account for any changes in p*K* values in D_2O compared to H_2O, so that the solvent effects were determined at the pH(D)-independent region of the profile. The p*K* values for *V*/*K* determined in D_2O were within error, equal to the values determined in H_2O. The solvent deuterium isotope effects were determined by a direct comparison of initial rates in assays similar in content to those used for the primary deuterium isotope effects. Control reactions were done in both H_2O and D_2O to be certain that the SGAT reaction was limiting in the coupled reaction with LDH.

Deuterium Exchange. To determine whether SGAT can catalyze the exchange of deuterium into the C_α position of L-serine, the following experiment was done. A reaction mixture was prepared, which contained 30 mM potassium phosphate at pD 7.5, 20 mM L-serine, and 15 μg of SGAT

in D_2O in a final volume of 8 mL. Prior to adding the enzyme to the reaction mixture, a 1 mL aliquot was removed and 7.5 μL of concentrated DCl was added to the aliquot to lower the pD to about 3. After the enzyme was added to the reaction mixture, 1 mL aliquots were removed at 20, 40, and 80 min and DCl was added to quench the reaction. The enzyme was removed from the reaction mixtures by ultrafiltration through a Centricon YM-10 membrane. Proton NMR spectra were collected using a Varian Mercury VX-300 MHz NMR.

Pre-steady-State Kinetic Studies. The pre-steady-state studies were done using an OLIS rapid scanning spectrophotometer. The dead time of the instrument is about 4 ms. Scans were typically collected at 16 ms intervals for 1−4 s depending upon the experiment. In some cases, where fast rate constants were involved, the data were also collected at a scan rate of 1000 scans/s. The range of absorbance monitored was from 310 to 540 nM. Typical experiments were carried out using SGAT at a concentration of 1 mg/mL (about 26 μM monomer concentration) after mixing. The pH was maintained, depending upon the experiment, at either pH(D) 8.0 or 6.8 with either 100 mM Hepes or 100 mM potassium phosphate. The reactants were in the same buffer, and the concentrations were as indicated in the text. For experiments done in D_2O, the reactants were first dissolved in D_2O, lyophilized to dryness, and redissolved in D_2O. SGAT in the PMP form was prepared by incubating 0.5 mM SGAT/PLP (monomer concentration) with 0.6 mM L-serine, 1 mM NADH, and 5 units of LDH to remove the hydroxypyruvate formed by SGAT from L-serine. The reaction mixture was incubated for a few minutes at room temperature and then dialyzed versus two 1 L volumes of 100 mM potassium phosphate buffer at pH 7.5 and 4 °C. The enzyme after dialysis had a UV−vis absorbance spectrum consistent with E/PMP (*5*). The time courses for the rapid kinetic experiments were analyzed using the equations supplied by OLIS for global fits of the kinetic data.

Data Analysis. Initial velocity data were fitted using BASIC versions of the Fortran computer programs developed by Cleland (*9*). Data for primary and solvent deuterium isotope effects were fitted using eq 1, while saturation curves collected to determine the substrate dependence of the pre-steady-state first-order rate constant for the SGAT reaction were fitted using eq 2.

$$v = \frac{VA}{K_a(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (1)$$

$$v = \frac{VA}{K_a + A} \quad (2)$$

In eqs 1 and 2, v and V are the initial and maximum velocities, respectively, K_a is the Michaelis constant for A, F_i is the fraction of deuterium in the labeled compound, and $E_{V/K}$ and E_V are the isotope effects minus 1 on V/K and V, respectively.

Pre-steady-state data were fitted using the equations contained in the software package associated with the OLIS stopped-flow instrument. In cases where a sequential process in which A is converted to B, the spectra were fitted globally to eq 3 and data, where three species were involved, A, B, and C, were fitted to eq 4.

$$Y = A_oe^{k_1t} + C_o \quad (3)$$

$$Y = A_oe^{k_1t} + B_oe^{k_2t} + C_o \quad (4)$$

In these equations, k_1 and k_2 are the first-order rate constants, t is time, A_o and B_o are amplitudes for the first-order processes, and C_o is the initial absorbance.

Table 1: Isotope Effects for SGAT from *H. methylovorum*

$^D(V/K_{L-serine})$	1.17 ± 0.18^a	$^{D_2O}(V/K_{L-serine})$	2.21 ± 0.85
$^D(V/K_{ketomalonate})$	0.9 ± 0.1	$^{D_2O}(V/K_{ketomalonate})$	2.04 ± 0.65
^DV	1.16 ± 0.08	^{D_2O}V	5.7 ± 0.8

[a] The values reported in the table are the average of at least two separate determinations.

RESULTS

Isotope Effect Studies. The steady-state primary and solvent deuterium isotope effect data are summarized in Table 1. The primary deuterium isotope effect is effectively 1 on $^D(V/K_{L-serine})$, $^D(V/K_{ketomalonate})$, and ^DV. In contrast, a solvent deuterium isotope effect of about 2 is observed on both $^D(V/K_{L-serine})$ and $^D(V/K_{ketomalonate})$. The solvent isotope effect is even larger on ^DV and is equal to about 5.5. No evidence for exchange of deuterium into the α carbon of L-serine was obtained after 80 min of incubation of SGAT with L-serine in D_2O (data not shown). The NMR spectrum of L-serine was identical to the control spectrum after 80 min of incubation with the enzyme.

Stopped-Flow Studies. Figure 1 shows the results of the rapid mixing of L-serine with the internal Schiff base form of SGAT at pH 8 in D_2O. The spectra show a decrease in the absorbance at 414 nm and an increase at 333 nm, representing the formation of E/PMP. There is no evidence of any reaction intermediates. The rate constant for formation of E/PMP is about 16.6 s^{-1}. A similar experiment carried out in H_2O also shows only the formation of E/PMP with an associated rate constant of about 33.9 s^{-1}. The observed solvent isotope effect is thus 2.04. The rapid mixing of SGAT with L-serine 2-D results in a rate constant for formation of E/PMP of 36.3 s^{-1}, essentially equal to the value using L-serine 2-H of 33.9 s^{-1}. The rate of formation of the E/PMP is dependent upon the L-serine concentration and is described by a hyperbola. A fit of the data using eq 2 gives estimates of the $K_{L-serine}$ (in the direction of formation of the external Schiff base) and a maximum rate constant at saturating serine of 0.15 ± 0.03 mM and 42 ± 3 s^{-1}, respectively. The $K_{L-serine}$ is similar to the value of 0.28 mM determined from steady-state kinetic studies (*5*).

The rapid mixing of E/PMP with hydroxypyruvate, at 22 °C in H_2O, results in a rapid first-order increase in absorbance at 414 nm and a concomitant decrease at 333 nm, indicating the formation of the internal Schiff base with an associated rate constant of about 24 s^{-1} (Figure 2). Rapid mixing of SGAT/PMP with hydroxypyruvate in D_2O is slower with a rate constant of about 14 s^{-1}. As is seen with hydroxypyruvate, the rapid mixing of glyoxylate with E/PMP leads to an increase at 414 nm and a concomitant decrease at 330 nm, indicating the formation of the internal Schiff base in a first-order process with an associated rate constant of 61 s^{-1}

Table 2: Pre-steady-State Rate Constants[a] and Isotope Effects for SGAT

				H_2O		D_2O		
enzyme form	pH	T (°C)	substrate	k_1 (s^{-1})	k_2 (s^{-1})	k_1 (s^{-1})	k_2 (s^{-1})	k_{H_2O}/k_{D_2O}
PLP	8	22	L-serine	33.9 ± 0.9[b]		16.6 ± 0.6		2.04
PLP	8	22	L-serine 2-*d*	36.3 ± 0.1				
PMP	6.8	22	ketomalonate	dead time	5.0 ± 1 (5.4 ± 0.5)[c]	87 ± 3 (81 ± 10)	0.96 ± 0.1 (1.2 ± 0.01)	5.02
PMP	6.8	22	glyoxylate	61 ± 7 (133 ± 1.2)		24.4 ± 1.9		2.5
PMP	6.8	22	hydroxypyruvate	24.4 ± 0.1		13.6 ± 0.5		1.8

[a] Rate constants are, in all cases, the average of at least two experiments at a scan rate of 62 scans/s. [b] In all cases, rate constants were determined from a fit of the data using eq 2, with the exception of the ketomalonate data acquired in D_2O, where the data were fitted to eq 3. [c] Rate constants in parentheses were determined at a scan rate of 1000 scans/s and are the average of five or six experiments.

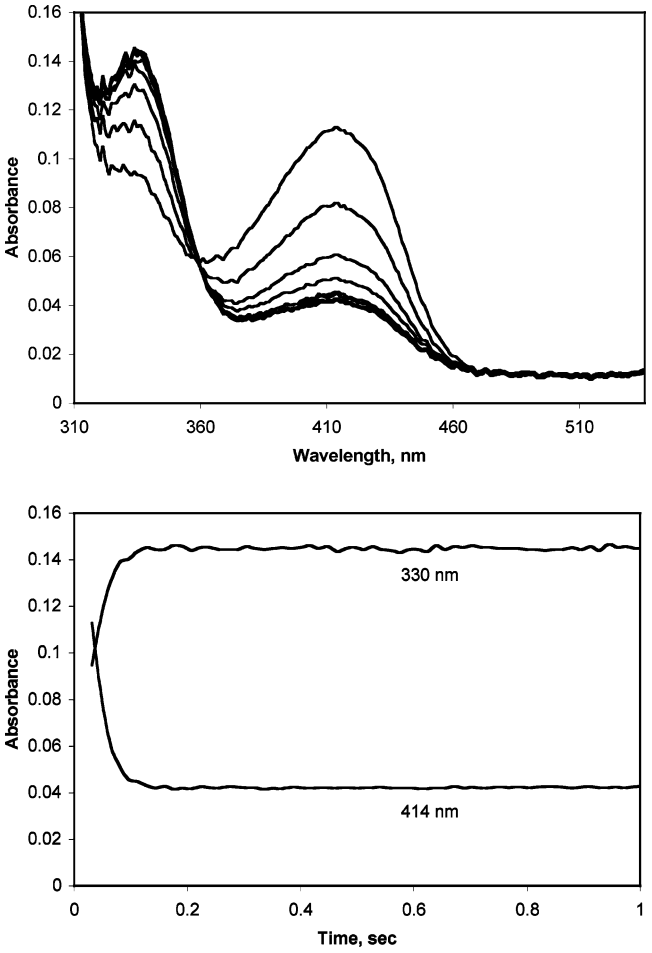


FIGURE 1: Spectral changes on rapid mixing of the PLP form of SGAT with L-serine in H_2O. The first 10 spectra are shown (upper panel) and time courses at the indicated wavelength (lower panel). The top spectrum at 414 nm is the first collected. Spectra were acquired every 16 ms. The final concentrations after mixing were SGAT, 26 μM (monomer concentration); L-serine, 2 mM; and potassium phosphate, 50 mM, at pH 8.

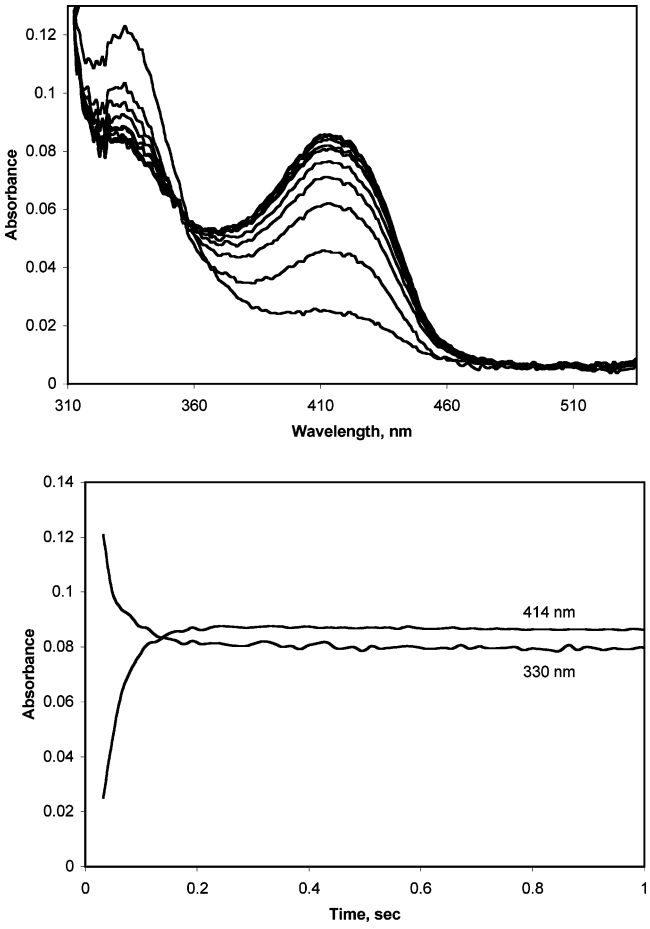


FIGURE 2: Spectral changes on rapid mixing of the PMP form of SGAT with hydroxypyruvate in D_2O showing the first nine spectra (upper panel) and time courses at the indicated wavelength (lower panel). The bottom spectrum at 414 nm is the first collected. Spectra were acquired every 16 ms. The final concentrations after mixing were SGAT, 26 μM (monomer concentration); hydroxypyruvate, 1 mM; and potassium phosphate, 50 mM, at pH 6.8.

(133 ± 1.2 s^{-1} at 1000 scans/s), and this is decreased to 24 s^{-1} in D_2O. In contrast to the rapid-mixing results with the natural keto substrates, hydroxypuruvate and glyoxalate, the alternative substrate ketomalonate gives evidence for an additional reaction intermediate when mixed with the E/PMP form of SGAT. Results for mixing ketomalonate with E/PMP are shown in Figure 3. Figure 3 clearly shows the build-up of an intermediate that absorbs at 490 nm and then decays to give spectra typical of the internal Schiff base. The rate of formation of the 490 species is fast in H_2O and occurs in the dead time of the instrument. The decay of the 490 nm species to the internal Schiff base is associated with a rate constant of 5 s^{-1}. In D_2O, the formation of the 490 intermediate is about 87 s^{-1} and decays to the internal Schiff base with a rate constant of about 1 s^{-1}, and thus, a solvent deuterium isotope effect of about 5 is obtained on the decay rate. Because the formation of the 490 nm intermediate is fast, the experiment was repeated at a scan rate of 1000 scans/s. Using the faster scan rate, the rate constants obtained in

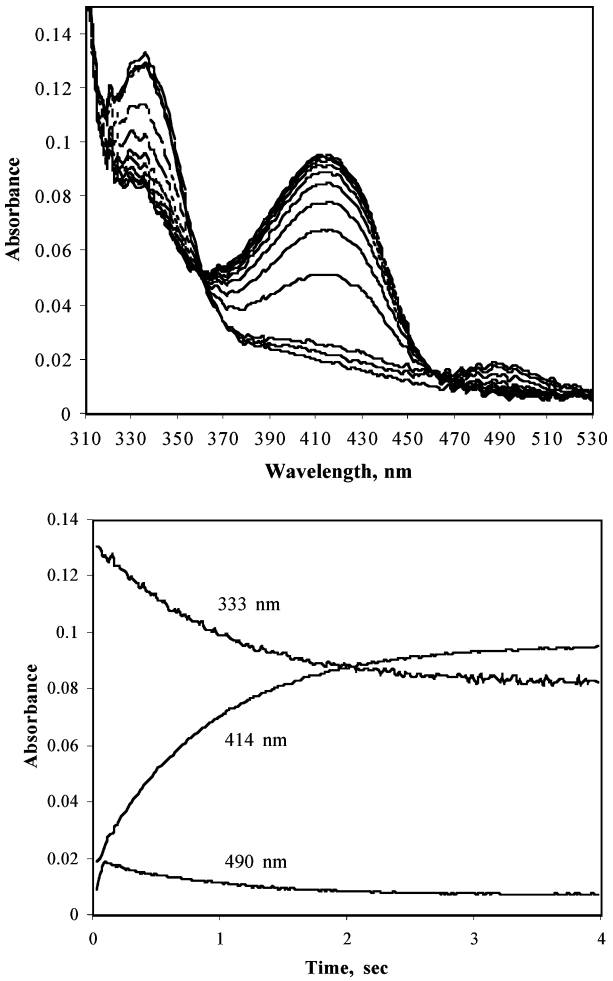


FIGURE 3: Rapid mixing of the PMP form of SGAT with ketomalonate in D_2O showing spectral changes (upper panel) and time courses at the indicated wavelength (lower panel). The bottom spectrum at 414 nm is the first collected. Spectra were acquired every 16 ms. Although these data were also collected at 1000 scans/s, the spectra acquired at the slower scan rate are shown for clarity. Final concentrations after mixing were as follows: SGAT, 26 μM (monomer concentration); ketomalonate, 2 mM; and potassium phosphate, 50 mM, at pH 6.8.

D_2O were 81 $\pm$ 10 s^{-1} for formation of the 490 nm species and 1.2 $\pm$ 0.01 for decay. At the faster scan rate, the formation of the 490 intermediate in H_2O was barely detectable and essentially occurred in the instrument dead time. The instrument dead time is about 4 ms; thus, the formation of the 490 intermediate in H_2O is equal to or greater than $0.693/0.004 = 173$ s^{-1}. All of the rate constants derived from the rapid mixing experiments are summarized in Table 2.

DISCUSSION

Studies with the Natural Substrates. The results from steady-state kinetics indicate that there is no primary deuterium isotope effect when using L-serine 2-D as the labeled substrate. In addition, the pre-steady-state rate constants are essentially identical when the internal Schiff base is mixed with either L-serine or L-serine 2-D. These data indicate that abstraction of the α proton from the external aldimine intermediate is not rate-limiting for the SGAT-catalyzed reaction. When SGAT is mixed with D-serine, the absorbance maximum at 414 nm is shifted to about 420 nm (data not shown), presumably as a result of formation of the external aldimine with D-serine that cannot go on to products because of the inability of the enzyme to abstract the α proton. The α proton would likely be oriented improperly for abstraction with D-serine. Presumably, the external Schiff base formed with L-serine would show a similar shift in absorbance maximum as seen with D-serine and would build up in the pre-steady state if abstraction of the α proton is slow. However, rapid mixing of the internal Schiff base with L-serine shows no indication of a shift in the absorbance maximum at 414, also suggesting no rate limitation by α-proton abstraction.

In contrast to the results for the primary deuterium isotope effects, a significant solvent deuterium isotope effect of about 2 is observed in both the pre-steady state and steady state with L-serine as the substrate. Several steps in the proposed mechanism could lead to a solvent isotope effect, but steps up to and including the formation of the quinonoid intermediate (**V** in Scheme 1) are ruled out because the observed primary deuterium isotope effect is unity. However, a possible solvent-sensitive step is protonation of C4′ of the quinonoid intermediate. If protonation of C4′ is a slow step, build-up of the quinonoid intermediate would be expected but is not observed. The most likely solvent deuterium-sensitive step is hydrolysis of the ketimine intermediate (**VI** in Scheme 1). If hydrolysis of the ketimine is slow, in the pre-steady state, observation of the ketimine intermediate may be expected. However, the ketimine likely absorbs at around 330 nm, very similar to E/PMP, and thus, observation of the ketimine intermediate would be difficult to detect.

Rapid mixing of E/PMP with hydroxypyruvate leads to an increase in absorbance at 414 nm and a decrease in absorbance at 330 nm. The 414 nm peak likely represents the ketoenamine tautomer of the internal aldimine. From the rapid mixing experiments, there is no indication of the build-up of intermediates such as the quinonoid or external aldimine. The solvent isotope effect from the rapid mixing experiments for the reaction of E/PMP with hydroxypyruvate is about 1.8, similar to the isotope effect of 2 in the opposite reaction direction. Because no intermediates are observed along the reaction pathway, the step that likely reflects the solvent isotope effect is formation of the ketimine from E/PMP and hydroxypyruvate. Data are consistent with rate limitation by hydrolysis of the ketimine in the direction of hydroxpyruvate formation from L-serine, as predicted by the Haldane relationship (*10*). The Haldane relationship for a ping-pong half-reaction is the ratio of the *V*/*K* values for the reactant and product, i.e., $K_{eq} = (V/K_{L-serine})/(V/K_{hydroxypyruvate})$ and $^{D_2O}K_{eq} = {}^{D_2O}(V/K_{L-serine})/{}^{D_2O}(V/K_{hydroxypyruvate})$. A small solvent deuterium equilibrium isotope effect is expected on the half-reaction, because fractionation factors for deuterium are similar in D_2O and the ketimine intermediate and in E/PMP and hydroxypyruvate (**VI** and **VII** in Scheme 1).

Glyoxylate, the aldo substrate for the second half-reaction, when rapidly mixed with E/PMP leads to spectral changes similar to those seen with hydroxypyruvate. There is an increase in the absorbance at 414 nm and a decrease at 330 nm, consistent with the rapid formation of the ketoenamine tautomer of the internal aldimine and without detection of any reaction intermediates. The rate of formation of the ketoenamine tautomer with glyoxylate is several times faster

than observed with hydroxypyruvate. Glyoxylate is the fastest reacting natural substrate of those tested. The solvent deuterium isotope effect is about 2.5 for the reaction of gyoxylate with E/PMP and is somewhat larger than the value of about 1.8 measured with hydroxypyruvate. Because the results with glyoxylate are similar to those obtained with hydroxypyruvate, the formation of the ketimine intermediate is likely also the slowest step in the formation of glycine.

Alternative Substrates. Ketomalonate is an alternative substrate that has been used in place of glyoxalate in steady-state kinetic studies of SGAT (*5*). The solvent deuterium isotope effect on $V/K_{\text{ketomalonate}}$ is about 2 in the steady state, but ^{D_2O}V is about 5.5 with L-serine and ketomalonate as the substrates. Studies conducted to examine the pH dependence of the SGAT reaction suggested that ketomalonate is a sticky substrate (*5*); that is, it goes on to form products more rapidly than it dissociates from the enzyme. The smaller value of $^{D_2O}(V/K_{\text{ketomalonate}})$ compared to ^{D_2O}V also suggests that ketomalonate is a sticky substrate, because it has a large forward commitment to catalysis that would suppress the value of $^{D_2O}(V/K)$. This will only be true if the keto half-reaction limits the overall reaction and the ketomalonate V/K is about 5-fold less than the L-serine V/K (*5*). The most interesting observation concerning the reaction of ketomalonate with SGAT appears in the pre-steady-state studies. Rapid mixing of E/PMP with ketomalonate initially leads to formation of an intermediate that absorbs at 490 nm, typical of the quinonoid intermediate. These results are in contrast to the results obtained with the natural substrates where no 490 nm peak is observed upon rapid mixing of hydroxypyruvate or glyoxylate with E/PMP. The formation of the quinonoid occurs in the instrument dead time in H_2O, and the decay of the intermediate leads to the appearance of a 414 nm species, likely the ketoenamine tautomer of the internal Schiff base. The formation of the quinonoid intermediate is slower in D_2O and can be measured in the pre-steady-state. Consequently, there is a solvent deuterium isotope effect of about 2 upon formation of the quinonoid intermediate. The decay of the quinonoid is the slowest step upon mixing ketomalonate with E/PMP, is about 6 times slower than the rate constant for the reaction of L-serine with E/PLP, and indicates that the second half-reaction is slow overall for the SGAT reaction with L-serine and ketomalonate as the substrates. The decay of the quinonoid intermediate is also solvent-sensitive, displaying a solvent isotope effect of about 5, similar to the value of about 5.5 observed on V for the steady-state reaction. Thus, with the natural substrates, hydrolysis of the ketimine is limiting, while protonation of the quinonoid intermediate at C_α to form the external Schiff base limits with ketomalonate.

On the basis of the steady-state and pre-steady-state rates and isotope effects, protonation of the quinonoid intermediate at C_α to form the external Schiff base completely limits the overall reaction with ketomalonate and L-serine as the substrates. A comparison of the previously determined pK of 8.2 from the $V/K_{\text{ketomalonate}}$ pH rate profile with the pK of 7.2 from the $pK_{\text{i oxalate}}$ (oxalate is an inhibitor competitive with ketomalonate) can be used to estimate the stickiness factor ($k_{\text{chem}}/k_{\text{off}}$) for ketomalonate from the expression app $pK_a = pK_a + \log(1 + k_{\text{chem}}/k_{\text{off}})$; $8.2 = 7.2 + \log(1 + k_{\text{chem}}/k_{\text{off}})$; $k_{\text{chem}}/k_{\text{off}} \sim 9$. A value can also be estimated from the isotope effect data, assuming that the value of 5.5 is the intrinsic isotope effect, using the value of 2 for $^{D_2O}(V/K_{\text{ketomalonate}})$ and the expression for the isotope effect, $^{D_2O}(V/K_{\text{ketomalonate}}) = (^{D_2O}k_{\text{chem}} + k_{\text{chem}}/k_{\text{off}})/(1 + k_{\text{chem}}/k_{\text{off}})$; $k_{\text{chem}}/k_{\text{off}} = 3.7$. Given the large error on the pK_a value obtained from the $pK_{\text{i oxalate}}$ profile (7.2 ± 0.6), the value of $k_{\text{chem}}/k_{\text{off}}$ is likely closer to 4 than 9. On the other hand, although values of $^{D_2O}(V/K_{\text{L-serine}})$ and $^{D_2O}(V/K_{\text{glyoxalate}})$ are quantitatively similar to the values obtained from pre-steady-state studies, neither of the isotope effects of about 2 is the intrinsic solvent deuterium isotope effects given the value of 5.5 on ^{D_2O}V. It is thus likely that protonation of the quinonoid intermediate also likely contributes to the overall rate limitation but with ketimine hydrolysis being the major rate-limiting step.

The quinonoid intermediate is not detected in the reaction of the enzyme with either glyoxylate or hydroxypyruvate. Ketomalonate is most similar in structure to hydroxypyruvate. The carboxylate group of ketomalonate that effectively substitutes for the β carbon of hydroxypyruvate will introduce a somewhat larger group and also a negative charge at this position in the molecule. Within the active site, ketomalonate or any intermediate formed from ketomalonate, will likely have a carboxylate group with its negative charge oriented to give either attraction or repulsion dependent upon enzyme side chains in its vicinity. Consequently, there could be both steric interference and/or electrostatic interactions that might affect the reactivity of the quinonoid intermediate. It is also possible that the quinonoid intermediate formed with ketomalonate could be resonance-stabilized, contributing to its stability. However, resonance stabilization will increase negative charge density on the carboxylates, and this may or may not be accommodated in the SGAT active site. In any case, the observation of a quinonoid intermediate on the reaction of SGAT with ketomalonate suggests that this intermediate also forms in the reaction of SGAT with the natural substrates but does not build up to a sufficient concentration.

Quinonoid in Other Systems. Although a quinonoid intermediate, the resonance-stabilized carbanion formed upon cleavage of a bond to C_α of the external Schiff base, is known to be the central intermediate in reactions catalyzed by PLP-dependent enzymes (*11*, *12*); demonstration of its presence in the reactions has been difficult. The presence of a kinetically competent quinonoid intermediate has most often been observed in reactions with alternative substrates or coenzymes and mutant enzymes (*13*−*16*). The formation of a quinonoid intermediate in the reaction of a carbonyl-containing substrate with a transaminase in the PMP form rarely has been reported. The formation of a quinonoid intermediate has been shown in the reaction of the PMP form of dialkylglycine decarboxylase with pyruvate in the transamination half-reaction. DGD differs from SGAT in that the rate-limiting step in the DGD L-alanine transamination half-reaction is protonation at C4′ to form the ketimine intermediate. The reaction of DGD−PMP with pyruvate deprotonation at C4′ is the slowest step, but there is some rate limitation by protonation at C_α, which leads to detection of the quinonoid intermediate. In contrast, with SGAT, the rate-limiting step with ketomalonate as a substrate is protonation at C_α.

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