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TRANSIENT KINETIC ANALYSIS OF L-SERINE INTERACTION WITH *E. COLI* D-3-PHOSPHOGLYCERATE DEHYDROGENASE CONTAINING AMINO ACID MUTATIONS IN THE HINGE REGIONS †

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Abstract

In *E. coli* D-3-phosphoglycerate dehydrogenase, the amino acid sequences G294–G295 and G336–G337 are found between structural domains and appear to function as hinge regions. Mutagenesis studies of these sequences showed that bulky side chains had significant effects on the kinetic properties of the enzyme. Placement of a tryptophanyl residue near the serine binding site (W139F/E360W) allows serine binding to be monitored by fluorescence quenching analysis. Pre-steady state analysis has demonstrated that serine binds to two forms of the free enzyme, E and E*. Conversion of Gly-336 to valine has its main effect on the K_d of serine binding to one form of the free enzyme (E) while maintaining the cooperativity of binding observed in the native enzyme. Conversion of Gly-294 to valine eliminates a rate limiting conformational change that follows serine binding to E. The conformational change between the two forms of free enzyme is maintained, but the Hill coefficient for cooperativity is significantly lowered. The data indicate that the cooperative transmission induced by serine binding is transmitted through the Gly294–Gly295 hinge region to the opposite serine binding interface and that this is most likely propagated by way of the substrate binding domain-regulatory domain interface. In the G294 mutant enzyme, both serine bound species, E•Ser and E*•Ser, are present in significant amounts indicating that cooperativity of serine binding does not result from the binding to two different forms. The data also suggest that the E* form may be inactive even when serine is not bound.

D-3-Phosphoglycerate dehydrogenase (PGDH[1], E.C. 1.1.1.95) catalyzes an early step in the biosynthesis of L-serine (1). The enzyme is feedback inhibited by L-serine, which binds at the interface between two regulatory domains and forms hydrogen bonds to both domains across the interface (2). *E. coli* PGDH is a tetramer of identical subunits and each subunit contains three structurally distinguishable domains: the nucleotide-binding domain, the substrate-binding domain and the regulatory domain (3). The regulatory domain is a member of the ACT domain family found mainly in enzymes involved in amino acid metabolism but also in some transcription regulators (4,5). In fact, the regulatory domain of PGDH was the first ACT domain to have its three dimensional structure determined and is considered the archetypical ACT domain (3). The enzyme can be considered a dimer of dimers where the fundamental dimer is formed between two nucleotide domains in the center of the structure

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[1]Abbreviations used are: PGDH, D-3-phosphoglycerate dehydrogenase; ACT, Aspartate kinase, Chorismate mutase, TyrA.

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with a regulatory domain at opposite ends. The tetramer is formed through the interaction of two pairs of regulatory domains forming an elongated toroid (Figure 1).

Each of the two regulatory domain interfaces contains two L-serine binding sites formed by the nearly 180° symmetry at the domain interface. However, previous studies have shown that maximal inhibition of catalytic activity occurs when only two of the four sites are occupied, one at each interface (6,7)). Serine binding to the regulatory domains is cooperative, with the first two serines to bind showing positive cooperativity and the last two displaying negative cooperativity. Thus, while four binding sites exist because of the symmetry at the regulatory domain interface, only two sites are operative in the metabolic regulation of enzyme activity.

Previous studies with hybrid tetramers of *E. coli* PGDH (6,7), where individual effector and catalytic sites were knocked out by mutagenesis, revealed the catalytic participation of each active site and the regulatory influence of each effector site. At any one time, only two of the four active sites are turning over. This half-of-the-sites activity is produced by one catalytic site on each side of the nucleotide binding domain interface. A flip-flop type mechanism was suggested where the two catalytic sites successively alternate with the other two in catalytic competence. Binding of a single effector molecule to the first site inhibits essentially all of the catalytic activity of the active site in its dimer as well as approximately a third of the activity of the active site in the other dimer. Binding of a second effector molecule at the opposite regulatory domain interface increases the catalytic inhibition to greater than 95%. Successive effector binding occurs but has little if any additional influence on inhibition of catalytic activity. Thus, the effector sites also exhibit a type of half-the-sites activity.

Each of the PGDH domains is relatively compact and they are connected by short stretches of polypeptide. The nucleotide-binding domain and the substrate-binding domain are connected by two polypeptides while the substrate binding domain is connected to the regulatory domain by a single polypeptide. Within these connecting polypeptides are Gly-Gly sequences which appear to function as hinge regions (8). These are G294–G295 between the nucleotide binding domain and the substrate binding domain and G336–G337 between the substrate binding domain and the regulatory domain. Structural analysis by X-ray crystallography (9) shows that there is a rotation of approximately 15° about the polypeptides connecting the nucleotide and substrate binding domains (G294–G295 region) when serine binds to the regulatory domain. A rotation about the polypeptide connecting the substrate-binding domain to the regulatory domain has not been observed.

Mutagenesis studies of the Gly-Gly sequences in the domain-connecting polypeptides showed that the introduction of bulky side chains at these positions had significant effects on the kinetic properties of the enzyme (8). Introduction of a valine residue at position 294 significantly reduced the catalytic activity of the enzyme and completely abolished the cooperativity of the serine inhibition of enzyme activity without significantly affecting the concentration of serine needed to produce 50% inhibition (IC_{50}). Introduction of a valine residue at position 336 increased the IC_{50} of serine by approximately 15–20 fold without affecting the level of cooperativity. Gly-294 and Gly-336 are highly conserved throughout the bacterial kingdom in this type of PGDH.

Serine binding can be monitored by fluorescence quenching after replacement of a glutamyl residue for a tryptophanyl residue in the vicinity of the serine binding site (E360W). The only native tryptophan residue in the protein was also removed to produce the double mutant W139F/E360W (10). Stopped-flow transient kinetic analysis of serine binding demonstrated that PGDH exists in at least two conformations (E and E*) and that serine can bind to both

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conformations. An additional conformational change could also be observed after serine bound to the predominate form, E. To address the question of whether either of these conformational changes is responsible for the cooperativity of serine inhibition of catalytic activity, stopped flow analyses of PGDH W139F/E360W/G294V and W139F/E360W/G336V were performed. The results show that both forms of the enzyme (E and E*) bind serine and result in inhibition of catalytic activity, but it is the conformational change that occurs after serine binding that results in the cooperativity of inhibition of catalytic activity.

Materials and Methods

E. coli PGDH mutant enzymes were expressed and isolated as previously described (11). The construction and expression of the W139F/E360W mutation has been reported previously. Additional mutations at G295 and G336 were introduced by PCR mutagenesis as previously described (8). Steady-state activity was monitored by following the change in absorbance at 340 nm by following the conversion of NADH to NAD^+, using α-ketoglutarate as the substrate (12). The concentration of native enzyme was determined using an E 1% of 6.7 at 280 nm (13).

Stopped-flow Fluorescence Spectroscopy

Pre-steady state kinetic analyses were performed with an Applied Photophysics Model SX-20 stopped-flow spectrometer. The reactions and all reagents were thermostated at 25° C with a circulating water bath. Serine binding was monitored by following the quenching of an engineered tryptophan residue (E360W) at the serine binding interface. The protein was excited at 295 nm and the fluorescence change was measured with a 320 nm cutoff filter (10). All reactions were performed in 20 mM potassium phosphate buffer, pH 7.5.

A total of 1,000 points were collected for each trace and at least 10 individual traces were averaged at each set of conditions. The binding transients were analyzed either with the Pro-Data Viewer fitting software provided with the instrument or with the data fitting function of KinTek Global Kinetic Explorer (14,15) and fit to a single or a sum of exponential functions defined as

$$Y=\sum_{i=1}^{n} A_i \exp(-k_{obs,i}\, t)+C \qquad \text{(Eq. 1)}$$

where Y is the fluorescence intensity at time t, $k_{obs,i}$ is the observed rate of the ith process with an amplitude of Ai and C is an offset value (16,17). The data were fit globally with KinTek Global Explorer to the models shown in Fig. 4 and the FitSpace program of KinTek Global Explorer evaluated the Upper and Lower bounds for the rate constants.

Plots of k_{obs} versus serine concentration, where k_{obs} decreased initially and then increased followed by a plateau, were fit to,

$$k_{obs}=(k_3+(k_{-3})(K_d*/K_d*+[L]))+((k_2[L])/(K_d+[L]))+k_{-2} \qquad \text{(Eq. 2)}$$

where the rate and dissociation constants correspond to those depicted in Figure 4

Plots of k_{obs} versus serine concentration, where k_{obs} decreased initially and then increased linearly, were fit to,

$$k_{obs}=(k_3+(k_{-3})K_d*/K_d*+[L]))+((k_1[L])+k_{-1} \qquad \text{(Eq. 3)}$$

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where the rate and dissociation constants correspond to those depicted in Figure 4.

The amplitude data from the serine binding transients were fit to,

$$A=A_m[L]^n/((K_{0.5})^n+[L]^n) \quad \text{(Eq. 4)}$$

where A is the amplitude, A_m is the maximum amplitude, $K_{0.5}$ is the serine concentration at half A_m, and n is the Hill coefficient.

Results

Previous transient kinetic analysis of L-serine binding to *E. coli* W139F/E360W PGDH (10) revealed that each ACT domain interface produced its own integrated fluorescent signal and the data fit best to two exponentials. The k_{obs} plots were complex in that they were each composed of two binding events and indicated that serine could bind to two forms of the enzyme with different affinities. Plots of k_{obs} versus ligand concentration displayed a decreasing k_{obs} at low ligand concentration followed by an increasing k_{obs} that plateaued at higher ligand concentrations. This characteristic was explained by a linked equilibria model where ligand can bind to two forms of the enzyme with different affinities (10). Before isomerization, the binding affinity is weak and does not compete effectively with the isomerized form until higher ligand concentrations are reached. Thus, the initial k_{obs} primarily reflect binding to the isomerized form, but as binding to the other form increases at higher ligand concentrations, the k_{obs} decrease as a result of a decrease in the concentration of the isomerized form. Eventually, the signal from the other form predominates and the k_{obs} start increasing.

L-serine binding to W139F/E360W/G336V produced transients that are best fit to only a single exponential (Figure 2). A plot of k_{obs} vs. serine concentration (Fig 3) again produced a complex plot similar in nature to those seen for the W139F/E360W (native) enzyme. At low concentrations of serine, the observed rate (k_{obs}) decreases with increasing serine concentration. As the concentration of serine is increased further, the observed rate starts to increase and gradually plateaus at a relatively high serine concentration. The main difference between the native and the G336V mutant enzyme is that the inflection occurs at a much higher concentration. The data were fit globally to a model (Figure 4) for serine binding to two isomerized forms of the enzyme as was done previously for the native enzyme. When the K_d values were constrained initially, the model produced a good fit. The K_d values were initially estimated from the k_{obs} plots and iteratively adjusted until the model was well constrained. The model produced the estimated rate constants shown in Table 1 and Figure 4.

L-Serine binding to W139F/E360W/G294V also produced transients that fit well to a single exponential (Fig. 5). A plot of k_{obs} vs. serine concentration (Fig. 6) again produced a complex plot where the observed rates decreased with increasing serine concentration at low levels. However, as the observed rates increased, they did not level off as seen for the native and G336V enzymes. Instead, the data could be fit to a straight line at higher serine concentrations indicating either the absence of a conformational change after binding or a binding step that was not rate-limited by a subsequent conformational change. When the data were globally fit to a model that eliminated the conformational change following ligand binding to E, the model was well constrained and produced the estimated rate constants shown in Table 1 and Figure 4.

When the amplitudes of the serine binding transients are plotted as a function of serine concentration, the $K_{0.5}$ and Hill coefficient values are similar to that determined from serine

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inhibition analysis (Table 2). The activity of the W139F/E360W/G294V mutation was very low and not able to be assayed accurately. This is probably due to the additional mutation near the active site to replace the native tryptophan residue. However, the overall structural integrity of the mutant enzyme does not appear to be compromised since both effector and substrate binding sites seem to be fully functional as judged by efficient binding to the 5'-AMP affinity column used for purification and the observation that the amplitudes of serine binding of the mutant and non-mutant enzyme are comparable. We previously demonstrated (8) that the introduction of the G294V mutation reduces the Hill coefficient to near 1. A similar reduction in the Hill coefficient is seen when the serine binding amplitudes are fit to equation 4.

Discussion

While the pre-steady state serine binding transients for W139F/E360W could clearly be fit best to two exponentials, the introduction of an additional mutation at G294 (W139F/E360W/G294V) or G336 (W139F/E360W/G336V) produced transients that fit best to a single exponential. Previous work (10) showed that modeling of the amplitudes for W139F/E360W PGDH demonstrated that each regulatory domain interface appeared to be producing its own fluorescent signal and that each signal consisted of a multiple binding event. The data was consistent with a model where ligand binding alternated from one interface to the other so that one signal represented the first and third binding event and the other signal represented the second and fourth binding event. This is consistent with other early work showing that maximal inhibition resulted from one ligand bound at each interface (6,7). The introduction of additional mutations appears to either eliminate one signal or decease the differences so they can't be distinguished. Since the triple mutant enzymes, W139F/E360W/G294 and W139F/E360W/G336, are still inhibited to greater than 95% by serine, the latter is the most likely interpretation.

The k_{obs} versus serine concentration plots for each of the triple mutants still show the initial decrease in k_{obs} that was observed for W139F/E360W PGDH and is consistent with serine binding to two forms of the enzyme. However, the effect of the G294V mutation is different than that for the G336V mutation. For W139F/E360W/G336V, a major effect of the G336V mutation is to significantly increase the overall K_d of serine binding to the predominant form of the enzyme (E), such that K_d is much greater than K_d*. It also appears to significantly decrease the rate of conversion of E to E* so that the formation of E* is disfavored. The value for k_3 was not well constrained and produced a lower bound approaching zero and an upper bound no greater than 0.2 s^{-1}. However, k_3 must be greater than zero or else the second form of the enzyme (E*) would not be present and the decreasing k_{obs} would not be observed. E*•Ser is the major species present at equilibrium (Figure 7). Note that the Hill coefficient of inhibition of activity for W139F/E360W/G336 of 1.8 is similar to that of 2.0 for W139F/E360W, indicating that the positive cooperativity is maintained. The amplitude plots also indicate a $K_{0.5}$ and Hill coefficient similar to that for the inhibition of catalytic activity (Table 2).

The k_{obs} versus serine concentration plot for W139F/E360W/G294 shows a remarkable difference to that of W139F/E360W/G336 in that the k_{obs} increases linearly at higher serine concentrations. Thus, the conformational change following serine binding to E is either eliminated or is no longer rate limiting. The K_d for serine binding to E is not affected compared to that for W139F/E360W, but the Hill coefficient for both inhibition of activity and binding is decreased to near 1. Also, the conversion of E to E* is more favorable than that for the G336V mutant. The data fit very well to a model where the conversion of E•Ser to E*•Ser is eliminated (Fig. 4) and suggest that the cooperative effect seen for serine inhibition is due to a conformational change following serine binding to a single form and

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not to the binding of serine to two forms of the enzyme. The model also predicts that significant amounts of both E•Ser and E*•Ser are present at equilibrium so that both complexes result in inhibition of activity (Figure 7).

The crystal structures of *E. coli* PGDH in the absence and presence of bound effector, L-serine, show a 15° rotation of the substrate binding domain relative to the nucleotide binding domain. This rotation takes place about a "hinge" region that includes G294. A similar change is not seen in the relative positions of the regulatory (ACT) domain and substrate binding domain. However, both structures, with and without serine, show the binding site cleft in an open position that is not conducive to catalysis. This suggests that the structures are somewhat constrained by crystal packing effects and do not necessarily adequately reflect the solution structures. Nonetheless, the locations of the mutated residues within the available structures can aid in the interpretation of the kinetic data. Mutation of G336, positioned in the polypeptide linking the regulatory and substrate domains, exerts its major effect on the interface where serine binds resulting in a decrease in binding affinity for serine. The subsequent conformational change, E•Ser to E*•Ser is most likely transmitted through the regulatory domain-substrate binding domain interface and not greatly affected by the G336V replacement. Recent work with the regulatory subunit of acetohydroxyacid synthase (18) shows a similar domain interface interaction involving an ACT domain. This work showed that transmission of the effector binding at the ACT domain to the active site was dependent on domain interactions at their interface.

In order for the conformational change to be transmitted from an initial binding event at one serine binding interface to the other serine binding interface at the other end of the molecule, it must travel by way of the nucleotide binding domain interface at the center of the molecule (Figure 1). This conformational transmission is eliminated or greatly reduced by mutation at G294 that is positioned in the hinge region between the substrate and nucleotide binding domains, suggesting that the conformational change is transmitted through this polypeptide linker.

From a kinetic point of view, the enzyme exists in two forms prior to serine binding, and both are capable of binding serine. The G294V mutation produced a situation where ES and E*S are no longer linked sequentially in the pathway and thus both forms are present in significant amounts at equilibrium (Figure 7). Since both forms are present and the Hill coefficients are significantly reduced, the data suggests that in the G294V conformers, the sites are largely independent and non-interacting. Therefore the cooperativity appears to be largely the result of ligand binding to E, and not because the effector can bind to two conformational forms of the enzyme.

In the context of the regulatory mechanism, mutation at Gly-294 eliminates the interaction between sites at opposite regulatory domain interfaces so that they now act as equal and independent sites. That is, binding at the first site fails to influence the second site because the molecular communication pathway has been defeated by the mutation of Gly-294 in the hinge. Thus, while the first effector molecule to bind essentially shuts down the catalytic site in its own dimer, it has no influence on the active sites in the other dimer. Now they are shut down only when effector binds to the dimer in which they reside.

In PGDH, E* may represent an inactive form of the enzyme. However, since E* is always significantly under-populated, it is not possible to make that determination based on the present data. Because the tetrameric enzyme contains four binding sites and we know that at least two sites need to be occupied for optimal inhibition, the kinetic parameters determined here likely reflect an aggregate binding. Therefore it is not possible to accurately model site occupancy as a function of ligand concentration, particularly at low serine concentration. On

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the other hand, the data and the models are not inconsistent with E* being a ligand free, inactive form that is stabilized by ligand binding. Previous work has shown that serine inhibits the enzyme by decreasing the population of active species through the formation of an inactive enzyme-substrate-serine complex (10). E* may represent a free form of the enzyme with essentially the same conformation (inhibited) as that found in the dead-end complex with serine.

This investigation has provided further insight into the mechanism of allosteric inhibition of *E. coli* D-3-phosphoglycerate dehydrogenase by further defining the roles of the glycine hinge regions found at the connecting polypeptides between structurally distinct domains. It has also served to better define the characteristics of an alternate conformer of the effector free enzyme that was recently observed. This work lays the groundwork for additional investigation into the nature and conformational state of the E* form.

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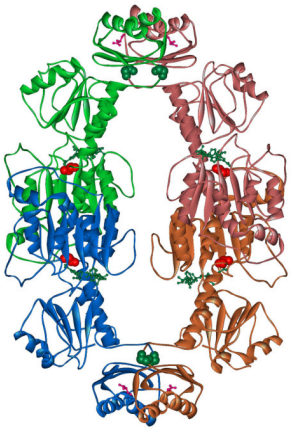

Figure 1. The structure of E. coli PGDH

The structure of *E. coli* PGDH is depicted in ribbon form. The four identical subunits are colored differently for clarity. The active site is shown with bound NADH (Green ball and stick). Bound serine is shown in Pink ball and stick configuration at the ACT domain interfaces. The locations of G294 (Red CPK model) and G336 (Green CPK model) are shown at the hinge regions between the substrate and nucleotide binding domains and the substrate and regulatory (ACT) domains, respectively.

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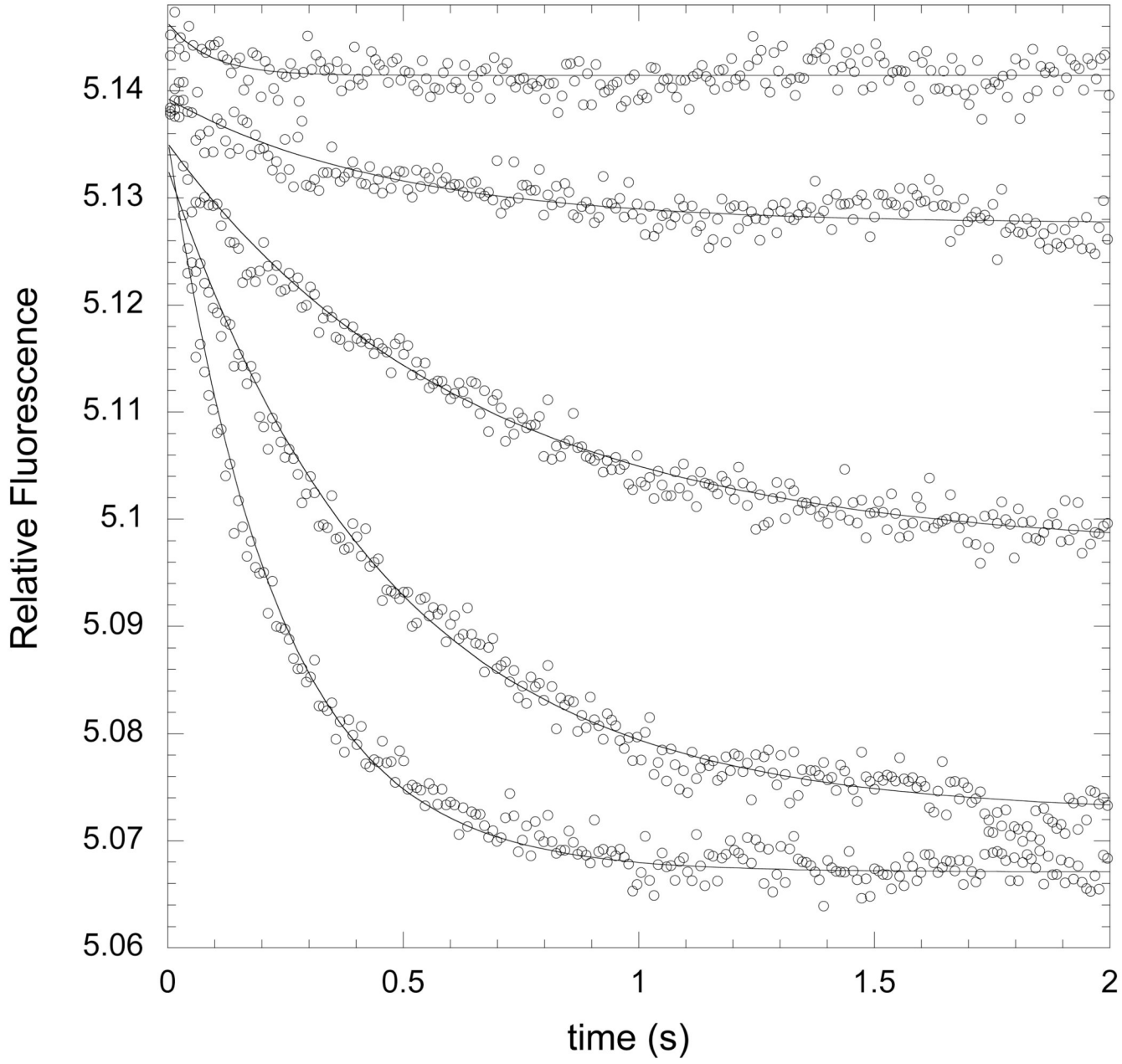

Figure 2. Time course of L-serine Binding to W139F/E360W/G336V PGDH

Protein concentration is 0.5 μM subunit. The transients are shown for 55, 90, 150, 300, and 1000 μM L-serine in descending order in the plot. Every third data point is plotted. The lines are fits of the data to equation 1 for a single exponential.

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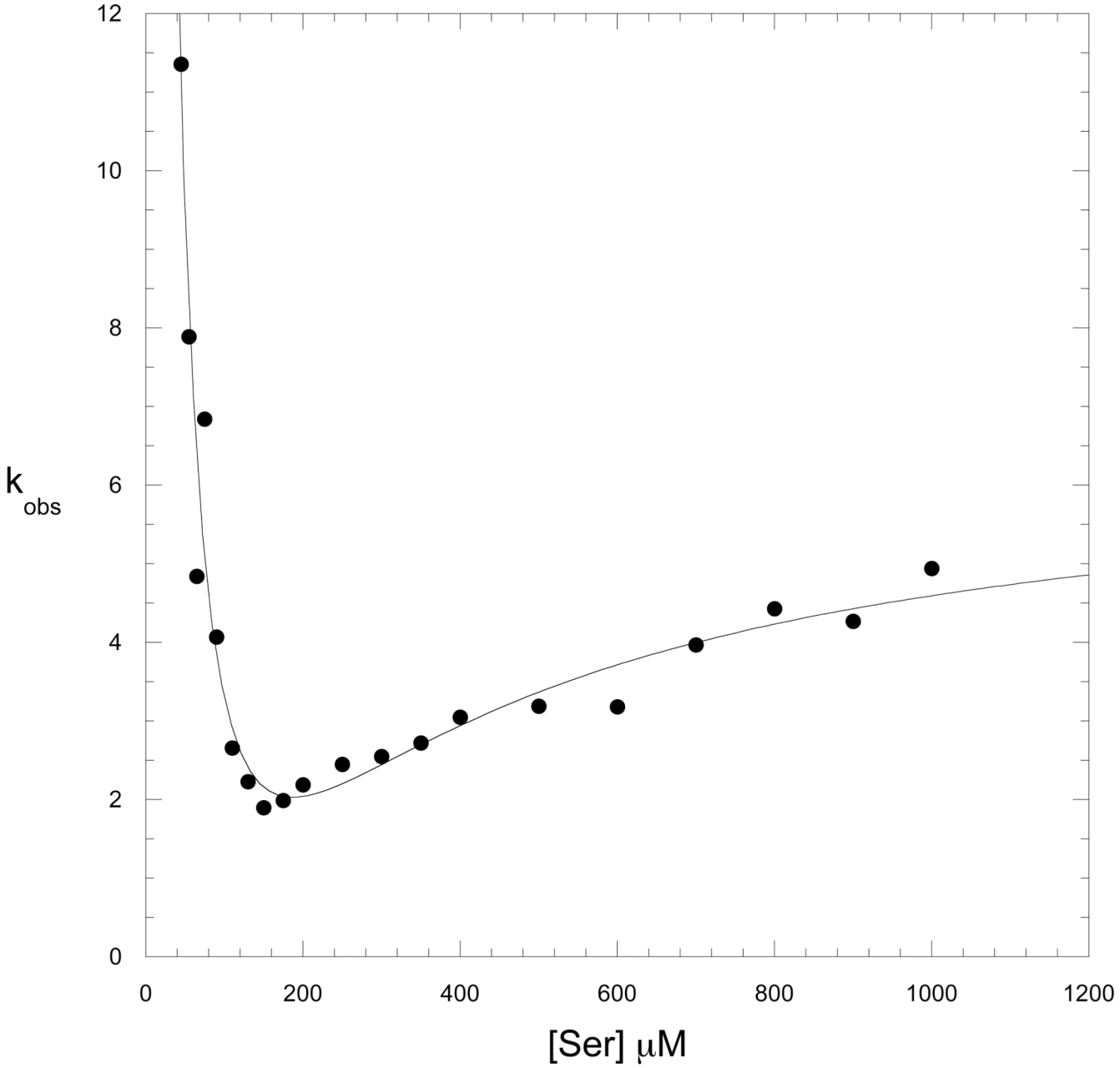

Figure 3. Observed rate constants for L-serine binding to W139F/E360W/G336V PGDH
The observed rate constants from fitting the transients are plotted versus L-serine concentration. The solid line is a fit of the data to equation 2.

Figure 4. Models used for global fitting of the serine binding transients obtained from W139F/E360W/G336V and W139F/E360W/G294V

The models are based on serine binding to two forms of the enzyme, E and E*, as previously described for W139F/E360W PGDH (10). $K_d = k_{-1}/k_1$ and $K_d^* = k_{-4}/k_4$.

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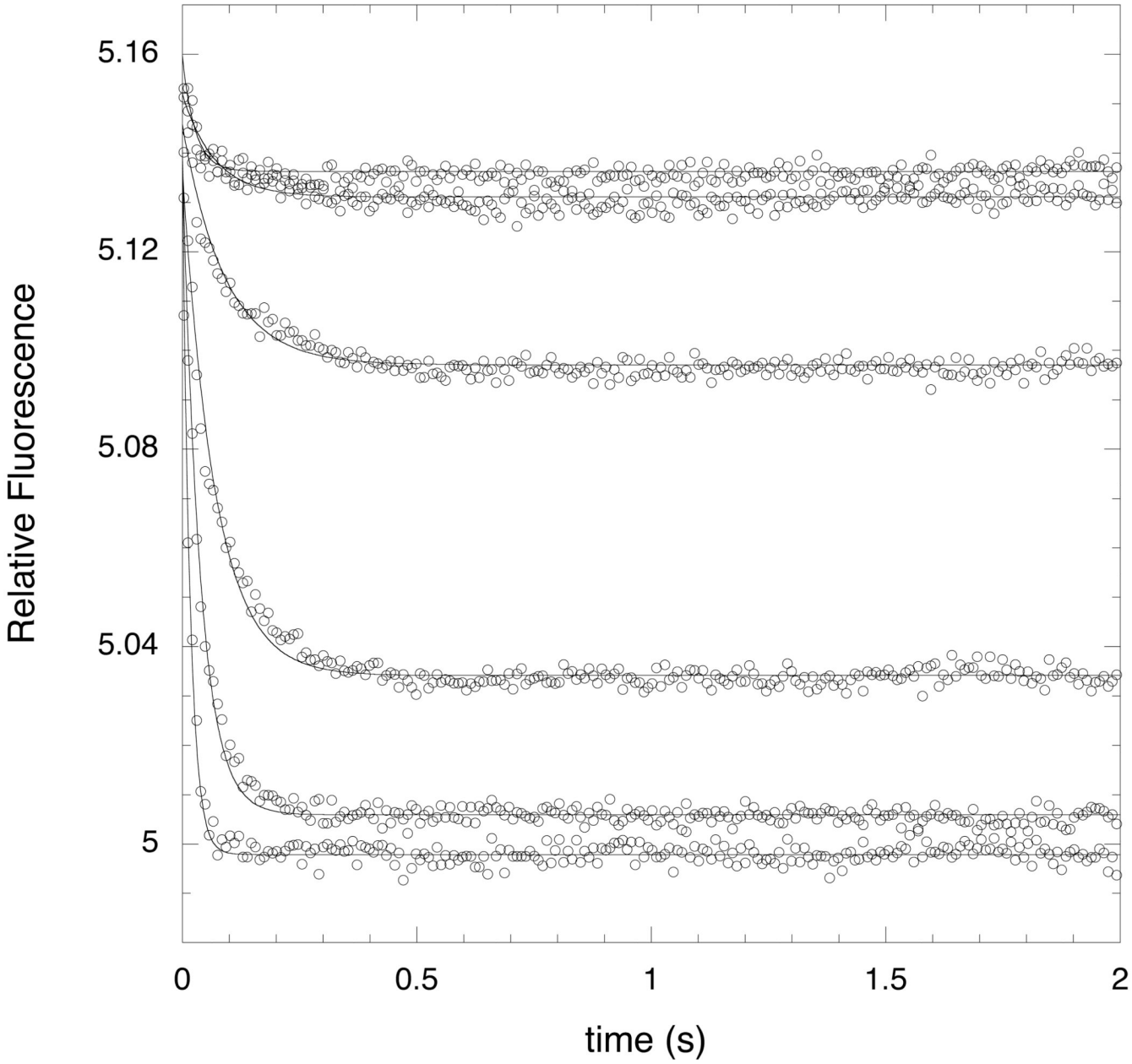

Figure 5. Time course of L-serine Binding to W139F/E360W/G294V PGDH

Protein concentration is 0.5 μM subunit. The transients are shown for 3.5, 5, 10, 25, 55 and 150 μM L-serine in descending order in the plot. Every third data point is plotted. The lines are fits of the data to equation 1 for a single exponential.

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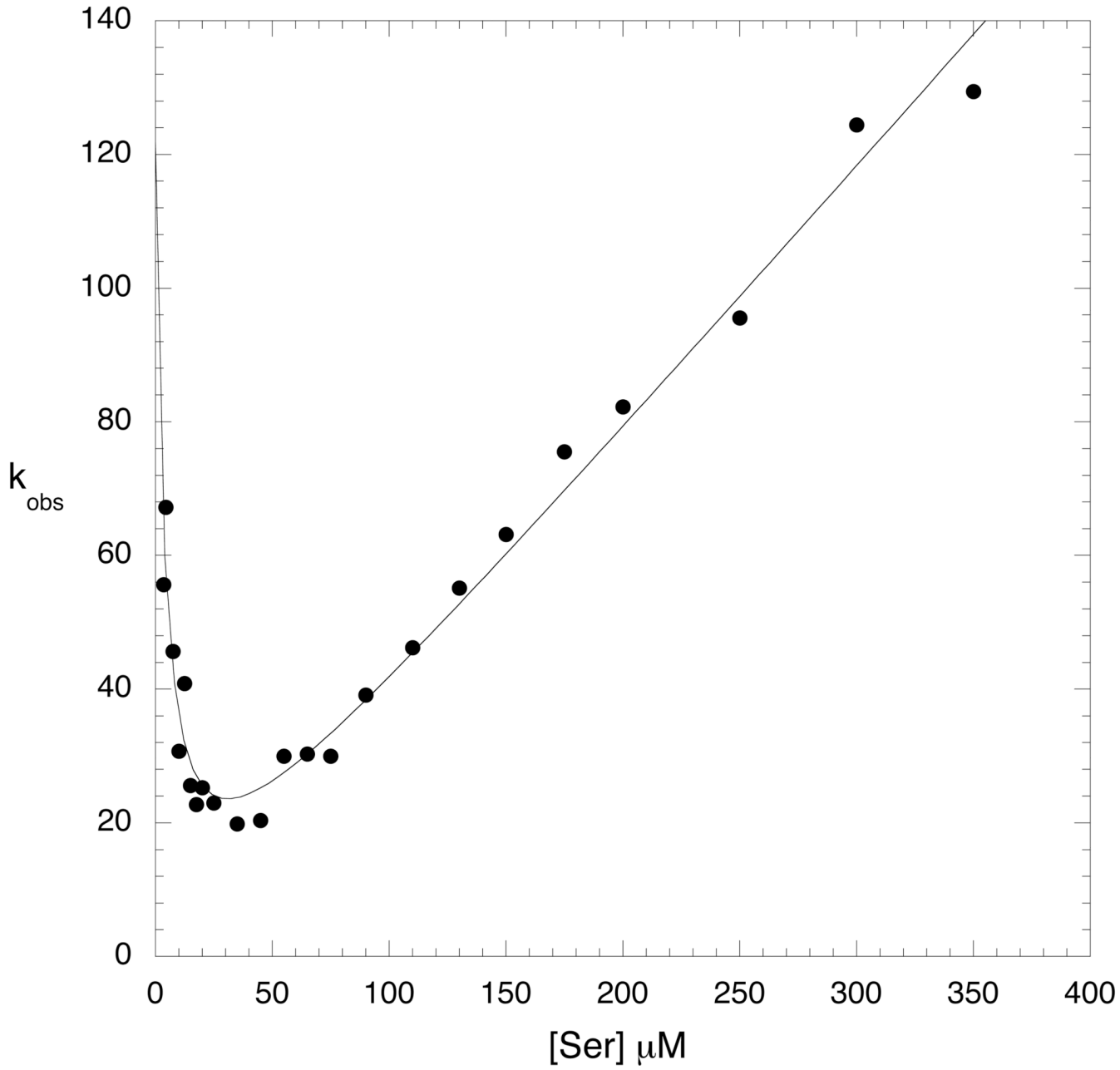

Figure 6. Observed rate constants for L-serine binding to W139F/E360W/G294V PGDH
The observed rate constants from fitting the transients are plotted versus L-serine concentration. The solid line is a fit of the data to equation 3.

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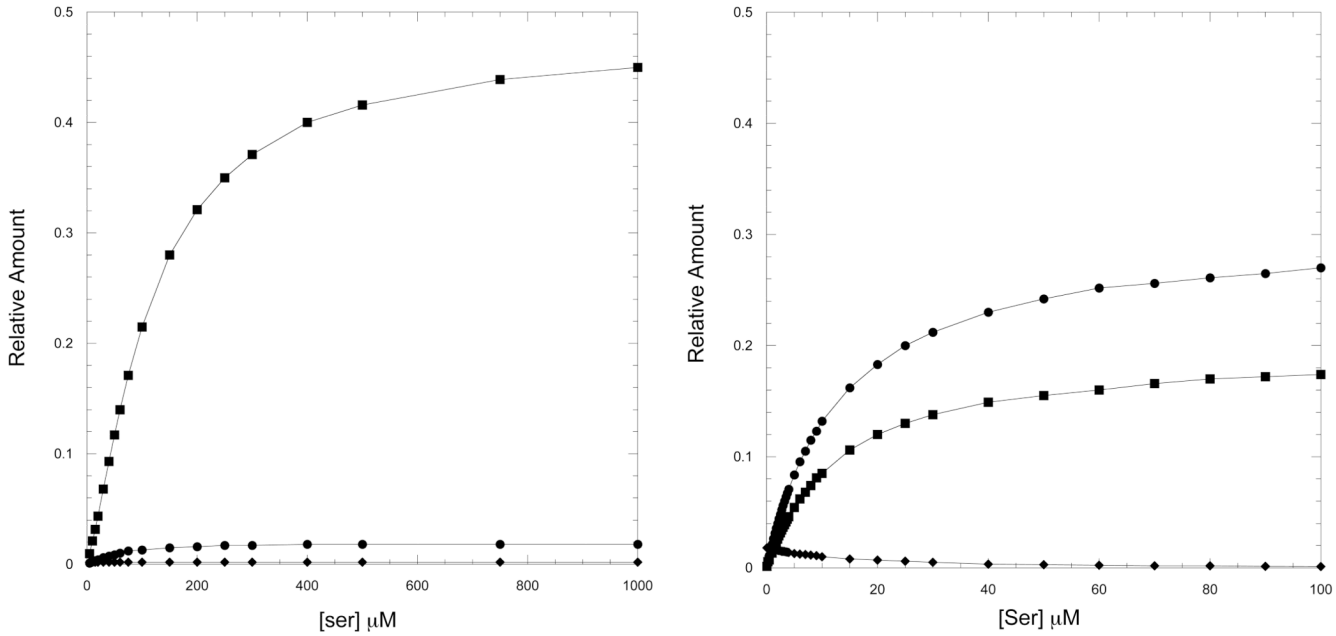

Figure 7. Simulated Distribution of Species for the Models Depicted in Figure 5 for W139F/E360W/G336V and W139F/E360W/G294V

W139F/E360W/G336V is on the left and W139F/E360W/G294V is on the right. The relative amount of E*•Ser (■), E•Ser (●), and E* (♦) are shown as a function of serine concentration. The simulation was performed with the subunit concentration set at 0.5 μM so that the maximum relative amount is 0.5. The simulation for W139F/E360W/G336V used a value of 0.1 for k_3.

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Table 1

Estimates of Kinetic Constants

	W139F/E360W 1[a] (W139F/E360W 2)	W139F/E360W G336V			W139F/E360W/G294V		
			LB[b]	UB[b]		LB[b]	UB[b]
K_d(μM)	~12[c] (~120)	~2060[c]			20		
k_1 ($\mu M^{-1}S^{-1}$)					0.339±0.004	0.337	0.384
k_{-1}(s^{-1})					6.88±0.09	6.73	7.85
k_2(S^{-1})	61.4±4 (161±8)	12.6±2	11	13.3			
k_{-2}(S^{-1})	≤10 (≤5)	0.4±0.05	0.2	0.5			
K_d*(μM)	~1[c] (~1[c])	~1[c]			~1[c]		
k_3(S^{-1})	≤20 (≤2)	0.0004±0.006	$4e^{-8}$	0.22	1.05±0.05	0.67	1.64
k_{-3}(S^{-1})	88±7 (166±22)	61.7±6.4	39.5	121	27.6±1.3	16.8	45.1

[a] From Reference 10

[b] LB, Lower Bound and UB, Upper Bound determined with FitSpace in KinTek Global Explorer

[c] Constrained during fitting

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Table 2

Kinetic Parameters Determined from Inhibition of Catalytic Activity and Serine Binding

	Inhibition of Activity		Binding Amplitudes	
Enzyme	**$K_{0.5}$ μM**	**n_H**	**$K_{0.5}$ μM**	**n_H**
W139F/E360W	15.3±0.4	2.2±0.1	10.6±0.1	2.4±0.1
W139F/E360W/G336V	258±8	2.3±0.1	151±2	2.9±0.1
W139F/E360W/G294V	15[a]	1.08±0.04[a]	35±4	1.4±0.2

[a]The values are for 4C/A G294V (ref. 8).