Sensitivity Analysis of a Proposed Model Mechanism for Newly Created Glucose-6-Oxidases

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DOI 10.1002/aic.12762
Published online September 14, 2011 in Wiley Online Library (wileyonlinelibrary.com).

Parametric sensitivity analysis of a proposed model mechanism can serve as a valuable diagnostic for studying the fundamental characteristics of an enzymatic reaction. As the first step toward understanding the kinetics of a series of newly engineered glucose-6-oxidases, the activity of which has never been found in nature, we have proposed a mechanistic kinetic model and performed a parametric sensitivity analysis. The mechanism of our model consists of two enzyme conformations, namely ''less active'' and ''more active,'' and is shown to be consistent with experimental observations of prolonged periods of enzyme induction. The extended lag phase phenomenon was found to be well described mechanistically by a slow rate of interconversion between the two enzyme conformations (relative to the rate of product formation), and this prediction was further supported by our experimental results. The proposed enzymatic model will serve as a blueprint with which to better understand the mechanistic behavior of newly generated glucose-6-oxidases. © 2011 American Institute of Chemical Engineers *AIChE J,* 58: 2303–2308, 2012
Keywords: glucose oxidase, galactose oxidase, lag time, kinetic mechanism, sensitivity analysis, mutagenesis

Introduction

Galactose oxidase (GaOx) is a copper-containing enzyme that catalyzes the oxidation of primary alcohols to aldehydes with the concomitant reduction of molecular oxygen to hydrogen peroxide. This enzyme has been extensively studied for several decades and the determination of its crystal structure[1,2] has paved the way for an improved understanding of its kinetic mechanism.[3–5] The copper at the active site is coordinated by two His and two Tyr residues, with a solvent serving as the fifth ligand of the square pyramid. One of these ligands, Tyr272, is the site of the O radical[5] which is stabilized by Tyr272-Cys228 crosslinking via a thioether bond. Meanwhile, Trp290 is believed to have an important role in stabilizing the radical by stacking interaction with Tyr272-Cys228 (delocalization of the unpaired spin density) and by entry restriction of other molecules to the active radical site (protection of the radical from solvents).[6] Studies have shown that GaOx has three redox states: an activated form (Y•-Cu(II), Ox-GaOx) containing an oxidized complex and reacting with alcohols; an inactive form containing Cu(II) but no radical (Y-Cu(II), semi-Red-GaOx); and, finally, an oxygen-reactive form containing Cu(I) but no radical (Y-Cu(I), Red-GaOx). With consideration of these structural and redox characteristics, appropriate chemical mechanisms have been suggested.[3,4]

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GaOx is known to exhibit activity with a broad range of substrates including galactose, but it has almost no demonstrated activity on glucose, an isomer of galactose.[7,8] The inability to use glucose as a substrate is due to a steric clash between Tyr495 and the C4-hydroxyl group of glucose.[2,9] Sun et al. generated GaOx mutants with glucose activities, but the demonstrated activity levels remained quite low.[8] Recently, we created glucose oxidases (GlcOx) from GaOx by computationally searching the sequence space of candidate mutations, combinatorially assembling the selected sequences, and experimentally screening the created library.[10] While characterizing these GlcOx to select the best enzyme, we routinely observed that newly engineered proteins incorporating the W290F mutation experienced significant lag times during catalysis in addition to relatively high losses of GaOx activity.

Lag periods in enzyme activity have previously been observed in copper-containing tyrosinases[11,12] iron-containing lipoxygenases,[13,14] and copper-containing copper, zinc superoxide dismutases.[15] Interestingly, we found that all of these enzymes contain metal ions and catalyze redox reactions involving molecular oxygen, but the lag was reported to be attributed to different factors.[13,15–17] Tyrosinases, exhibiting two catalytic activities which yield quinones from monophenols via diphenols, were believed to incorporate an autocatalytic hydroxylase step.[16,17] Two alternate models have been suggested to explain the lag period associated with lipoxygenases, including: (1) the product activation model in which the inactive enzyme form in the Fe (II) state is oxidized to the active Fe (III) form by the product and (2) the substrate inhibition model in which the substrate is displaced from the regulatory site by the product.[13] Finally, submicromolar concentrations of transition metal ions have

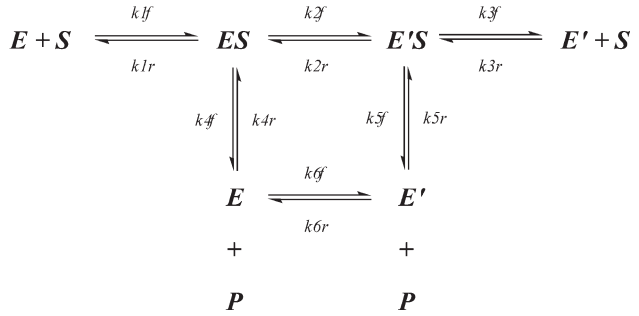

Figure 1. A proposed model mechanism[18] for GlcOx.

been found to interfere with copper,zinc superoxide dismutases, leading to an increased lag period.[15]

To better understand the nature of this observed lag phenomenon, we suggest a reaction mechanism to describe the function and activity of our newly engineered GlcOx. This mechanism was previously introduced to explain single substrate hysteretic enzyme cases in which two enzyme forms possessing different kinetic parameters lead to the occurrence of lag phenomena.[18] As the first step toward supporting the validity of this proposed mechanism for GlcOx, we have performed a parametric sensitivity analysis to investigate how the coexistence of multiple enzyme conformations ("less active" and "more active") may explain the observed occurrence of delayed enzyme kinetics. We have also investigated the capacity by which changes in the values of the rate constants in the established mechanism can generate the observed lag phenomenon. Sensitivity analysis of a proposed model mechanism can serve as a valuable diagnostic to study the fundamental characteristics of an enzymatic reaction. Whereas this technique does not allow us to directly assess the suitability of the proposed model structure, inferences regarding which parameters are most responsible for the prediction of an objective criterion may be deduced by identifying the parameters to which that output is most sensitive. Thus, the associated predictions can provide fundamental insight into the relative significance of the individual mechanisms, which compose the overall model. As will be demonstrated for the present case, this can be of particular value for enzyme models incorporating multiple, simultaneous phenomena.

Materials and Methods

Kinetic studies

Three representative GlcOx (Des3-2, Des3-6, and Des3-7) mutants previously constructed that contain the intact Trp290 residue[10] were compared with the W290F-containing mutants M-RQW[8] and Des3-10.[10] The vector, which contains the GaOx mutant M-RQW, was kindly provided by Professor Frances Arnold at Caltech,[8] and the M-RQW gene was subcloned into the 6xHis-tag-containing pET21b as described previously.[10] The kinetics of the purified GlcOx on glucose (220 mM) was monitored using the HRP-ABTS coupled assay[5] as described previously.[10] The absorbance change rate was determined at 405 nm using a spectrophotometer (Beckman Coulter, Fullerton, CA), and the extinction coefficient of 36.8 mM^{-1} cm^{-1} was used to calculate the concentration of product formed.[10]

Table 1. Parameter Estimates Used for Solution of the Model Presented in Figure 1

Parameter	Estimate	Units
k1f	1	μM^{-1} s^{-1}
k1r	250	s^{-1}
k2f	0.05	s^{-1}
k2r	0.1	s^{-1}
k3f	50	s^{-1}
k3r	1	μM^{-1} s^{-1}
k4f	5	s^{-1}
k4r	0	μM^{-1} s^{-1}
k5f	50	s^{-1}
k5r	0	μM^{-1} s^{-1}
k6f	0.0001	s^{-1}
k6r	0.001	s^{-1}

Table 2. Nomenclature Used in This Work

Parameter	Units	Definition
kif, *kir*	s^{-1} or μM^{-1} s^{-1}	Forward and reverse rate constants associated with reaction step *i*
s_{ij}		Sensitivity coefficient associated with state variable *i* and parameter *j*
x		Model state variable
p		Model parameter
y		Model output or measured variable
t	s	Time
Θ_{lag}		Lag time
E	μM	Enzyme concentration, less active conformation
E′	μM	Enzyme concentration, more active conformation
S	μM	Substrate concentration
E′S	μM	ES complex, more active form
ES	μM	ES complex, less active form
P	μM	Product concentration
Subscripts		
0, *f*		Denote initial and final conditions, respectively
max		Denotes maximum value

Numerical methods

Solution of the selected model, as well as the associated dynamic sensitivity equations, was performed using Matlab® and the intrinsic ordinary differential equation solver *ode15s*. Parameter estimates for solution of the model of interest (Figure 1) were obtained from Bates and Frieden,[18] and are listed in Table 1. Nomenclature used in this work is summarized in Table 2. Lag times (Θ_{lag}) associated with each experimental data set were approximated by first evaluating the tangent of each $\Delta P/\Delta P_{max}$ (calculated as $\left(\frac{P-P_0}{P_f-P_0}\right)$) curve at the point where the slope approaches its steady-state value. The tangent curve was then extrapolated to determine the value at which it intersects with the *x*-axis. This value was designated as Θ_{lag}. To improve the comparisons between experimental data and model predictions, t_{max} was selected as one-quarter of the maximum lag time observed among all simulations ($\Theta_{lag,max}$).

Parametric sensitivity analysis

Although sensitivity coefficients may be estimated by numerous, previously described techniques, the "Direct Method," originally described by Dickinson and Gelinas,[19] was used for the present study as it is considered to be of high computational rigor. According to the Direct Method, the differential equations which comprise a dynamic model may be supplemented with appropriate differential sensitivity equations. This allows the complete set to be solved simultaneously and yields predictions of both the state variables and

the sensitivity coefficients, s_{ij},[20,21] defined as the partial derivatives of the state variables (x) with respect to the associated parameters (p):

$$s_{ij} = \frac{\partial x_i}{\partial p_j} \quad \text{(a)}$$

To generate the set of sensitivity equations, we first consider an enzyme kinetic model of the following general form:

$$\frac{dx}{dt} = g(x,t,p), \quad y = h(x,p), \quad x(t_0) = x_0 \quad \text{(b)}$$

where t is time, the state variables (E, E′, S, E′S, ES, and P) are given by x, the parameters by p, and relevant output, or measured, variables are represented by y. In this case, the differential sensitivity equations can be defined as:

$$\frac{\partial s_{ij}}{\partial t} = \sum_{k=1}^{n} \frac{\partial g_i}{\partial x_k} s_{kj} + \frac{\partial g_i}{\partial p_j} \quad \text{(c)}$$

whereas the sensitivities associated with the output responses may then be evaluated as:

$$\frac{\partial y_i}{\partial p_j} = \sum_{k=1}^{n} \frac{\partial h_i}{\partial x_k} s_{kj} + \frac{\partial h_i}{\partial p_j} \quad \text{(d)}$$

To facilitate comparison during analysis, scaling effects can be removed by normalizing the sensitivity coefficients as $\frac{p_j}{y_i}\frac{\partial y_i}{\partial p_j}$.

Results

Evaluation of established mechanism and formulation of proposed mechanism

GaOx is known to exist in three different forms: an activated Ox-GaOx and two semi-reduced or reduced forms (semi-Red-GaOx or Red-GaOx), which each require oxidation before becoming activated. The inactive semi-Red-GaOx form does not participate in conversion reactions, only Red-GaOx does. GaOx follows a ping-pong mechanism consisting of two sequential steps, where the first step (Ox-GaOx + an alcohol → Red-GaOx + an aldehyde) is known to be rate-limiting.[3,4] thus, the second step, which involves reaction with oxygen (Red-GaOx + O_2 → Ox-GaOx + H_2O_2), can usually be omitted from the general mechanism.

It is possible that the enzymes we generated with the W290F mutation still follow the established mechanism for wild-type GaOx, but that changes in the value of the rate constants (i.e., model parameters) with glucose are such that (i) both reaction steps must now be considered and a single substrate-single enzyme model is no longer valid and (ii) the 2-substrate model now leads to lag behavior. To test this, we performed an array of numerical simulations assuming first order reactions with respect to both glucose (for the first reaction step) and oxygen (for the second reaction step), consistent with both the low overall reaction rates observed and previous reports (simulation results not shown).[4,10] Beginning with the rate constants reported for the established two-step mechanism,[4] we found that lag behavior appeared only when each of the rate constants were decreased by more than three to six orders of magnitude. However, even under these conditions, the observed lag time was predicted to be on the order of seconds, rather than minutes as we have observed experimentally. In addition, considering that two mutants with similar specific activity (5.8 ± 0.6 $M^{-1}s^{-1}$ for Des3-6 and 5.6 ± 0.8 $M^{-1}s^{-1}$ for Des3-10) showed different lag behavior (Des3-6 without lag period; Des3-10 with lag period), we concluded that a new reaction mechanism is likely required to explain such different behaviors.

Enzymes are dynamic and have an infinite number of possible conformations. However, to simplify our analysis, we have assumed a model mechanism[18] consisting of only two enzyme conformations: a "less active" GlcOx (E, with low affinity toward the substrate and a low turnover rate) and a "more active" GlcOx (E′, with high affinity toward the substrate and a high turnover rate) (Table 1 and Figure 1). In this case, the "less active" pool includes a group of previously uncharacterized conformations of the enzyme that results from the W290F mutation, and each maintains some catalytic activity. We have assumed that the first, substrate reaction step is limiting; and, given an experimental protocol designed to eliminate oxygen limitations, we have omitted the second reaction step from the model mechanism for GlcOx. We have also included the following key assumptions: (1) a rapid equilibrium is achieved for the substrate-binding steps, (2) product formation steps are irreversible, and (3) no product inhibition occurs (Table 1 and Figure 1). In contrast to a typical allosteric model,[22] this proposed mechanism is capable of explaining the phenomenon of an extended lag period that occurs when the interconversion steps between ES (or E) and E′S (or E′) is slow relative to the product formation steps.[18] When substrate interacts with the enzyme, the enzyme-substrate (ES) complex concentration immediately reaches a high value, but the E′S concentration increases only gradually, resulting in a slower rate of product (P) formation. As the system then approaches the steady state, the E′S concentration reaches its maximum value with the concomitant product generation at the maximum rate.

Lag time phenomena and sensitivity analysis of the proposed model mechanism

One practical utility of sensitivity coefficients is in their ability to describe the changes (both in magnitude and direction) that occur to the predicted values of the state variables as associated with incremental perturbations in the parameter values. As with state variables of a dynamic model, sensitivity coefficients may also vary dynamically. Thus, it is important to characterize the dynamic sensitivity of a model to understand how, and under what temporal regions, each parameter may play a role in the overall predictions.

The presented model was solved using initial conditions of E and S of 1 and 1000 μM, respectively, whereas the initial values for all remaining state variables were taken to be 0. The initial conditions associated with each of the sensitivity coefficients were also appropriately determined using the initial values of the state variables. For simplification, the initial substrate concentration was assumed to be sufficiently excessive that its value should not change appreciably over the simulated range. For experiments, we used the initial substrate concentration of at least 220 mM, and the produced product concentration was never above 0.04 mM, justifying this assumption. As a result, $\frac{dS}{dt}$ was set to a value of zero for all times for the present study.

The effects of variations in *k2f* (and, appropriately, *k2r* as a constant equilibrium constant was maintained for this reaction step) on the overall model predictions was also investigated, and the results are compared in Figure 2. As *k2f* was

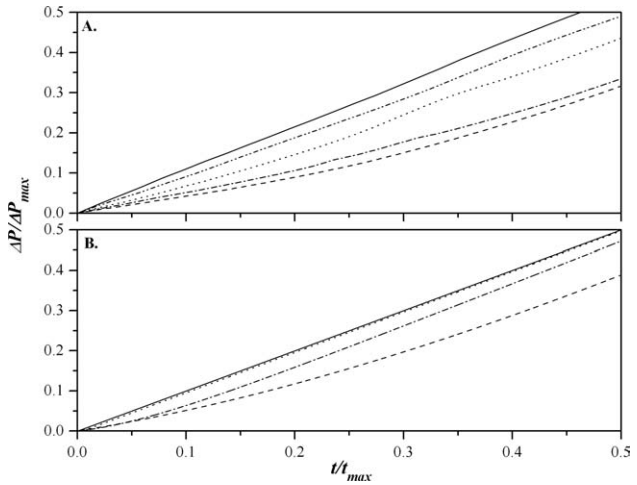

Figure 2. Comparison of the normalized change in product concentration as a function of dimensionless time as obtained experimentally (A.) for Des3-10 (dashed), Des3-2 (dot-dashed), Des3-7 (solid), Des3-6 (dot-dot-dash), and M-RQW (dotted), and as predicted with the presented model (B.) for *k2f* values of 0.005 (dashed), 0.05 (dot-dashed), 0.5 (dotted), and 5 s^{-1} (solid).

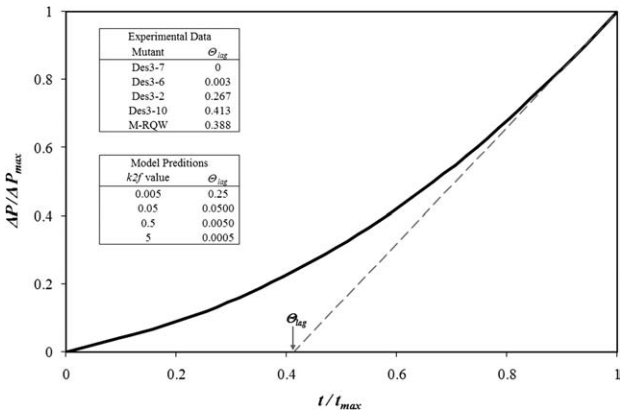

Figure 4. Approximation of the lag time, Θ_{lag}, associated with mutant Des3-10. Inset tables summarize the experimental lag times associated with each of the studied mutants, as well as the lag times associated with the model predictions as approximated via the same procedure.

decreased, the predicted lag phase associated with product (P) synthesis was found to increase. As is further illustrated in Figure 3, among the range of *k2f* values simulated, although initial rates of product formation were found to vary dramatically, each was found to approach the same steady-state value. This is similarly consistent with the results of Figure 2, wherein as t/t_{max} approaches its maximum value, each of the predicted curves arrives at the same constant slope.

The lag times associated with the experimental data and model simulations could also be directly approximated according to the method described above. These results are shown in Figure 4, together with an illustration of this approach. Whereas a range of lag times between 0 and 0.40 was observed to be obtained experimentally, we find that a similar range could be generated by varying the value of *k2f* from its nominal value of 0.005 s^{-1} to as high as 5 s^{-1}. Interestingly, the lag times of M-RQW or Des3-10, both of which contain the W290F mutation, were higher than that of Des3-2, Des3-6, or Des3-7, which each contain the intact Trp290 (Figure 4). Trp290 is known to play an important role in stabilizing the O radical of Tyr272.[5,6] Thus, the mutation of this residue is expected to affect the interconversion steps between ES (or E) and E′S (or E′). Delayed accumulation of the more active enzyme conformation would be expected to result in increased lag times (higher Θ_{lag} values).

The dynamic sensitivity of the selected model was subsequently characterized with respect to each of the parameters of Table 1. The predicted results with respect to P, the state variable of the highest interest to this study, are plotted in

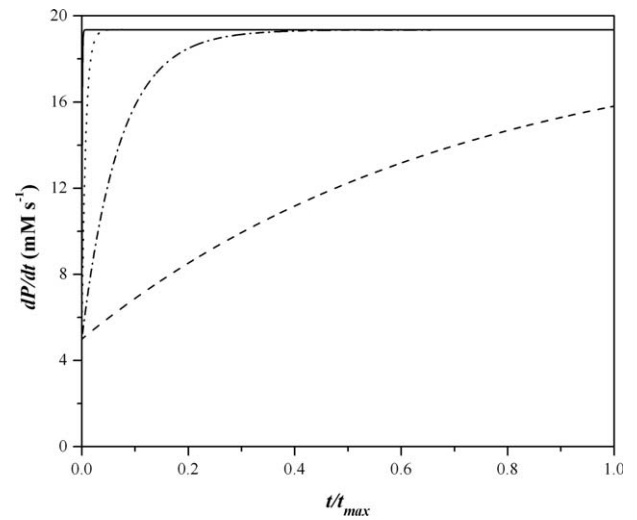

Figure 3. The rate of change in product concentration (P) with respect to time predicted as a function of dimensionless time for *k2f* values of 0.005 (dashed), 0.05 (dot-dashed), 0.5 (dotted), and 5 s^{-1} (solid).

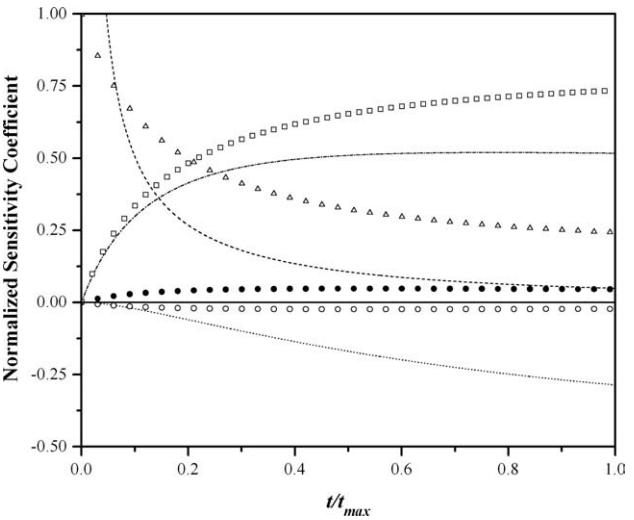

Figure 5. Normalized sensitivity coefficients predicted as a function of dimensionless time for *k1f* (dashed line), *k2f* (dot-dash line), *k2r* (dotted line), *k3f* (open circles), *k3r* (solid circles), *k4f* (open triangles), and *k5f* (open squares).

Figure 5. As is clearly seen, the impact of the predictions for individual parameters is found to vary greatly with respect to the state of the system. For instance, although the model is initially quite sensitive to the parameters *k1f* and *k4f*, as the system proceeds toward its steady-state, the influence of *k1f* diminishes and approaches zero. Conversely, while the model is initially predicted to be insensitive to both *k2f* and *k5f*, this condition quickly changes as these parameters become increasingly important to the prediction of *P*, particularly at the steady state. Similarly, predictions of *P* were also found to be increasingly sensitive to *k2r*, however in a direction opposite to that of *k2f* (indicative of their opposing reaction directions). Meanwhile, predicted values of *P* were found to be only mildly sensitive to both *k3f* and *k3r*. Finally, for all practical purposes, *P* was predicted to be insensitive to each of *k1r*, *k4r*, *k5r*, *k6f*, and *k6r* over the entire simulated range, including the lag period (data not shown).

From initialization ($t/t_{max} = 0$) through the preliminary stage of the reaction (i.e., the lag phase), predicted values of P were found to be rather sensitive to *k4f* and much less so to *k5f* (Figure 5). This prediction suggests that, during this stage, most of the product formation occurred through *k4f* and was thus derived from *ES*, not E′S. Toward the steady-state, however, we have predicted the opposite to be true. As seen in Figure 5, predicted values of P become increasingly sensitive to *k5f* and less so to *k4f* (though *k4f* still remains a significant parameter). Accordingly, this would suggest that, at steady-state (i.e., after the lag phase ends) more P is derived via the flux through *k5f* from E′S than is from ES. Overall, these predictions support the hypothesis that activation of the ES complex is an important step associated with the proposed mechanism and the function of this enzyme.

Discussion

Whereas the presented sensitivity analysis results are characteristic of the parameter values given in Table 1 (i.e., the location in the parameter space), the model and its sensitivity coefficients may also be solved for different sets of parameter values. Although we are free to explore the parameter space, we will confine our investigation to only physically relevant sets of parameters. From its initial value of 0.0001 s^{-1}, predictions of *P* remained almost completely insensitive to *k6f*. However, had *k6f* been 1000-times greater (i.e., estimated as 0.1 s^{-1}) we would have then found that *P* would be sensitive to *k6f* throughout the initial transient period, until approaching steady-state. From a mechanistic perspective, as *k6f* increases so does the rate of formation of E′ which, in turn, can lead to increased rates of E′S formation (through *k3r*, see Figure 1), and ultimately to *P* (by *k5f*). Thus, as the value of *k6f* increases, so does its importance relative to the formation of *P* (data not shown). On the other hand, if *k5f* were to be increased by a factor of 1000, for example, we would then find that predictions of *P* would be rendered essentially insensitive to the value of this parameter. In other words, *k5f* would become so large that small changes to its value would then have little overall effect on the flux through this step. Under such conditions, it would also follow that the model would become increasingly sensitive to those reactions, which supply the precursor E′S. Accordingly, our predictions suggest that the sensitivity of *P* to *k3r* and *k2f* would become greatly elevated under these conditions (data not shown).

No naturally-occurring glucose oxidase has yet been found which acts upon the C-6 hydroxyl group of glucose. Sun et al. generated glucose 6-oxidase (M-RQW),[8] but the kinetics of this mutant has never been studied intensively. We have generated and kinetically analyzed a set of glucose-6-oxidases and found that the W290F mutation was highly correlated with an observable delay in the initiation of glucose oxidation. Whereas the exact cause of this lag phenomenon remains unknown, here we have proposed a mechanistic model to describe the enzyme kinetics and performed sensitivity analysis as the first step toward understanding this behavior. We have also found that changes in the magnitude of the first order rate constants alone do not result in significant lag period for the established, wild-type mechanism with galactose. Our analyses have shown that the interconversion step between ES and E′S, which is responsible for activation of the ES complex, may be primarily responsible for the observed delay. Trp290 is known to protect the active site by restricting the entry of other molecules and by stabilizing the radical on Tyr272.[5,6] When Trp290 is replaced by Phe, a smaller residue, we expect a more accessible and less stable radical site, resulting in a much slower accumulation of E′S (as predicted by a larger *k2r* or, alternatively, a smaller *k2f*). As illustrated in Figure 2, the predicted results based on our proposed model mechanism are consistent with this explanation. It has also been observed that the W290H mutant displays almost the same value of K_m (on galactose) as that of the wild type GaOx, but that its k_{cat} value is more than three orders of magnitude lower than that of the wild type GaOx.[5] The fact that the steady-state model predictions were found to be more sensitive to the parameters associated with enzyme turnover (i.e., *k4f* and *k5f* which are both related to k_{cat}) than those parameters associated with the equilibrium steps (i.e., *k1f*, *k1r*, *k3f*, and *k3r*, which are all related to K_m) might be connected to this experimental result. Similarly, the newly created GlcOx containing the W290F mutation (M-RQW and Des3-10)[8] showed a very low GaOx activity.[10] This phenomenon may likewise be explained by the coexistence of the two enzyme conformations and the increased destabilization of the E′S form by the W290F mutation, leading to slower turnover. Finally, the observation that by arbitrarily changing the equilibrium constant of Reaction 1 (Figure 1, where K1 = *k1r*/*k1f*) from a nominal value of 250 to 1/250 μM imposed no significant effect to our predictions of the lag profile or the normalized sensitivity coefficients (data not shown) further supports our finding that the model predictions are less sensitive to the equilibrium steps associated with the substrate, enzyme, and substrate-enzyme complexes than the turnover steps.

Conclusions

We constructed and characterized mutants of GaOX to utilize glucose as a substrate, which may find use in biosynthetic pathways.[10] As the first step toward understanding these new enzymes, we proposed a kinetic model and performed sensitivity analysis. With this model, we were able to explain the relationship between the lag phenomenon and one of the key amino acid residues for catalysis. Whereas detailed kinetic, spectroscopic, and structural studies will be required to broaden our understanding of these newly created GlcOx enzymes, especially with respect to different

conformations (E and E′), our current work would serve as the first blueprint to this end.

Acknowledgments

This work was supported by the Synthetic Biology Engineering Research Center (SynBERC) funded by the National Science Foundation (Grant No. EEC-0540879). We acknowledge Prof. Frances Arnold at Caltech for providing plasmid DNA containing the M-RQW gene.

Literature Cited

1. Ito N, Phillips SE, Stevens C, Ogel ZB, McPherson MJ, Keen JN, Yadav KD, Knowles PF. Novel thioether bond revealed by a 1.7 A crystal structure of galactose oxidase. *Nature* 1991;350:87–90.
2. Ito N, Phillips SE, Yadav KD, Knowles PF. Crystal structure of a free radical enzyme, galactose oxidase. *J Mol Biol*. 1994;238:794–814.
3. Humphreys KJ, Mirica LM, Wang Y, Klinman JP. Galactose oxidase as a model for reactivity at a copper superoxide center. *J Am Chem Soc*. 2009;131:4657–4663.
4. Whittaker MM, Ballou DP, Whittaker JW. Kinetic isotope effects as probes of the mechanism of galactose oxidase. *Biochemistry* 1998;37:8426–8436.
5. Baron AJ, Stevens C, Wilmot C, Seneviratne KD, Blakeley V, Dooley DM, Phillips SEV, Knowles PF, McPherson MJ. Structure and Mechanism of Galactose Oxidase - the Free-Radical Site. *J Biol Chem*. 1994;269:25095–25105.
6. Saysell CG, Barna T, Borman CD, Baron AJ, McPherson MJ, Sykes AG. Properties of the Trp290His variant of Fusarium NRRL 2903 galactose oxidase: Interactions of the GOase(semi) state with different buffers, its redox activity and ability to bind azide. *J Biol Inorg Chem*. 1997;2:702–709.
7. Sun LH, Petrounia IP, Yagasaki M, Bandara G, Arnold FH. Expression and stabilization of galactose oxidase in Escherichia coli by directed evolution. *Prot Eng*. 2001;14:699–704.
8. Sun LH, Bulter T, Alcalde M, Petrounia IP, Arnold FH. Modification of galactose oxidase to introduce glucose 6-oxidase activity. *Chembiochem* 2002;3:781–783.
9. Wachter RM, Branchaud BP. Molecular modeling studies on oxidation of hexopyranoses by galactose oxidase. An active site topology apparently designed to catalyze radical reactions, either concerted or stepwise. *J Am Chem Soc*. 1996;118:2782–2789.
10. Lippow SM, Moon TS, Basu S, Yoon SH, Li X, Chapman BA, Robison K, Lipovsek D, Prather KL. Engineering enzyme specificity using computational design of a defined-sequence library. *Chem Biol*. 2010;17:1306–1315.
11. Palumbo A, Misuraca G, D'Ischia M, Prota G. Effect of metal ions on the kinetics of tyrosine oxidation catalysed by tyrosinase. *Biochem J*. 1985;228:647–651.
12. Xue CB, Luo WC, Jiang L, Xie XY, Xiao T, Yan L. Inhibition kinetics of cabbage butterfly (Pieris rapae L.) larvae phenoloxidase activity by 3-hydroxy-4-methoxybenzaldehyde thiosemicarbazone. *Appl Biochem Biotechnol*. 2007;143:101–114.
13. Wang ZX, Killilea SD, Srivastava DK. Kinetic evaluation of substrate-dependent origin of the lag phase in soybean lipoxygenase-1 catalyzed reactions. *Biochemistry* 1993;32:1500–1509.
14. Schilstra MJ, Veldink GA, Vliegenthart JF. Kinetic analysis of the induction period in lipoxygenase catalysis. *Biochemistry* 1993;32:7686–7691.
15. Winterbourn CC, Peskin AV, Parsons-Mair HN. Thiol oxidase activity of copper, zinc superoxide dismutase. *J Biol Chem*. 2002;277:1906–1911.
16. Gukasyan GS. Study of the kinetics of oxidation of monophenols by tyrosinase. The effect of reducers. *Biochemistry (Mosc)*. 2002;67:277–280.
17. Molina FG, Munoz JL, Varon R, Lopez JN, Canovas FG, Tudela J. An approximate analytical solution to the lag period of monophenolase activity of tyrosinase. *Int J Biochem Cell Biol*. 2007;39:238–252.
18. Bates DJ, Frieden C. Treatment of enzyme kinetic data. 3. The use of the full time course of a reaction, as examined by computer simulation, in defining enzyme mechanisms. *J Biol Chem*. 1973;248:7878–7884.
19. Dickinson RP, Gelinas RJ. Sensitivity analysis of ordinary differential equation systems - direct method. *J Comput Phys*. 1976;21:123–143.
20. Mauch K, Arnold S, Reuss M. Dynamic sensitivity analysis for metabolic systems. *Chem Eng Sci*. 1997;52:2589–2598.
21. Patnaik PR. Dynamic sensitivity of a chemostat for a microbial reaction with substrate and product inhibition. *Appl Math Model*. 1994;18:620–627.
22. Monod J, Wyman J, Changeux JP. On the nature of allosteric transitions: a plausible model. *J Mol Biol*. 1965;12:88–118.

Manuscript received Apr. 19, 2011, and revision received July 31, 2011.