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Dimerization and Bifunctionality Confer Robustness to the Isocitrate Dehydrogenase Regulatory System in *Escherichia coli* *[S]

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Background: Robustness of the *Escherichia coli* isocitrate dehydrogenase (IDH) regulatory system has been experimentally demonstrated.
Results: A biochemically and structurally realistic mathematical model of the IDH system was developed.
Conclusion: The model identifies features of the system, including homodimerization of IDH and bifunctionality of its regulatory enzyme, that lead to robustness.
Significance: Mathematical modeling can connect enzymology and systems biology.

An important goal of systems biology is to develop quantitative models that explain how specific molecular features give rise to systems-level properties. Metabolic and regulatory pathways that contain multifunctional proteins are especially interesting to study from this perspective because they have frequently been observed to exhibit robustness: the ability for a system to perform its proper function even as levels of its components change. In this study, we use extensive biochemical data and algebraic modeling to develop and analyze a model that shows how robust behavior arises in the isocitrate dehydrogenase (IDH) regulatory system of *Escherichia coli*, which was shown in 1985 to experimentally exhibit robustness. *E. coli* IDH is regulated by reversible phosphorylation catalyzed by the bifunctional isocitrate dehydrogenase kinase/phosphatase (IDHKP), and the level of IDH activity determines whether carbon flux is directed through the glyoxylate bypass (for growth on two-carbon substrates) or the full tricarboxylic acid cycle. Our model, which incorporates recent structural data on IDHKP, identifies several specific biochemical features of the system (including homodimerization of IDH and bifunctionality of IDHKP) that provide a potential explanation for robustness. Using algebraic techniques, we derive an invariant that summarizes the steady-state relationship between the phospho-forms of IDH. We use the invariant in combination with kinetic data on IDHKP to calculate IDH activity at a range of total IDH levels and find that our model predicts robustness. Our work unifies much of the known biochemistry of the IDH regulatory system into a single quantitative framework and highlights the importance of constructing biochemically realistic models in systems biology.

During growth on a two-carbon substrate, *Escherichia coli* diverts significant carbon flux to the glyoxylate bypass, an anapleurotic pathway that circumvents two decarboxylation steps in the tricarboxylic acid cycle (1). The flux through the glyoxylate bypass is determined by the level of active isocitrate dehydrogenase (IDH),[2] a key tricarboxylic acid cycle enzyme that is regulated by reversible phosphorylation by the bifunctional isocitrate dehydrogenase kinase/phosphatase (IDHKP). Phosphorylation by IDHKP inactivates IDH, limiting the synthesis of α-ketoglutarate and directing flux through the bypass. When *E. coli* is transferred to a substrate containing more than two carbons, IDHKP dephosphorylates IDH to reactivate the tricarboxylic acid cycle as the primary metabolic pathway. Maintaining precise control of IDH activity is thus essential for the ability of *E. coli* to switch between growth on two-carbon and other substrates. Phosphorylation of IDH by IDHKP has also served as a classic model system for studying the role of protein post-translational modification in complex cellular processes, and a number of fundamental biochemical discoveries were made from examination of the IDH regulatory system. IDH was the first substrate identified for a prokaryotic protein kinase, establishing that protein phosphorylation is not restricted to eukaryotes (2). IDHKP was one of the earliest examples of a multifunctional enzyme to be discovered and characterized (3), and the IDH regulatory system provided the original experimental basis for enzymatic zero-order ultrasensitivity (4).

Because of the physiological importance of the glyoxylate bypass and their unusual biochemical characteristics, IDH and IDHKP have been extensively studied using methods from classical biochemistry and molecular biology, as reviewed in Refs. 5–7. In a study of particular interest, in 1985, LaPorte *et al.* (8) provided experimental evidence that the IDH regulatory system exhibits robustness: the ability of a biochemical network to perform its correct function even as levels of the chemical species in the network fluctuate. During growth on acetate, IDH

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[2] The abbreviations used are: IDH, isocitrate dehydrogenase; IDHKP, isocitrate dehydrogenase kinase/phosphatase; ACR, absolute concentration robustness.

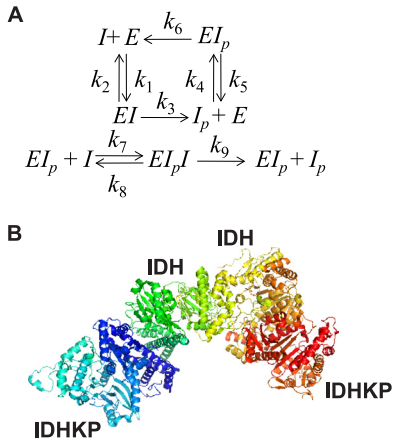

FIGURE 1. **The model of Shinar *et al.* (15) of the IDH regulatory system and the recent structure of IDHKP.** *A*, Shinar *et al.* (15) proposed the above chemical reaction network to describe how IDHKP (*E*) interacts with active (*I*) and inactive (I_p) IDH to regulate the *E. coli* glyoxylate bypass. IDHKP can form two different binary complexes with IDH, *EI*, in which *E* acts as a kinase, and EI_p, in which *E* acts as a phosphatase. Two molecules of IDH can also form a ternary complex (EI_pE) with IDHKP. To simplify the analysis of their model, Shinar *et al.* (15) assumed that EI_pE does not have phosphatase activity and can only form in an ordered fashion (*i.e.* EI_p can bind to *I*, but *EI* cannot bind to I_p). *B*, structure of two IDHKP molecules (*blue*/*cyan* and *red*/*orange*) bound to an IDH dimer (*green* and *yellow*) obtained by Zheng and Jia (19). Their structural results indicate that a ternary complex can be formed between two IDHKP molecules and one IDH dimer, but not between one IDHKP and two IDH molecules, as in the above network.

activity varied minimally even as the total amount of IDH present fluctuated 1.8-fold (natural variation between strains) and more than 20-fold (between natural levels and overproduction from a plasmid) (8).

This type of robust behavior is not unique to the IDH regulatory system. Similar experimental observations have been made for the nitrogen assimilation system and the EnvZ/OmpR two-component osmoregulatory system of *E. coli* and the carbon fixation pathway of C_4 plants (9–11). A distinctive feature of these systems is that (like the *E. coli* glyoxylate bypass) each contains a bifunctional enzyme that catalyzes opposing post-translational modifications. These experimental results strongly suggest that bifunctional enzymes tend to confer robustness to biochemical processes. There have been long-standing efforts to understand this connection between bifunctionality and robustness theoretically, beginning with the suggestion by Russo and Silhavy (12) that the robustness of the EnvZ/OmpR system is due specifically to the bifunctionality of EnvZ and the Batchelor and Goulian model (10) of that system. More recently, a desire to explore the bifunctionality-robustness connection from a quantitative, systems biology perspective has motivated the development of mathematical models of the EnvZ/OmpR, IDH regulatory, nitrogen assimilation, and carbon fixation systems (13–17). Each of these studies relies on mathematical and numerical analysis of the underlying chemical reaction networks to derive expressions that predict robust behavior.

The recent study by Shinar *et al.* (15) of IDH robustness is representative of these systems-level analyses of bifunctional enzymes. They proposed a detailed chemical reaction network to describe how IDHKP interacts with IDH to regulate the glyoxylate bypass (Fig. 1*A*). At the center of their network is the ternary complex EI_pI, where *E* denotes IDHKP and *I* and I_p denote active and inactive IDH, respectively. The putative formation of such a ternary complex is a direct consequence of having a bifunctional enzyme that can simultaneously bind two molecules of its substrate. Using mass-action kinetics, Shinar *et al.* (15) derived a mathematical expression that shows that the ternary complex gives rise to approximately robust behavior in the IDH regulatory system. On the basis of this result, they concluded that their model provides an explanation for the experimental observation of robustness by LaPorte *et al.* (8).

The reaction network of Shinar *et al.* (15) (Fig. 1*A*) makes two major assumptions about the biochemistry of the IDH regulatory system. Formation of the ternary complex EI_pI requires that one molecule of IDHKP be able to simultaneously bind one phosphorylated and one unphosphorylated IDH, which implies that IDHKP must have separate active sites for its kinase and phosphatase activities. Additionally, although IDH is known to form an obligate homodimer *in vivo* (18), Shinar *et al.* (15) opted to model it as a monomer. When Shinar *et al.* proposed their model, the crystal structure of IDHKP had not been determined, and the exact configuration of its active sites was an open question. However, detailed structural information about IDHKP has since been published (19), revealing that the enzyme employs a single site for both its kinase and its phosphatase activities and switches between them using a conformational shift (Fig. 1*B*). It follows from these data that the ternary complex proposed by Shinar *et al.* (15) cannot form and that their model needs to be amended. In this study, we show that these structural data and other biochemical information about IDH and IDHKP lead to a different model of the *E. coli* IDH regulatory system. Our model assumes that the IDHKP kinase and phosphatase reactions are catalyzed from the same active site and that dimerization of IDH is an important feature of the system. From mass-action analysis of our model, we derive a polynomial expression, an invariant, that relates the steady-state levels of the phospho-forms of the IDH dimer. The key invariant is obtained using algebraic techniques that do not require assigning numerical values to any of the parameters (*i.e.* the rate constants in the system). This approach allows us to develop a model that accurately reflects the biochemical complexity of the IDH regulatory system. We then study the behavior of the invariant numerically, given several experimentally justified assumptions about the system. Under these conditions, we find that the invariant predicts the robustness of active IDH to changes in total IDH levels, in agreement with existing experimental results.

EXPERIMENTAL PROCEDURES

Evidence for a Shared Active Site on IDHKP and Dimerization of IDH—Here we outline the biochemical justification for the two major assumptions (shared active site on IDHKP and dimerization of IDH) of our model. Prior to the determination of the crystal structure of IDHKP, a number of mutagenesis studies suggested that the kinase and phosphatase activities might share a single active site. For instance, Stueland *et al.* (20) found that mutation of a lysine residue in the ATP binding domain severely compromised both the kinase and the phosphatase activity of IDHKP. This result suggested that the two

activities rely on the same ATP binding site, making it unlikely that the active sties could be located in structurally distinct regions. (This hypothesis was also supported by a photoaffinity labeling study (21).) A kinetic study of two IDHKP mutants provided further evidence for the existence of a shared active site (22). The V_{max} for the phosphatase reaction was shown to be inversely related to the kinase V_{max}, suggesting that the activities are closely linked and possibly differentiated by a conformational shift (22). Supporting these earlier experiments, the IDHKP structure of Zheng and Jia (19) conclusively demonstrated that the active site is shared. IDHKP was found to have two domains, a regulatory domain and a catalytic domain where ATP binds and the kinase and phosphatase activities take place. AMP, a potent inhibitor of IDHKP kinase activity, binds between the domains and induces a conformational change to promote phosphatase activity. This structural information reveals that it is impossible for an IDHKP molecule to act simultaneously as a kinase and a phosphatase.

Additionally, *E. coli* IDHKP is known to bind only to intact, dimeric IDH (23). This absolute substrate specificity (which has recently been characterized on a structural level (24)) is so strong that replacing *E. coli* IDH with *Bacillus subtilis* IDH, which dimerizes and exhibits extensive sequence and structural homology with *E. coli* IDH, reduces the K_m of IDHKP 60-fold for kinase activity and 3,450-fold for phosphatase activity (25). In addition to characterizing isolated IDHKP, Zheng and Jia (19) also reported the structure of an IDH homodimer with an IDHKP bound to each half of the dimer. This structure revealed that the substrate recognition loop of IDHKP interacts extensively with both halves of the dimer, providing an explanation for why IDHKP fails to recognize monomeric IDH. These data strongly suggest that dimerization of IDH is essential to its function in the *E. coli* glyoxylate bypass.

Model of IDH Regulation in E. coli—We now describe our model of the IDH regulatory system and the corresponding reaction network (see Fig. 2). The model makes several standard assumptions. We assume that all reactions follow a Michaelis-Menten scheme. Accordingly, we do not explicitly account for molecules of ATP or the ATPase activity of IDHKP. ATP is assumed to be maintained in excess at a constant concentration, and levels of ADP and P_i are also assumed to be constant. These assumptions are discussed in detail in Ref. 26. We treat IDH as a homodimer that can take one of three forms, I_{oo} (neither monomer phosphorylated), I_{op} (one monomer phosphorylated), and I_{pp} (both monomers phosphorylated), and we assume that all IDH is dimerized (18). Because no cooperative effects are known to occur between the two halves of the IDH homodimer (27), we consider each phosphorylation to be an independent event and do not distinguish between the monophosphorylated forms (*i.e.* $I_{op} = I_{po}$). Each IDH monomer can bind to one IDHKP (denoted as E), leading to the formation of three different ternary complexes: $EI_{oo}E$, $EI_{op}E$, and $EI_{pp}E$. Thus, in our notation, the enzyme-substrate complex studied by Zheng and Jia (19) is $EI_{oo}E$. Each of these ternary complexes has one (dimeric) IDH attached to two molecules of IDHKP, the opposite of the ternary complex in the model of Shinar *et al.* (15), which consisted of one IDHKP bound to two units of IDH. Our model includes all possible binary and ternary complexes that can form between IDHKP and the various states of IDH. Additionally, IDHKP is allowed to act as either a kinase or a phosphatase when bound in a ternary complex. Because the two halves of the IDH dimer are independent, we assume that simultaneous double modifications (*e.g.* $EI_{oo}E$ going to $EI_{pp}E + E$ or $EI_{pp}E$ going to $EI_{oo}E + E$) are unlikely. We exclude such reactions from the model.

We also assume that the conformational change required for IDHKP to switch between kinase and phosphatase activity occurs on a much faster time scale than the catalytic steps ($k_{cat} = 9.8 \times 10^{-2}\ s^{-1}$ for kinase activity and $k_{cat} = 6.5 \times 10^{-2}\ s^{-1}$ for phosphatase activity, calculated from data in Ref. 22). Therefore, we do not include a reaction representing the shift between the IDHKP kinase and phosphatase activities. The two conformations are assumed to be in rapid equilibrium before the formation of each binary or ternary complex. Although the kinetics of the IDHKP shift have not been studied, similar conformational changes for other enzymes are known to occur many orders of magnitude faster than the IDHKP catalytic steps, supporting our assumption (28).

Mass-action Analysis and Algebraic Calculations—Mass-action analysis of the reaction network in Fig. 2 yields a system of 10 ordinary differential equations (listed in Table 1). At steady state, these 10 differential equations give 10 polynomial equations, $f_1 = 0, \ldots, f_{10} = 0$, that involve the 10 species. For clarity, the species are written as $x_1, \ldots, x_{10}$ (defined in Table 1) in Table 1 and in the supplemental material.

Conclusions are based on mathematical analysis of the polynomial system and calculations involving the derived algebraic expressions. Symbolic algebraic and numerical calculations were performed using Mathematica 8.0 (Wolfram Research). A printout of a Mathematica notebook detailing the calculations is provided in the supplemental material. The actual notebook is available on request from the authors. Computation of reaction network deficiency was performed in MATLAB 7.12.0 (The MathWorks, Inc.) using a custom program developed by Guy Shinar and Eran Eden (Reaction Network Analyzer V1.0).

Gröbner basis calculations were performed following the procedure described by Manrai and Gunawardena (29). To derive Equation 2, a lexicographically ordered Gröbner basis was computed for the 10 polynomial equations in Table 1 with the variables ordered as $x_4, x_5, x_6, x_7, x_8, x_9, x_1, x_3, x_{10}, x_2$.

Parameter Estimation—Parameters for the numerical calculations were determined from kinetic data on IDHKP obtained by Miller *et al.* (22). The parameters in the model ($k_1, \ldots, k_{20}$) fall into six categories, on-rate, off-rate, and catalytic rate for IDHKP acting as a kinase, and the same for IDHKP acting as a phosphatase. We assumed that the on-rates are determined by the diffusion-limited interaction of IDH and IDHKP and set both at $1 \times 10^8\ M^{-1}\ s^{-1}$, a typical value for protein-protein diffusion-limited interactions (30). We set the catalytic rate constants to the k_{cat} values measured for IDHKP kinase and IDHKP phosphatase (22). Finally, we calculated the off-rates from the on-rates, catalytic rates, and Michaelis-Menten constants of Miller *et al.* (22) according to the formula

$$K_m = \frac{k_{off} + k_{cat}}{k_{on}} \qquad \text{(Eq. 1)}$$

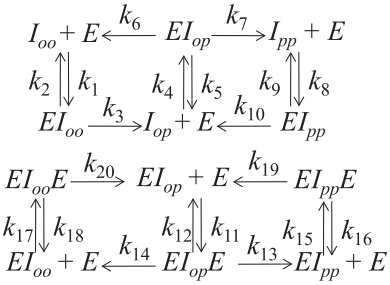

FIGURE 2. **Proposed model of the IDH regulatory system incorporating substrate dimerization.** In our model, IDH is treated as a homodimer that can bind two molecules of IDHKP, whereas IDHKP is assumed to have a single active site and be incapable of simultaneously binding two molecules of IDH. IDH and IDHKP can form a variety of binary and ternary complexes, all of which are represented in the network. No restrictions are placed on the activity of IDHKP in the ternary complexes or the order of complex formation. Accordingly, this is the most complete network that is consistent with what is currently known about the biochemistry of IDH and IDHKP.

RESULTS

Derivation of the Key Invariant—As described under "Experimental Procedures," we applied steady-state mass-action kinetics to the reaction network in Fig. 2 to obtain the system of polynomial equations in Table 1. From algebraic analysis of this polynomial system, we find that there is a polynomial expression, an invariant, involving only these three variables that holds in any positive steady state of the system (*i.e.* any steady state in which all 10 variables take strictly positive values). It has the form

$$I_{oo}^2 + a_2 I_{op}^2 + a_3 I_{pp}^2 + a_4 I_{oo} I_{op} + a_5 I_{oo} I_{pp} + a_6 I_{op} I_{pp} = 0 \quad \text{(Eq. 2)}$$

where $a_2, \ldots, a_6$ are algebraic combinations of the rate constants, normalized so that the coefficient of I_{oo}^2 is 1. The full expressions for the coefficients are given in the supplemental material. The above invariant provides important constraints on the levels of the three IDH phospho-forms at steady state and sheds light on the experimentally observed robustness of the IDH regulatory system, as discussed in the following section.

Equation 2 is calculated by a method previously described in Ref. 29, as shown in the supplemental material. Briefly, by algebraically combining the polynomial equations, $f_1 = 0, \ldots, f_{10} = 0$, it may be possible to eliminate some of the variables and derive equations that only depend on a specified subset of variables $x_{i1}, \ldots, x_{ik}$. Gröbner bases provide a systematic way to do this and can be thought of as a polynomial generalization of Gaussian elimination for linear systems (29). Crucially, such elimination can be done with the rate constants treated as uninterpreted algebraic symbols that do not need to be given numerical values. The coefficients of the equations are then rational algebraic expressions in the symbolic rate constants. For instance, it might be possible to obtain a linear equation of the form $I_{oo} = K$, where K is some rational algebraic expression in the rate constants. Such an equation would imply that the concentration of unphosphorylated IDH is constant at steady state, irrespective of how much total IDH (T) or total IDHKP is present. This is "absolute concentration robustness" (ACR), as defined by Shinar and Feinberg (31). Although a demonstration of ACR would account for the robustness found experimentally, we show using Gröbner bases that there is no such equation involving only I_{oo} (supplemental material). Instead, the invariant in Equation 2 emerges as the simplest algebraic relationship between the three IDH phospho-forms (I_{oo}, I_{op}, and I_{pp}) that holds in any positive steady state.

Numerical Prediction of Robustness from the Main Invariant—In this section, we show numerically that, given several reasonable assumptions, our invariant predicts the robustness of I_{oo} to T. For the simulations, we use parameters estimated from the kinetic data of Miller *et al.* (22) as described under "Experimental Procedures" (Table 2). Our assignment of parameter values assumes that reactions are not influenced by the phosphorylation state or bound IDHKP on the other half of the IDH dimer. As such, reactions in a particular class (*e.g.* all the kinase catalytic reactions) are assumed to be chemically similar and are given the same rate constant (k_{cat}, for the kinase catalytic rate). This reduces the number of independent parameters from 20 to six. We first consider how the invariant constrains I_{oo} at various I_{op} and I_{pp} values with the parameters fixed as defined in Table 2. Fig. 3*A* shows a plot of I_{oo} as a function of the two other phospho-forms. I_{oo} varies approximately linearly with I_{op} but remains nearly constant at any fixed I_{op} even as I_{pp} changes. This behavior can be confirmed from examination of $\partial I_{oo}/\partial I_{pp}$. As shown in Fig. 3*B*, $\partial I_{oo}/\partial I_{pp}$ is close to zero for all I_{op} and I_{pp} values, confirming that I_{oo} remains constant when I_{op} is fixed.

These results suggest that our model predicts the robustness of active IDH when I_{op} remains constant. The limited experimental data on monophosphorylated IDH appear to support this requirement. Borthwick *et al.* (32) attempted a systematic characterization of IDH extracted from *E. coli* growing on glycerol. They readily isolated both fully active (doubly unphosphorylated) and fully inactive (doubly phosphorylated) IDH, but found no monophosphorylated IDH. The authors speculated that the absence of detectable I_{op} might be due in part to disproportionation of the phosphate during purification. Such behavior, however, would imply that the phosphates in singly phosphorylated dimers are markedly more labile than those in doubly phosphorylated dimers, which survived purification in abundance. Given that the halves of the IDH dimer are known to be independent, it appears unlikely that the phospho-state of one half could influence the stability of a phosphate on the other half. We believe instead that the inability of Borthwick *et al.* (32) to isolate monophosphorylated IDH reflects that very low but nonzero levels of I_{op} are present at steady state. I_{op} must be present in a nonzero concentration because otherwise I_{pp} could only be formed through simultaneous double phosphorylations, which is also unlikely. This view is further supported by the identification by LaPorte *et al.* (8) of only two phosphoforms during their overexpression experiments and the apparent absence of any physiological role for monophosphorylated IDH. On the basis of this evidence, we suggest that I_{op} is maintained at a consistently low (and therefore approximately constant) level, which we denote as ϵ.

We can now make use of the conservation law for IDH species. Cellular IDHKP levels have been measured to be roughly 1000-fold lower than IDH levels (32, 33). As such, we can write the approximate conservation law

TABLE 1

Mass-action analysis of the IDH regulatory system

Table 1 lists the 10 ordinary differential equations that describe the behavior of the chemical reaction network underlying our model. At steady state, these differential equations all equal zero and become the system of algebraic equations that is studied in detail in this paper. For clarity, the 10 species and complexes appearing in the reaction network are written as $x_1, \ldots, x_{10}$ in the table and in the supplemental material, and the 10 steady-state polynomial equations are referred to as $f_1, \ldots, f_{10}$.

species	variable	ordinary differential equation	name
E	x_1	$dx_1/dt = -k_1x_1x_2 + (k_2 + k_3)x_4 - k_4x_1x_3 + (k_5 + k_6 + k_7)x_6 - k_{15}x_1x_5 +$ $k_{17}x_1x_4 - k_{11}x_1x_6 + (k_9 + k_{10})x_5 - k_8x_1x_{10} + (k_{18} + k_{20})x_7 +$ $(k_{12} + k_{13} + k_{14})x_8 + (k_{16} + k_{19})x_9$	f_1
I_{oo}	x_2	$dx_2/dt = -k_1x_2x_1 + k_2x_4 + k_6x_6$	f_2
I_{op}	x_3	$dx_3/dt = k_3x_4 - k_4x_1x_3 + k_5x_6 + k_{10}x_5$	f_3
EI_{oo}	x_4	$dx_4/dt = k_1x_1x_2 - (k_2 + k_3)\,x_4 + k_{14}x_8 - k_{17}x_1x_4 + k_{18}x_7$	f_4
EI_{pp}	x_5	$dx_5/dt = k_8x_1x_{10} - (k_{10} + k_9)\,x_5 + k_{13}x_8 - k_{15}x_1x_5 + k_{16}x_9$	f_5
EI_{op}	x_6	$dx_6/dt = k_4x_1x_3 - (k_6 + k_5 + k_7)\,x_6 - k_{11}x_1x_6 + k_{12}x_8 + k_{19}x_9 + k_{20}x_7$	f_6
$EI_{oo}E$	x_7	$dx_7/dt = k_{17}x_1x_4 - (k_{20} + k_{18})\,x_7$	f_7
$EI_{op}E$	x_8	$dx_8/dt = k_{11}x_1x_6 - (k_{12} + k_{13} + k_{14})\,x_8$	f_8
$EI_{pp}E$	x_9	$dx_9/dt = k_{15}x_1x_5 - (k_{19} + k_{16})\,x_9$	f_9
I_{pp}	x_{10}	$dx_{10}/dt = k_7x_6 - k_8x_1x_{10} + k_9x_5$	f_{10}

TABLE 2

Parameter values used in the simulations

Table 2 gives the numerical values for the rate constants used in the calculations with the main invariant. k_{cat} values for the kinase and phosphatase activities of IDHKP are taken from the kinetic data of Miller *et al.* (22). On-rates were estimated assuming diffusion-limited binding, and off-rates were calculated from the other parameters and Michaelis-Menten constants published by Miller *et al.* (22).

parameter	kinase	rate constants	parameter	phosphatase	rate constants
k_{on}	$1 * 10^8\ M^{-1}s^{-1}$	k_1, k_4, k_{11}, k_{17}	κ_{on}	$1 * 10^8\ M^{-1}s^{-1}$	k_8, k_{15}
k_{off}	$23\ s^{-1}$	k_2, k_5, k_{12}, k_{18}	κ_{off}	$40\ s^{-1}$	k_9, k_{16}
k_{cat}	$9.8 * 10^{-2}\ s^{-1}$	k_3, k_7, k_{13}, k_{20}	κ_{cat}	$6.5 * 10^{-2}\ s^{-1}$	$k_6, k_{10}, k_{14}, k_{19}$
K_M	$2.3 * 10^{-7}\ M$		κ_M	$4 * 10^{-7}\ M$	

$$T = I_{oo} + I_{op} + I_{pp} \qquad \text{(Eq. 3)}$$

in which the complexes between IDH and IDHKP have been neglected. Using Equation 3, we write I_{pp} in Equation 2 in terms of total IDH (T), I_{oo}, and I_{op}, set all I_{op} terms to ϵ, and solve for I_{oo}. This procedure yields a long expression for I_{oo} as a function of only T, given in the supplemental material. As shown in Fig. 3*C*, I_{oo} varies minimally with T assuming the parameter values given in Table 2 and some fixed value for ϵ. In contrast, using the conservation law to eliminate I_{oo} from the invariant and I_{pp} as a function of T reveals that I_{pp} is linearly dependent on total IDH. These computational results are in agreement with the experimental data on IDH robustness (Fig. 3*D*) and demonstrate that our model provides an explanation for the observed robustness.

The Role of the Rate Constants in the Robustness—Inspection of Equation 2 suggests that robustness arises for the following reason. When the rate constants are assigned the values given in Table 2, $|a_5|$ and $|a_6|$, are at least 30 times larger than $|a_2|$, $|a_3|$, and $|a_4|$ (Table 3). This suggests that the first four terms in Equation 2 can be neglected, leaving only

$$a_5 I_{oo} I_{pp} + a_6 I_{op} I_{pp} = 0 \qquad \text{(Eq. 4)}$$

which simplifies to

$$I_{oo} = -\frac{a_6}{a_5} I_{op} \qquad \text{(Eq. 5)}$$

Equation 5 indicates that I_{oo} will remain constant at any fixed I_{op} regardless of the value of I_{pp}, in agreement with the behavior observed from inspection of Fig. 3*A*.

The coefficients $a_2, \ldots a_6$ are complicated polynomials, normalized by a_1, in the six independent parameters, k_{on}, k_{off}, and k_{cat} for the kinase and κ_{on}, κ_{off}, and κ_{cat} for the phosphatase. We recall that a polynomial is a sum of monomials. For a_2, a_3, and a_4, we found on inspection that each monomial in the corresponding polynomial has a relatively small numerical value when evaluated at the reference parameter values in Table 2. All monomials lie in the range $[-200, 200]$, and no one monomial is dominant. In contrast, the coefficients a_5 and a_6 each have a pair of monomials that are much larger (by a factor of nearly 50) than all the others. Setting aside the two monomials in each of these pairs, the remaining monomials in a_5 and a_6 all lie in the range $[-200, 200]$. Taking the two dominant monomials in a_5 yields the expression $k_{cat}\alpha$, and doing the same in a_6 yields the expression $-\kappa_{cat}\alpha$ where

$$\alpha = \kappa_{cat}\kappa_{off}k_{off}^4\kappa_{on}k_{on}(\kappa_{off}k_{on} - k_{off}\kappa_{on}) \qquad \text{(Eq. 6)}$$

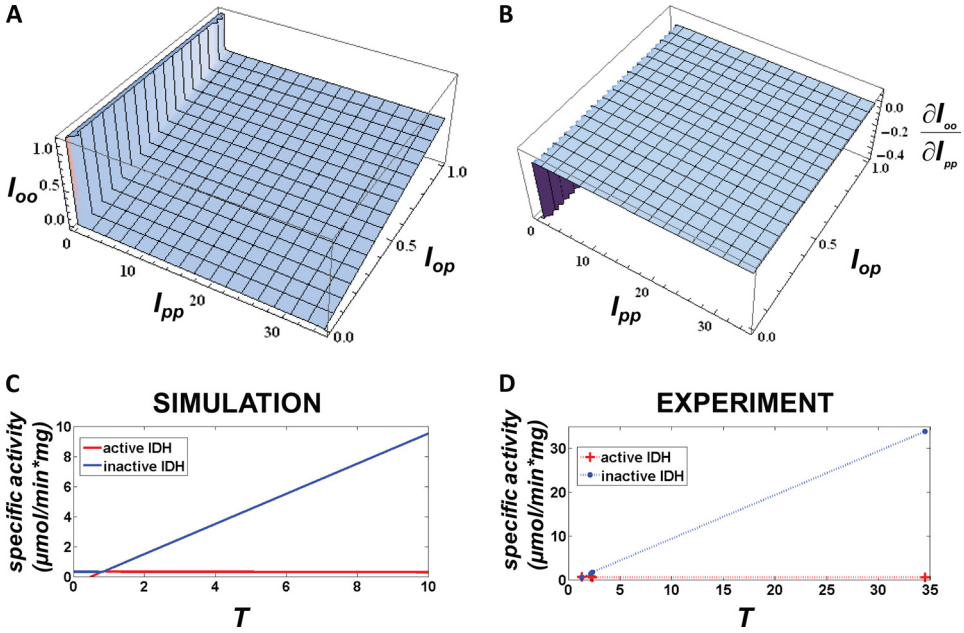

FIGURE 3. **Calculations with the key invariant predict robustness of active IDH.** *A*, three-dimensional plot of I_{oo} as a function of I_{op} and I_{pp} assuming the parameter values given in Table 2. *B*, three-dimensional plot of $\partial I_{oo}/\partial I_{pp}$ showing that it is approximately zero. *C*, plot of I_{oo} and I_{pp} as a function of *T* for a fixed I_{op} value as calculated from the invariant. *D*, plot of LaPorte *et al*. (8) experimental values for active and inactive IDH at various *T* values between 1.1 and 34.5 units. The *straight line* that interpolates between the widely separated data points is only suggestive. However, the simulation in *C* shows straight line behavior in the model. IDH activity was determined by monitoring reduction of NADP; one unit of IDH corresponded to the reduction of 1 μmol of NADP/min (8).

TABLE 3

Normalized coefficient values for Equation 2

Table 3 gives the numerical values for the six coefficients in Equation. 2 normalized to the value of a_1 when the rate constants have the values specified in Table 2.

coefficient	value
a_1	1
a_2	31.1
a_3	11.7
a_4	−234.8
a_5	10872.1
a_6	−7173.8

At the reference parameter values in Table 2, the expression $k_{cat}\alpha$ equals 10,786, and the expression $-\kappa_{cat}\alpha$ equals −7,154, which are within 1% of the exact values of a_5 (10,872) and a_6 (−7,174), respectively. We see from this that the dominance of a_5 and a_6 over the other coefficients a_2, a_3, and a_4, which formed the basis for the explanation of robustness offered above (Equation 5), arises largely from the structure of α, in which the on- and off-rates play a particularly significant and highly multiplicative role. We have not been able to interpret α further, but this analysis does relate the origin of the robustness to the biochemical rate constants.

Sensitivity Analysis—We undertook a sensitivity analysis to investigate whether the robust behavior demonstrated in the previous sections is due to the exact numerical values chosen for the parameters. We found that the six parameters, if allowed to vary, do not have to be confined to a smaller dimensional space around the reference values in Table 2 for robustness to be preserved. Mathematically, this indicates that the region of parameter space in which robustness is found is an open subset of six-dimensional space, at least in the vicinity of the reference values.

We independently and randomly chose values for the on-rates, off-rates, and catalytic rates from the uniform probability distribution on the interval $[\nu/2, 2\nu]$, where ν is the reference value for the corresponding parameter given in Table 2. We did this 200,000 times. We then calculated for each set of parameter values the Michaelis-Menten constants of the kinase and the phosphatase using Equation 1. We found that if the chosen parameter sets were further restricted to those whose Michaelis-Menten constants lay in the interval $[0.8\nu, 1.2\nu]$, which reduced the number of parameter sets from 200,000 to ~18,000, then robustness was preserved. The interval $[0.8\nu, 1.2\nu]$ was the maximum range for the Michaelis-Menten constants for which robustness was obtained with all parameter combinations tested. For each parameter set selected as described, we calculated the mean $\partial I_{oo}/\partial I_{pp}$ over a grid of I_{op} and I_{pp} values and found it to be very close to zero in each case (*i.e.* on the order of 10^{-4}). This behavior corresponds to what we found at the reference parameter values.

Restricting parameter sets to a range of Michaelis-Menten constants still serves to establish that the region of robust parameters is six-dimensional and hence that the robustness of I_{oo} to changes in *T* is not due to an accident in the choice of the reference values. The Michaelis-Menten constants are known from experimental measurements, and restricting them to have physiologically realistic values seems appropriate. However, we did find that there is less latitude for change in the Michaelis-Menten constants, which need to be maintained within a smaller interval around their reference values than do the on-, off-, and catalytic rates.

We note one further issue regarding the invariant in Equation 2. Because the invariant is quadratic, there could be two positive solutions for I_{oo} as a function of I_{op} and I_{pp}, depending on the values of the coefficients $a_2, \ldots, a_6$. This would be dif-

ficult to interpret physiologically for the IDH regulatory system. The phenomenon of bistability has been reported previously for systems with two or more phosphorylation sites (34, 35). However, bistability usually requires three steady states, of which two are stable (hence "bistable") and one is unstable (36). A second positive solution for the invariant may correspond instead to a steady state in which one or more other components have negative values, which would be unphysiological. We found, however, only a single positive solution for I_{oo} at the reference parameter values in Table 2 and in all the parameter sets that we tested in the sensitivity analysis. This behavior can also be attributed to the large negative value for a_6 with the reference parameter values. A quadratic equation has exactly one positive real root if and only if its constant term is negative. Hence, if the invariant in Equation 2 is treated as a quadratic equation in I_{oo}, it has exactly one positive real root if, and only if,

$$a_2 I_{op}^2 + a_3 I_{pp}^2 + a_6 I_{op} I_{pp} < 0 \qquad \text{(Eq. 7)}$$

Large negative a_6 values for biochemically reasonable parameters ensure that the above inequality is satisfied and a single positive solution for I_{oo} is always found.

DISCUSSION

Connection to the Shinar-Feinberg Theorem—Shinar and Feinberg (31) recently proved an important theorem for determining when a biochemical network exhibits robustness. Their result hinges on the deficiency of the network, a non-negative integer that is a measure of the dynamical complexity of the network and depends only on its structure and not on its rate constants. Their theorem states that any network that has deficiency one and meets several other conditions will exhibit ACR (described above). The deficiency of the network describing our model (Fig. 2) is two, so the Shinar-Feinberg Theorem cannot be applied to analyze its robustness. The Shinar-Feinberg Theorem is useful because it enables one to study the robustness of a biochemical system without undertaking extensive algebraic calculations of the sort performed in this investigation. However, the failure of our network to meet the required conditions highlights the need for additional theoretical techniques, like Gröbner basis calculations, to characterize robustness and other systems-level properties in complicated biochemical networks.

Comparison with Other Models—We also considered two hypothetical models of the IDH regulatory system that relax one of the major assumptions of our model (dimerization of IDH and bifunctionality of IDHKP). Algebraic analysis of these simplified reaction networks reveals that they are described by fundamentally different invariants than the invariant (Equation 2) derived from our model. Treating IDH as a monomer regulated by a bifunctional IDHKP leads to the simple reaction network shown in Fig. 4*A* for the interconversion of active (I) and inactive (I_p) IDH. A Gröbner basis calculation on the corresponding reaction network leads to the following invariant (see supplemental material)

$$\frac{I}{I_p} = \frac{(k_2 + k_3)k_4 k_6}{k_1 k_3 (k_5 + k_6)} \qquad \text{(Eq. 8)}$$

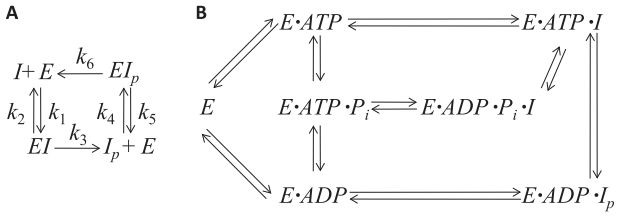

FIGURE 4. **Alternate models of the IDH regulatory system that do not explain robustness.** *A*, hypothetical model in which IDH is treated as a monomer converted between active (I) and inactive (I_p) forms by bifunctional IDHKP (E). *B*, Miller *et al.* (22) kinetic model of the IDH regulatory system. The model focuses on how IDHKP uses ATP and the relationship between the kinase and phosphatase reactions.

As such, the model with monomeric IDH predicts that the ratio of active to inactive IDH is kept robust at steady state (*i.e.* the ratio is set exclusively by parameter values). As seen in Fig. 3*D*, this behavior is the opposite of what is observed experimentally; the measured ratio of active to inactive IDH dropped from 1.2 to 0.02 when total IDH was increased from 1.3 to 34.5 units (8). Equation 8 suggests that a bifunctional enzyme (that like IDHKP has a shared active site) acting on a monomeric substrate can ensure that the substrate is partitioned proportionately between the modified and unmodified forms even as total enzyme or total substrate levels fluctuate. This type of robustness is potentially useful in certain biological contexts, but is clearly incompatible with the IDH regulatory system, which relies on phosphorylating more and more IDH to maintain constant IDH activity when total IDH is increased.

A second hypothetical model treats IDH as a dimer but splits IDHKP into a monofunctional kinase (K) and monofunctional phosphatase (F). Suppose that the two catalytic domains K and F are placed on the same polypeptide to form K × F, in such a way that the two domains do not influence each other (*i.e.* substrate may be bind to either domain with the same rates, irrespective of what is bound to the other). We have shown in a separate study[3] that the dynamical behavior of such a bifunctional K × F is identical to that of the two separate, monofunctional enzymes K and F, with the single difference that there is an additional conservation law that $K_{tot} = F_{tot}$. We show that this kind of bifunctionality leads to the property of "robust upper bounds," which does not correspond to the robustness observed experimentally in the IDH system. Furthermore, in this bifunctional scenario, nothing prevents the formation of a ternary complex in which both K and F are simultaneously bound to their respective substrates. Such behavior is not possible with IDHKP, as discussed earlier. Hence, we can rule out a model in which K and F operate as monofunctional enzymes.

We have already discussed the differences between our model and the model of Shinar *et al.* (15) in detail. Fig. 4*B* shows one other previously developed model of the IDH regulatory system. Miller *et al.* (22) proposed a kinetic model of IDHKP that describes the relationship between its kinase and phosphatase activities and the role of ATP, ADP, and pyrophosphate in both reactions. Their model is biochemically realistic and is useful for understanding certain aspects of the mechanism of

[3] T. Dasgupta, D. H. Croll, J. A. Owen, M. G. Vander Heiden, J. W. Locasale, U. Alon, L. C. Cantley, and J. Gunawardena, submitted for publication.

IDHKP. However, their network, which has deficiency zero, cannot explain ACR of active IDH. This conclusion can be drawn from another recent theorem of Shinar and Feinberg (37), that no network with deficiency zero can exhibit ACR. Additionally, we were unable to find an invariant leading to approximate robustness for the network. These results provide further evidence that the dimerization of IDH, which is not accounted for in the model of Miller *et al*. (22), is important to the robustness of the system.

Should IDHKP Be Treated as a Dimer?—Purified IDHKP is known to dimerize *in vitro* through the formation of a disulfide bond between two cysteine residues (33, 38). Nevertheless, we opted to model IDHKP as a monomer. IDHKP has not been shown to form a homodimer *in vivo*, and there is no clear indication that dimerization of IDHKP is physiologically important. This is in sharp contrast to IDH, whose dimerization is essential for the binding of IDHKP. The complex of two IDHKP monomers bound to an IDH dimer was crystallized in the presence of the strong reducing agent dithiothreitol, which was added to prevent the formation of any disulfide bonds that might impede crystallization (39, 40). Accordingly, a functional $EI_{oo}E$ complex was able to form even when IDHKP dimerization was inhibited. We speculate that the observed IDHKP homodimers formed because large numbers of IDHKP monomers were brought into close contact under favorably oxidative conditions during purification and that dimerization is unlikely to occur inside cells. Formation of new disulfide bonds *in vitro* is not unusual; dimerization of hepatitis B surface antigen produced in yeast is one example (41).

Regulation of IDHKP—Our model focuses on the regulation of IDH by IDHKP to understand how robustness in the glyoxylate bypass is implemented. To focus the model on IDH regulation, we assume that the kinase and phosphatase conformations of IDHKP are in rapid equilibrium and that both are therefore available as needed for reactions. This approach, however, ignores the regulation of IDHKP (*e.g.* how the cell increases kinase or phosphatase activity in response to a change in carbon substrate availability). IDHKP is regulated by a variety of cellular metabolites (including AMP), and the structural basis for the action of many IDHKP regulators is now understood (19). An interesting avenue for future research is the development of an integrated, dynamical model that explains how total IDHKP is partitioned between kinase and phosphatase activity by small-molecule regulators and how this partitioning enables responsiveness to changing environmental conditions. Experiments in which cells are subjected to rapid, oscillatory changes in carbon substrate could provide additional information for such a model (42, 43).

Experimental Tests of the IDH Regulatory Model—Further experimental work is needed to test all the predictions of the model and to fully characterize IDH robustness. Detection of a rare phospho-form such as I_{op} should now be possible with the advent of mass spectrometry-based proteomics (44). Levels of all three phospho-forms should be accurately quantified using mass spectrometry in *E. coli* strains with and without IDH overexpressed, enabling confirmation that the phospho-form relationship and robustness predicted from the invariant are correct. These measurements should be made at more total IDH levels than were considered by LaPorte *et al*. (8). Other experimental tests could be used to demonstrate directly that robustness is dependent on the bifunctional nature of IDHKP. For instance, robustness could be studied in a strain in which IDHKP has been deleted and replaced with a monofunctional kinase and monofunctional phosphatase. A comparable experiment has been performed with the bifunctional adenylyltransferase that regulates glutamine synthetase in *E. coli* (16).

Conclusions—We have developed a model of the *E. coli* IDH regulatory system that is firmly grounded in the current structural, mechanistic, and kinetic data on IDH and IDHKP and that condenses much information about this classic biochemical system into a succinct quantitative framework. We have used new methods of algebraic analysis, together with experimentally supported assumptions, to show how the experimental observations of robustness by LaPorte *et al*. (8) can be explained. Our model suggests that two features of the system, the bifunctionality of IDHKP and the dimerization of IDH, are important to achieving robustness. This approach highlights the value of incorporating detailed enzymatic data, which are often neglected in modeling studies, into quantitative systems biology models.

Many complex biological models are studied by numerical simulation of the underlying ordinary differential equations. This approach leaves all conclusions completely dependent on the numerical values assigned to parameters. In the case of mass-action models, these parameters are the rate constants of the individual reactions, which unlike phenomenological kinetic constants (*e.g.* K_m or k_{cat}) generally cannot be measured directly. We have previously termed the reliance of many systems biology models on hard-to-measure constants the "parameter problem" (45). Our approach to deriving the main invariant circumvents the parameter problem by treating all constants symbolically and studying the model through exact mathematical analysis, not simulations. This method enabled us to obtain a simple expression for the relationship between the three IDH phospho-forms without making assumptions about parameter values. We then used the invariant to make specific, numerical predictions about the robustness of active IDH and the requirements for maintaining robust behavior. These results would have been difficult or impossible to arrive at just by simulating the parent system. The exact characterization of a complicated biochemical model (through derivation of the main invariant) was made possible through the application of recently developed algebraic techniques. Future work should aim to identify other analytical tools that can be exploited to efficiently study systems of biological interest without simulations.

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