Global Kinetic Analysis of Mammalian E3 Reveals pH-dependent NAD\(^{+}/\text{NADH}\) Regulation, Physiological Kinetic Reversibility, and Catalytic Optimum\(^*\)\(^\dagger\)\(^\ddagger\)

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Mammalian E3 is an essential mitochondrial enzyme responsible for catalyzing the terminal reaction in the oxidative catabolism of several metabolites. E3 is a key regulator of metabolic fuel selection as a component of the pyruvate dehydrogenase complex (PDHc). E3 regulates PDHc activity by altering the affinity of pyruvate dehydrogenase kinase, an inhibitor of the enzyme complex, through changes in reduction and acetylation state of lipoamide moieties set by the NAD\(^{+}/\text{NADH}\) ratio. Thus, an accurate kinetic model of E3 is needed to predict overall mammalian PDHc activity. Here, we have combined numerous literature data sets and new equilibrium spectroscopic experiments with a multitude of independently collected forward and reverse steady-state kinetic assays using pig heart E3. The latter kinetic assays demonstrate a pH-dependent transition of NAD\(^{+}\) activation to inhibition, shown here, to our knowledge, for the first time in a single consistent data set. Experimental data were analyzed to yield a thermodynamically constrained four-redox-state model of E3 that simulates pH-dependent activation/inhibition and active site redox states for various conditions. The developed model was used to determine substrate/product conditions that give maximal E3 rates and show that, due to non-Michaelis-Menten behavior, the maximal flux is different compared with the classically defined \(k_{\text{cat}}\).

Dihydrolipoamide dehydrogenase (E3) is one of three catalytic subunits (E1, E2, and E3) found in a number of multienzyme catabolic complexes (1). E3 is responsible for catalyzing the terminal redox reaction, which is the reversible two-electron oxidation of dihydrolipoamide to lipoamide with the concomitant reduction of NAD\(^{+}\) to NADH (2–4). E3 is a component of the pyruvate dehydrogenase complex (PDHc),\(^2\) which oxidizes pyruvate to produce CO\(_2\), acetyl-CoA, and NADH (5). The PDHc is at the interface of glycolysis, the tricarboxylic acid cycle, and is reciprocally regulated relative to fatty acid \(\beta\)-oxidation (6, 7).

Its hub-like position in central metabolic pathways has endowed the PDHc with a major regulatory role in mammals with links to metabolic maladaptations (8) related to heart failure (9–11), diabetes (12, 13), and the Warburg effect in cancer cells (14–16). An imbalance between glucose/fatty acid substrate selection is a hallmark of the aforementioned diseased states (6, 17). The PDHc is also known to be a critical factor in substrate selection, which is modulated by multiple factors (13, 18).

Mammalian PDHc regulation is governed in large part by its own specific pyruvate dehydrogenase kinase (PDK) and phosphatase (PDP) (19–21). PDK and PDP bind to the lipoamide-containing L sites (22–25) located on E2 and the E3-binding protein, a PDHc structural subunit. PDK binding, and thus PDHc activity, is greatly affected by the redox and acetylation state of lipoamide. When reduced, acetylated lipoamide moieties favor PDK binding (23, 24, 26, 27). The redox state of lipoamide moieties in E2 and the E3-binding protein is governed by the forward reductive acetylation activity of E1 and the NADH/lipoamide activity of E3. It has been postulated that E3 activity has primary kinetic control of the lipoamide redox state, because E1 activity is rate-limiting for the overall PDHc reaction (8). Thus, PDK/PDHc regulation via the NAD\(^{+}/\text{NADH}\) ratio, a general indicator of intracellular redox (28) and bioenergetic status (29), is mediated through the E3 component of the complex.

In vitro E3 component kinetics and redox regulation have been studied from multiple organisms and have demonstrated similar kinetic patterns but with remarkably different regulatory sensitivities to NAD\(^{+}/\text{NADH}\) ratio and pH (30–34). Mammalian and bacterial E3 components are the most disparate examples, with E3 from *Escherichia coli* demonstrating the greatest observed NAD\(^{+}/\text{NADH}\) catalytic sensitivity (30). Greater NAD\(^{+}/\text{NADH}\) sensitivity in *E. coli* E3 (Ec-E3) may be reasoned from a regulatory standpoint because *E. coli* PDHc lacks the PDK/PDP enzymes, which provide another layer of PDHc regulation in mammals (5, 35). Despite smaller NAD\(^{+}/\text{NADH}\) kinetic effects in mammalian versus bacterial E3s, PDK activity in the mammalian PDHc is very sensitive to NAD\(^{+}/\text{NADH}\) (26, 36, 37), mediated through E3.

NAD\(^{+}/\text{NADH}\) effects are intimately related to pH, as they may be more appropriately modeled as the ratio of NAD\(^{+}/(\text{NADH} + H^+)\), due to the release of a proton during NAD\(^{+}\) reduction (38). Thus, E3 NAD\(^{+}/\text{NADH}\) regulation is pH-de-
pendent. The reverse E3 reaction consumes NADH, lipoamide, and H\(^+\), while producing NAD\(^+\) and dihydrolipoamide. Typically, in the reverse direction, initial amounts of added NAD\(^+\) to in vitro assays provide an activating effect in acidic pH (30, 39, 40), whereas, ever increasing amounts of NADH cause E3 substrate inhibition (30). These observations span mammalian and bacterial E3s but are most prominent in the archetypical bacterial E3 from *E. coli*. Although the magnitude of NAD\(^+\)/(NADH + H\(^+\)) kinetic regulation differs from *E. coli* (30), human (33), rat (34), and spinach enzymes (32), we have previously shown that these differences are explained by a single thermodynamically constrained model (41).

Prior E3 analysis (41) demonstrates that NAD\(^+\) is mainly an activator in the reverse reaction, with *E. coli* (30), spinach (32), and human liver (33). Reed (34) also showed, using rat liver E3, that varying amounts of NAD\(^+\) lead to kinetic patterns deviating from a simple ping-pong mechanism (34). Even though the rat liver E3 kinetic study showed no NAD\(^+\) activation (34), NAD\(^+\) activation was demonstrated with human liver E3 enzyme (33, 41). Interestingly, NAD\(^+\) activation was also reported early on by Massey and Veeger (39) using pig heart E3, but little primary data were shown. Muiswinkel-Voetberg and Veeger (40) demonstrated the pH dependence of the initial velocity of pig heart E3 with the reverse reaction and showed activation of the rates by adding 0.1 mM NAD\(^+\). However, these data did not include physiologically relevant pH ranges (pH \(\geq 7\)).

There are other available data on the pH-dependent regulation of mammalian E3 (mam-E3) (42–47); however, none address the NAD\(^+\) pH-dependent kinetic regulation. After modeling E3 kinetic data from a number of organisms, we found that lacking mam-E3 data, especially reverse progress curve type data and NAD\(^+\)/(NADH + H\(^+\)) effects, weakened mam-E3 model parameterization and predictability in physiological conditions (41).

Therefore, we have collected a large array of pH-dependent progress curves and initial velocity data, in the reverse direction, using different amounts of NAD\(^+\) and lipoamide with pig heart E3. We also include independent data sets with the forward reaction that vary pH, NAD\(^+\), and dihydrolipoamide. Furthermore, we use spectroscopic methods to determine the redox state of the enzyme through FAD absorbance and fluorescence to gain further insight into E3 regulation.

Efforts to globally fit this data set with our previously defined model (41) revealed the need to modify the model. The result is a new 4-state thermodynamically constrained redox model of pig heart E3, which is able to reproduce a wide array of kinetic and equilibrium data. A unique example includes progress curves demonstrating, to our knowledge for the first time, both reverse E3 acidic NAD\(^+\) activation and alkaline NAD\(^+\) inhibition in a single consistent data set.

We also demonstrate the physiological kinetic reversibility of E3, which supports E3 kinetic control over the PDHc. Furthermore, in light of the observed non-Michaelis-Menten behavior of E3 (30, 39), we use non-linear optimization to search conditions that produce maximal E3 flux under physiological constraints. Our result demonstrates the difference between the maximum flux and \(k_{cat}\) (48).

**FIGURE 1.** *Mammalian E3 pH-dependent Activation/Inhibition.* A, pig heart E3 kinetic model consists of four redox states as follows: oxidized (S\(_1\)), hydride-reduced disulfide (S\(_2\)), hydride-reduced FAD (S\(_3\)), and a hydride-reduced disulfide and FAD state (S\(_4\)). Each enzyme redox state is depicted in the corresponding bubble captions, which illustrate the disulfide (S-S), flavin (FAD), and active site base (His) chemical forms as part of the E3 enzyme (Enz). In the model, S\(_2\) has the ability to undergo (de)protonation, where the middle schematic in the bubble caption represents the charge transfer complex required for hydride transfer between the thiolate redox center and FAD cofactor to advance the mechanism between S\(_2\) and S\(_3\) enzyme states (3). Dihydrolipoamide, NAD\(^+\), lipoamide, NADH, and protons are represented by A, B, P, Q, and H\(^+\), respectively. Fractional occupancies, the fraction of a substrate or product bound to a given enzyme state, are represented by \(f\), and subscripts indicate the substrate/product bound to a specific state (S\(_i\) through S\(_4\)). Substrates and products are considered to bind randomly within each redox state and in rapid equilibrium compared with chemical steps. Our assumption of rapid equilibrium binding is supported by previous studies (3, 43, 46). B, x-ray structure of human E3 (Protein Data Bank code 1ZMD) (4) illustrates the general substrate/product binding situation common to E3s, where the red face of the FAD cofactor is exposed, and the si face is guarded by an active site disulfide. In the human E3 structure (4), material binding pocket is annotated according to the bound NADH (green) and FAD (yellow) cofactors shown in stick representation. The active site disulfide (Cys-45 and -50 in humans) and histidine (residue 452 in humans) are shown as yellow and red sticks, respectively. This figure was made using PyMOL (72) and Protein Data Bank code 1ZMD.
Mammalian E3 pH-dependent Activation/Inhibition

Experimental Procedures

Reagents—All chemicals and buffers were purchased from Sigma. Pig heart E3 was purchased from Calzyme (San Luis Obispo, CA) without further purification. All experiments were conducted using Nanopure water. Pig heart E3 was reconstituted in 50 mM potassium phosphate and 0.3 mM EDTA at pH 7. Active site concentration was assessed by monitoring the enzyme-bound FAD spectrum and calculating the concentration based on the molar absorptivity of 11.3 mM⁻¹ cm⁻¹ at 455 nm (47). Stock solutions were aliquoted and stored at −20 °C.

Fluorescence Binding Assays—Fluorescence binding assays were conducted in 50 mM potassium phosphate, 0.3 mM EDTA, and adjusted to have an ionic strength of 170 mM using KCl at pH 7. Pig heart E3 (1.2 μM, 2 ml volume) solution was titrated with incremental amounts of a 100 mM NAD⁺ stock solution at 25 °C. The binding assay was monitored by pig heart E3 FAD fluorescence excited at 455 nm. Pig heart E3 FAD fluorescence emission spectra were collected from 480 to 650 nm using an LS 55 luminescence spectrometer (PerkinElmer Life Sciences). Excitation and emission slit widths were set at 5 and 8 nm, respectively. Fluorescence emission spectra were corrected for dilution.

DL-lipoamide titrations of pig heart E3 (0.75 μM, 2 ml volume) were conducted in the same general conditions as the NAD⁺ titration described above; although DL-lipoamide (47 mM) stock was solubilized in a 100% ethanol solution. The final percentage of ethanol in the titration was ≤20%. The DL-lipoamide binding assays were monitored by pig heart E3 protein fluorescence (tryptophan) excited at 280 nm and by pig heart E3 FAD fluorescence excited at 455 nm. E3 protein fluorescence was corrected for dilution and was also corrected for inner filter effects using Equation 1 (49).

\[
F(\lambda)_{\text{corrected}} = F(\lambda)_{\text{observed}} \times \frac{\left(\frac{\Delta A(\lambda)_{\text{excitation}} + \Delta A(\lambda)_{\text{emission}}}{2}\right)}{A(\lambda)_{\text{emission}}} \quad (\text{Eq. 1})
\]

Fluorescence binding assays were analyzed using their full spectra according to supplemental Equation S1 and its solution shown in supplemental Equation S2. A detailed description of this method is provided in the supplemental material under Fluorescence Binding Assay Method.

Pig Heart E3 Kinetic Assays—Pig heart E3 kinetic assays were conducted in various initial solutions of NAD⁺ (0, 0.1, and 0.5 mM), NADH (0.25 and 0.5 mM), DL-lipoamide (0.25, 1, and 3 mM), and pH (4.5, 5.25, 5.65, 6.25, 7, 8, and 8.5). Assays were conducted using 2 nM enzyme, 0.3 mM EDTA, 0.67 mg/ml BSA (50), at 25 °C in 170 mM ionic strength adjusted using KCl. Final ethanol concentrations, due to the DL-lipoamide 100% ethanol solvent, were held constant at 7.5% throughout all assays. Assay pH was buffered using 50 mM citrate (pH 4.5), MES (pH 5.25, 5.65, and 6.25), MOPS (pH 7), and Tris (pH 8 and 8.5). To mitigate unwanted buffer effects, we chose to use the structurally similar MES and MOPS buffers around physiologically relevant pH. We show that MOPS and Tris, although structurally dissimilar, produce no difference in E3 rate at the same pH (supplemental Fig. S10).

Assays were conducted on a Varioskan Flash multimode plate reader (Thermo Fisher Scientific, Waltham, MA) using a 96-well format with 200-μl assay total volumes, which were followed at 340 nm. Calibration curves for NADH and DL-lipoamide were carried out on the plate reader to account for each of their molar absorptivities in the dihydrolipoamide dehydrogenase reaction. Enzyme assays were initiated by the automated injection of 100 μl (half of the total assay volume) of E3 to each well.

Literature-derived FAD Anaerobic Absorbance Tittrations—Pig heart E3 FAD absorption spectra in Fig. 4, A, D, and E, were obtained using the graph-digitizing software ScanIt 1.06 (amsterCHEM) from Fig. 3 in Ref. 46 and Figs. 1 and 4 in Ref. 47, respectively. Further necessary modifications to these obtained data are described in the supplemental material, under E3 FAD Spectral Analysis. The spectral data in Fig. 4, D and E, were used to estimate the fraction of enzyme that exists in the three major E3 redox states of oxidized, 2e⁻-reduced charge transfer complex, and 4 e⁻-reduced state by applying supplemental Equation S3 and its solution supplemental Equation 4. A detailed discussion of how these spectral data were used to extract enzyme fractional states is also provided in the supplemental material, under E3 FAD spectral analysis.

Kinetic Modeling—The model schematic is shown in Fig. 1A, where the overall forward E3 reaction is shown in Reaction 1.

\[
A + B \rightleftharpoons P + Q + H^+ \quad \text{Reaction 1}
\]

In Reaction 1, A, B, P, Q, and H⁺ represent dihydrolipoamide, NAD⁺, lipoamide, NADH, and a proton, respectively. This model assumes that pig heart E3 is composed of four major
redox states, including fully oxidized (S1), hydride-reduced disulfide (S2), hydride-reduced FAD (S3), and a hydride-reduced disulfide and FAD state (S4). We also assume that substrates, products, and protons bind randomly and are in rapid equilibrium with each redox state. Binding polynomials are used to determine the fraction of bound and free forms of the enzyme (Equation 2).

Dihydrolipoamide/lipoamide (A/P) and NADH/NAD⁺ (Q/B) pairs are considered to bind to two separate sites on E3 as is the case reduced disulfide and FAD state (S4). We also assume that subdivision of this in regard to the kinetic model can be found in the datasets that were obtained from the literature. A discussion of this in regard to the kinetic model can be found in the supplemental material under Lipoamide Stereochemistry.

As demonstrated previously (41), we have allowed equilibrium dissociation constants to vary depending on the redox status of the enzyme to more accurately account for redox effects on the enzyme-ligand interaction. Different enzyme redox states are indicated in the binding polynomials by use of their numbered states (in Equation 2, x = states 1, 2, 3, or 4). Binding polynomials (Equation 2) for protons (Dh), representing the active site thiolate and base, are accounted for and assumed to be most significant in the instance of the hydride-reduced disulfide state (S2).

\[
D_h = \left(1 + \frac{H^+}{K_{thiolate}}\right)\left(1 + \frac{H^+}{K_{base}}\right)
\]

\[
D_{xA} = 1 + A \frac{P}{K_A} + P \frac{Q}{K_P} + Q \frac{P}{K_Q} + Q \frac{P}{K_Q}
\]

\[
D_{xQ} = 1 + B \frac{Q}{K_B} + Q \frac{Q}{K_Q}
\]

\[
f_{xA} = \frac{A}{D_{xQ}} \frac{f_{Bx}}{f_{Qx}} = \frac{B}{D_{xQ}} f_{Px} = \frac{Q}{D_{xQ}} f_{Qx} = \frac{P}{D_{xQ}} f_{Qx} = \frac{H^+}{K_{base}}
\]

Fractional occupancies (Equation 3) are produced by taking the ratio of the specific binding state that advances the mechanism from all other substrates (51).

The kinetic equations are shown in matrix form in the supplemental Equation S5 and were solved analytically by computing the pseudo inverse of the state-transition matrix using MATLAB (2014b) Symbolic Math Toolbox. The solution to supplemental Equation S5 provides the distribution equations for each enzyme state, which are used to create a flux expression for the net production of NADH (Q) (Equation 4).

\[
J = Et[S_1] f_{Bx} k_5 - [S_1] f_{Qx} k_6[H^+] + [S_2] f_{Qx} k_6 - [S_2] f_{Qx} k_7
\]

**Model Constraints**—Thermodynamic loop constraints were derived using the two inner loops (Fig. 1A), described in detailed elsewhere (41). The resulting constraints are shown in Equation 5 and were used to eliminate two rate constants from the flux expression, k5 and k7.

\[
K_{eq} = \frac{k_5 k_6 k_7}{k_5 k_6 k_7 A k_B D_2 D_{3Q}}
\]

The overall equilibrium constant for the dihydrolipoamide dehydrogenase reaction (Equation 6) is required for the above constraint and was normalized to pH 7 by multiplying the right hand side of Equation 6 by 107. Previously (41), the apparent
Mammalian E3 pH-dependent Activation/Inhibition

In this experiment, pig heart E3 was anaerobically reduced with dithionite (initial red spectrum) and then titrated with NAD\(^+\) (0, 0.151, 0.303, 0.602, and 1.757 mM; red to black lines) at a pH of 5.8. B, semi-log plot of the singular values from a singular value decomposition of the spectra in A. C, fraction of NAD\(^+\) bound to the 2e\(^-\) reduced E3 state was calculated assuming the spectra in A are composed of only bound and unbound species and solving for this fraction using linear least squares. The resulting fractions, or \(a\) values, are shown as a function of total NAD\(^+\) D, pig heart E3 FAD absorption spectra were obtained from Fig. 4 in Ref. 47. In this experiment, oxidized pig heart E3 was anaerobically titrated with dihydrolipoamide (0, 10.6, 21.2, 31.8, 44.3, 65.3, and 84.67 mM; red to black lines) at a pH of 5.8. E, pig heart E3 FAD absorption spectra were from oxidized (light green), 2e\(^-\) reduced (dark green), and 4e\(^-\) reduced (black) obtained from Fig. 1 in Ref. 47. F, pig heart E3 absorption spectra of the different redox states in E were used to solve (see “Experimental Procedures”) the fraction of oxidized, 2e\(^-\), and 4e\(^-\) reduced enzyme states as a function of the dihydrolipoamide titration in D. Expressed fraction at equilibrium constant at pH 7 was estimated to be 0.0766, using Equation 7, from the midpoint potentials of the lipoamide/dihydrolipoamide (−0.287 V) and NAD\(^+\)/NADH (−0.320 V) couples determined at 25 °C (2, 47).

\[
K_{eq} = \frac{[POH^+]_{eq}}{[AB]_{eq}} \quad \text{(Eq. 6)}
\]

\[
K_{eq} = \frac{n_{F}T}{F} e^{-\Delta F_{m}/RT} \quad \text{(Eq. 7)}
\]

In Equation 7, \(n\) is the number of electrons transferred; \(F\) is Faraday’s constant (96,485 C mol\(^{-1}\)); \(\Delta F_{m}\) is the midpoint potential difference; \(R\) is the gas constant (8.315 J K\(^{-1}\) mol\(^{-1}\)), and \(T\) is temperature in Kelvin. As was discussed previously (41), there are indeterminate errors associated with midpoint potentials (52) that are amplified through the exponential relationship between \(\Delta F_{m}\) and \(K_{eq}\) (Equation 7). Also in relation to this fact, we have found additional pH-dependent equilibrium data for E3 in the Goldberg et al. database (53), which reports an apparent equilibrium constant of 0.27 at pH 7. We allowed variation in \(K_{eq}\) to encompass experimental variability, where the boundary for the apparent \(K_{eq}\) at pH 7 was set between 0.03 and 0.4. Details of model fitting are described in the supplemental material under Fitting.

**pH-dependent E3 Flux Optimization via NAD\(^+\)/NADH and Lipo/DHL Ratios—**NAD\(^+\)/NADH and Lipo/DHL ratios can be optimized to produce a physiological maximal flux by using a fixed total amount of both lipoamide and NAD of 10 and 3 mM, respectively. These values were obtained from literature estimations (1, 54), and with specified NAD\(^+\)/NADH and Lipo/DHL ratios were used to solve for the corresponding oxidized and reduced lipoamide and NAD concentrations needed to satisfy the conditions. For instance, in the case of NAD, the total NAD concentration (NAD\(_{eq}\)) and ratio are given in Equation 8. Substitution and rearrangement yields Equation 9 and concentrations for both NAD\(^+\) and NADH.

\[
NAD_{eq} = \frac{NAD^+ + NADH}{NADH} \quad \text{(Eq. 8)}
\]

\[
NADH = \frac{NAD_{eq}}{1 + \text{ratio}} = \frac{NAD^+ - NADH}{NAD^+} \quad \text{(Eq. 9)}
\]

Using the above constraints, the NAD\(^+\)/NADH and Lipo/DHL ratios were optimized using the best-fit rate and equilibrium dissociation constants (Table 1) for both mam-E3 and Ec-E3 (Figs. 11–14); the Ec-E3 kinetic model and parameters have been determined in a prior publication using a 3-state redox model (41).

Substrate/product ratios were optimized using the same global and local minimizers as the data fitting described in the supplemental material. Initial ratio values were randomly chosen between the following boundaries. The NAD\(^+\)/NADH ratio was given the upper bounds of 10\(^2\) (Ec-E3) and 25 (mam-E3) and lower bounds of 10\(^{-2}\) (Ec-E3) and 25\(^{-1}\) (mam-E3). The Lipo/DHL ratio was given the upper and lower bound of 10\(^{12}\) and 10\(^{-12}\), respectively, for both Ec-E3 and mam-E3.

NAD\(^+\)/NADH boundaries were chosen to encompass a range that more than spans observed in vivo estimates (55–57).
Mammalian E3 pH-dependent Activation/Inhibition

FIGURE 5. Mammalian E3 pH-dependent NAD\(^+\) activation/inhibition. A, pig heart E3 reverse reaction progress curves in acidic conditions (pH 5.25) in different initially added amounts of NAD\(^+\) (0, 100, and 500 \(\mu\)M), shown as blue, green, and red circles, respectively. Model simulations are shown as dashed (3-state model) and solid (4-state model) lines of the corresponding data marker color. B, pig heart E3 reverse reaction progress curves at basic conditions (pH 8) in different initially added amounts of NAD\(^+\) (0, 100, and 500 \(\mu\)M), shown as blue, green, and red circles, respectively. Model simulations are shown as dashed (3-state model) and solid (4-state model) lines of the corresponding data marker color. C, human liver E3 pH-dependent forward initial rates (black circles) taken from Fig. 5 of Ref. 33. Model simulations are shown as black dashed (3-state model) and solid (4-state model) lines. D, pig heart E3 pH-dependent reverse initial rates in different initially added amounts of NAD\(^+\) (0, 100, and 500 \(\mu\)M), shown as blue, green, and red circles, respectively, with 4-state model simulations shown as solid lines. E, pig heart E3 pH-dependent reverse initial rates (as in D) with 3-state model simulations shown as dashed lines of the corresponding data marker color. F, difference plots (observed turnover (NAD\(^+\) added) — observed turnover (no NAD\(^+\) )) of data and simulations shown in D corresponding to the 4-state model. G, difference plots (observed turnover (NAD\(^+\) added) — observed turnover (no NAD\(^+\) )) of data and simulations shown in E corresponding to the 3-state model. A–E, error bars represent standard deviations of the data from at least three experimental repeats, where error bars in F and G represent the propagation of error from the difference of the observed rates.

FIGURE 2 summarizes an analysis of the enzyme spectra (60). In this method, we assume that the first state fractions that result from infinite substrate concentrations were calculated by taking the limit of each enzyme state distributions as substrates approach infinity (Figs. 11, A and B, and 12, A and B, bottom).

As described by Cornish-Bowden (48), the \(k_{cat}\) is not a true flux maximum but a limit in mathematical terms. These derivations and calculations were used to compare with the true optimizations of flux in NAD\(^+\)/NADH, Lipo/DHL, and pH (Figs. 11, C and D, and 12, C and D), albeit with a finite NAD and lipoamide pool of 3 and 10 \(\mu\)M, respectively (1, 54). These flux optimizations also differed from those shown in Figs. 13 and 14 by allowing a much wider range of NAD\(^+\)/NADH and Lipo/DHL to further probe E3 flux optima (upper bound, 10\(^{12}\); lower bound, 10\(^{-12}\), for both ratios). In addition, pH was an adjustable parameter bound between 4 and 9.

Results

Equilibrium Fluorescence Titrations—Fig. 2 summarizes an equilibrium titration of oxidized E3 (S\(_{z}\) in Fig. 1A) with NAD\(^+\). Utilizing the entire spectral dataset, accurate estimates of the fraction of NAD\(^+\) bound to the enzyme are computed by assuming linear superposition of NAD\(^+\) bound and free enzyme spectra (60). In this method, we assume that the first to allow a wide range that may apply to normal, abnormal, or diseased states (58). Lipo/DHL ratios are less documented and thus are given less stringent bounds. Enzyme fractional states (Figs. 7 and 11–14) were calculated with defined NAD\(^+\)/NADH, Lipo/DHL, and pH values using distribution equations (59).

Substrate/product optimization was carried out by either allowing pH to vary (Figs. 11 and 12; Table 2) or fixing pH at different values (Figs. 13 and 14). Optimizations that allowed pH to vary (Figs. 11 and 12) are shown in comparison with \(k_{cat}\) calculations described below and were given very wide boundaries for NAD\(^+\)/NADH and Lipo/DHL (upper bound, 10\(^{12}\); lower bound, 10\(^{-12}\)) to probe flux optima beyond physiological constraints.

\(\text{pH-dependent } k_{cat} \text{ and Corresponding Enzyme Fractional State Calculation—}\)Best-fit kinetic parameters (Table 1) using mam-E3 data (Fig. 6), for the 4-state redox model, and best-fit kinetic parameters for Ec-E3 described in Moxley et al. (41) were used to calculate the pH-dependent \(k_{cat}\) in both the forward (Figs. 11A and 12A; top) and reverse (Figs. 11B and 12B) directions. The forward and reverse \(k_{cat}\) values were derived by taking the limit of the flux expression as the substrates, in either direction, approach infinity (48). The corresponding enzyme state fractions that result from infinite substrate concentrations were calculated by taking the limit of each enzyme state distribution equation as substrates approach infinity (Figs. 11, A and B, and 12, A and B, bottom).

As described by Cornish-Bowden (48), the \(k_{cat}\) is not a true flux maximum but a limit in mathematical terms. These derivations and calculations were used to compare with the true optimizations of flux in NAD\(^+\)/NADH, Lipo/DHL, and pH (Figs. 11, C and D, and 12, C and D), albeit with a finite NAD and lipoamide pool of 3 and 10 \(\mu\)M, respectively (1, 54). These flux optimizations also differed from those shown in Figs. 13 and 14 by allowing a much wider range of NAD\(^+\)/NADH and Lipo/DHL to further probe E3 flux optima (upper bound, 10\(^{12}\); lower bound, 10\(^{-12}\), for both ratios). In addition, pH was an adjustable parameter bound between 4 and 9.

Results
spectrum represents the unbound species, and the final spectrum, in the titration, represents the bound species. We solve for the term $\alpha$ ([supplemental Equation S2](#)), which provides the fraction of bound protein, and a fit to each spectrum (Fig. 2B). From here, $\alpha$ as a function of NAD$^+$ (Fig. 2C) can be globally fitted with all other data sets (see below) and provides a very sensitive experiment reporting on NAD$^+$ binding affinity to the E3-oxidized state (or $S_1$ in Fig. 1A). Details of this method are explained in the [supplemental material](#), under Fluorescence Binding Assay.

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**Mammalian E3 pH-dependent Activation/Inhibition**

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| A | B | C |
|---|---|---|
| pH = 5.25 | pH = 6.25 | pH = 8 |

| D | E | F |
|---|---|---|
| pH = 5.25 | pH = 6.25 | pH = 8 |

| G | H | I |
|---|---|---|
| Turnover (min$^{-1}$) | Turnover (min$^{-1}$) | Turnover (min$^{-1}$) |

| J | K | L |
|---|---|---|
| pH | pH | pH |

| M | N | O |
|---|---|---|
| Oxidized | 4e reduced | Turnover (min$^{-1}$) |

| P | Q | R |
|---|---|---|
| Alpha vs [Lipoamide] (mM) | Alpha vs [NAD$^+$] (mM) | Alpha vs [NAD$^+$] (mM) |
**TABLE 1**

| Parameter | Redox-independent | Redox-dependent |
|-----------|------------------|-----------------|
| $K_i$     | $7.37 \times 10^7$ | $2.68 \times 10^7$ |
| $k_r$     | $4.94 \times 10^3$ | $2.12 \times 10^3$ |
| $k_l$     | $5.49 \times 10^3$ | $1.88 \times 10^3$ |
| $k_0$     | $1 \times 10^6$   | $1 \times 10^6$   |
| $k_0$     | $1 \times 10^6$   | $1 \times 10^6$   |
| $k_2$     | $3.17 \times 10^7$ | $4.26 \times 10^7$ |
| $k_3$     | $1.2 \times 10^7$ | $4.87 \times 10^6$ |
| $k_4$     | $2.2 \times 10^7$ | $1.6 \times 10^7$ |
| $k_{rh}$  | $4 \times 10^7$   | $4 \times 10^7$   |
| $pK_{h1}$ | $1.58 \times 10^{-1}$ | $1.58 \times 10^{-1}$ |
| $pK_{h2}$ | $9 \times 10^{-2}$ | $9 \times 10^{-2}$ |

| State 1 | State 2 | State 3 | State 4 |
|---------|---------|---------|---------|
| $K_{eq}$ | $12$ | $10^3$ | $49$ | $81$ | $2.7$ |
| $K_{eq}$ | $55$ | $2.4 \times 10^9$ | $1.9 \times 10^9$ | $1.5 \times 10^9$ |
| $K_{eq}$ | $2.8 \times 10^7$ | $2.5 \times 10^7$ | $1.2 \times 10^7$ | $2.7 \times 10^7$ |
| $K_{eq}$ | $34$ | $1 \times 10^5$ | $312$ | $3$ | $40$ |
| $K_{eq}$ | $3.15$ | $0.15$ | $0.63$ | $0.13$ |

$^a$ Parameter values are from the global fitting of the data in Fig. 6 to a 4-state redox model where the equilibrium dissociation constants ($K_{eq}$ in micromolars) were either independent or dependent on the E3 redox state. Parameter sensitivities are in parentheses and calculated according to a previously described method.

$^b$ Rate constants were constrained within the bounds of $10^{-3}$ to $10^3$ min$^{-1}$, and equilibrium dissociation constants were bounded between $1$ and $1 \times 10^9$ M$^{-1}$. $pK_{h1}$ and $pK_{h2}$ were bounded between 9 and 4, respectively.

$^c$ The rate constants $k_r$ and $k_l$ were eliminated from the flux expression using thermodynamic loop constraints (Equation 5) described under “Experimental Procedures.”

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**Analysis of Literature-derived Equilibrium UV-visible Titrations**—Additional information on ligand affinity and the redox equilibrium properties of pig heart E3 was obtained by analyzing existing FAD spectra of a NAD$^+$ equilibrium titration on the E3 charge transfer complex (Fig. 4A) (46) and a dihydrolipoamide titration of oxidized E3 (Fig. 4D) (47). The NAD$^+$ equilibrium titration (Fig. 4A) was conducted on the E3 charge transfer complex at pH 5.8 (apparent $K_{eq} = 0.0048$) (46) and reports on the binding affinity of NAD$^+$ to the enzyme state $S_4$ (Fig. 1A). A singular value decomposition of the spectral data illustrates that the spectra are mostly composed of two species (Fig. 4B), where we assume that these species represent NAD$^+$.

Fluorescence enhancement between 400 and 500 nm was believed to be attributed to DL-lipoamide fluorescence (Fig. 3D). This conclusion was reached by exciting (280 nm) a solution of DL-lipoamide alone as a function of increasing DL-lipoamide, which showed increases in fluorescence between 400 and 500 nm. Fluorescence beyond 400 nm was attributed to E3 FAD fluorescence (Fig. 3E) by conducting a separate titration exciting E3 FAD fluorescence at 455 nm, whereas while titrating with DL-lipoamide showed little effect on the spectra. This result is consistent with the lipoamide-binding site being distal to the isoalloxazine ring of the FAD cofactor. This result coincides with the structural data (Fig. 1B) and a two-site mechanism (one site each for NAD$^+$/NADH and Lipo/DHL) that we used to model the kinetic data (see “Experimental Procedures” for model description).

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**FIGURE 6.** Global fitting of mammalian E3 reverse progression curves, reverse/forward initial velocity, and equilibrium titration data to a 4-state redox model with redox-dependent equilibrium dissociation constants. A–F, pig heart E3 reverse reaction progress curve data were collected in different initially added amounts of NAD$^+$ (0, 100, and 500 μM), shown as blue, green, and red circles, respectively. Model simulations (4-state model) are shown as solid lines of the corresponding data marker color. All time-dependent assays shown in A–F contained 500 μM initially added NADH. A–C and D–F contained 0.25 and 1 mM DL-lipoamide, respectively. pH was held fixed at 5.25, 6.25, and 8 shown in A–C and D–F, respectively. G–L, pig heart E3 pH-dependent reverse initial velocity data were collected in different initially added amounts of NAD$^+$ (0, 100, and 500 μM), shown as blue, green, and red circles, respectively. Model simulations (4-state model) are shown as solid lines of the corresponding data marker color. Initial rates shown in G–I were obtained in 500 μM initially added NADH and 0.25 (G), 1 (H), and 3 mM (I) DL-lipoamide. Initial rates shown in J–L were obtained with 250 μM initially added NADH and 0.25 (J), 1 (K), and 3 mM (L) DL-lipoamide. M, pig heart E3 fractional redox states were obtained from the dihydrolipoamide equilibrium titration shown in Fig. 4, D–F. The oxidized, 2e$^-$ reduced, and 4e$^-$ reduced states as a function of DL-lipoamide are shown as blue, green, and red circles, respectively. N, human liver E3 forward initial rate data as a function of pH was obtained from Fig. 5 of Ref. 33 and fitted along with all other datasets in this figure. O, forward initial rate data (circles) as a function of NAD$^+$ in different fixed concentrations of dihydrolipoamide (25 (blue), 40 (green), 50 (red), 100 (magenta), 250 (cyan), 500 (yellow), and 750 μM (black)) were taken from the top of Fig. 1 in (63) and simulated (lines) with globally fitted parameters along with all other datasets shown in this figure. P–R, α values (blue circles) obtained from Figs. 2C, 3C, and 4C were simulated (blue lines) with globally fitted parameters assuming rapid equilibrium binding of each ligand described by their corresponding enzyme state fractional occupancies (Equation 3). Error bars represent standard deviations of the data from at least three experimental repeats, and error bars for literature-derived data sets (M–O) were assigned a 10% error according to the maximum ordinate value.
free and bound charge transfer complex. These spectra were then analyzed, as the fluorescence titrations described above, to obtain a binding curve as a function of NADH (Fig. 4C).

The redox status of pig heart E3 in the presence of increasing concentrations of dihydrolipoamide was monitored through FAD absorbance spectra (Fig. 4D) (47). Using absorbance spectra of known E3 major redox states (Fig. 4E), the fractions of E3 redox states as a function of dihydrolipoamide (Fig. 4F) can be determined from the titration spectra. (See supplemental material under E3 FAD spectral analysis.)

Mammalian E3 Activation/Inhibition and Modeling—We previously demonstrated (41) that a 3-state redox model consisting of oxidized, 2e−, and 4e− reduced states can simulate NADH activation effects as they vary among different E3 homologs. E. coli and spinach E3 data sets each showed that NADH acts only as an activator in acidic pH, which is then neutralized at increasing pH values (see Figs. 3F and 4C in Ref. 41), although the model best-fit for spinach E3 simulated a small amount of NADH inhibition at pH values above 7.5 (see Fig. 4C in Ref. 41).

Although a reasonable amount of data exist for E. coli (30) and spinach (32) E3 at more neutral and basic pH values, using various NADH concentrations, data sets in these conditions using mammalian sources of E3 are lacking (41). To gain more insight, we used the best-fit parameters for a human liver E3 kinetic data set, discussed previously (41), to simulate higher NADH-containing conditions as a function of pH (supplemental Fig. S1B). These simulations predict NADH inhibition at near neutral to basic pH values (supplemental Fig. S1B).

In light of these observations and lack of available mammalian E3 data, we collected pH-dependent kinetic data with pig heart E3 using a wider pH range that encompasses acidic, neutral, and basic values (Fig. 5). In doing so, we also observed activation by NADH at acidic pH (Fig. 5, A and D) and inhibition as the pH approaches more basic values (Fig. 5, B and D). This effect is more clearly seen in a difference plot (Fig. 5F), where positive and negative values indicate activation and inhibition, respectively. Altogether, these data demonstrate a pH-dependent switch between E3 NADH activation and inhibition near a pH of 6 (Fig. 5F).

The previously described 3-state redox model (41), parameterized using human liver E3 data (33), is able to capture the pH dependence of both the forward and reverse E3-catalyzed reaction and predicts product inhibition of NADH at near neutral to more basic pH values (supplemental Fig. S1). But the model fails to reproduce our data from the pig heart enzyme to the 3-state redox model (Fig. 5C, dashed line, and see 3-state redox model fitting).

To circumvent the lack of fit to our data (Fig. 5, circles) to the 3-state redox model (Fig. 5, dashed lines), we first implemented a 6-state model that accounted for a documented (44) change in oligomeric state as a function of pH with pig heart E3. This model is described in supplemental Fig. S3. We discovered that this model can fit the core pH-dependent NADH activation/
inhibition phenomenon with pH dependence of the forward reaction (supplemental Fig. S4), but it is significantly more complicated, adding six rate constants to the core 3-state model.

The simpler 4-state mechanism of Fig. 1A is better supported by the literature (47, 61, 62) and active site structure (Fig. 1B). This model, which accounts for the oxidation/reduction of the FAD cofactor (see FAD cofactor in Fig. 1B), is able to better fit the data (Fig. 5, solid lines). We also accurately fitted this data set using a 4-state redox model where the equilibrium dissociation constants ($K_d$) are not allowed to vary with the redox state of the enzyme (supplemental Fig. S2, solid lines), which suggests that the dissociation constants do not need to change as a function of E3 redox state to explain NAD$^+/\text{NADH}$-positive and -negative regulation.

Global Fitting of Kinetic and Spectroscopic Data to a 4-State Redox Model—A 4-state redox-dependent model simulates the pH-dependent NAD$^+$ activation/inhibition phenomenon of the reverse E3 reaction and forward pH-dependent data (Fig. 5, solid lines). To better characterize the NAD$^+$ activation/inhibition phenomenon, we gathered more reverse E3 kinetic data, while also incorporating the fitting of the equilibrium spectroscopic experiments (Fig. 6).

In addition, to more accurately simulate forward E3 kinetics, we obtained forward pig heart E3 initial velocity data (Fig. 6O) from Ref. 63, which varied NAD$^+/\text{NADH}$ in different concentrations of dihydrolipoamide. Dihydrolipoamide equilibrium titration data (47), described above, were also incorporated into this large data set by numerically integrating individual titration points to long times, which provides model consistency with FAD redox states as a function of dihydrolipoamide (Fig. 6M). Equilibrium titrations, described in Figs. 2 and 4, were incorporated into the fitting using fractional occupancies (Equation 3) to simulate the binding of NAD$^+$ to enzyme states S1 (Fig. 6Q) and S2 (Fig. 6R) and DL-lipoamide binding to enzyme state S1 (Fig. 6P).

Fig. 6 shows the best fit of the 4-state redox-dependent $K_d$ model. We also fit this large data set using a 4-state redox-independent $K_d$ model (supplemental Fig. S5), so that equilibrium dissociation constants for substrates and products do not depend on the redox state of the enzyme. The 4-state redox-independent model primarily has trouble fitting the forward
initial rates as a function of pH with all other data sets (supplementary Fig. S5).

The average difference between model fits and mean data shown in Fig. 6 is 12.6%. By computing the difference between model simulations and the error/uncertainty range of the data, the average deviation between model and data is 3.1%. Of 455 simulation points, 90 lie outside the range of uncertainty in the data. However, although the model fits are good, they are imperfect and do show biased error with respect to data in Fig. 6, G–L. The fits to these data could be improved by adding additional states to the model. The potential impact of these imperfections in the model fits is explored below.

Because our experiments use a DL-lipoamide mixture, a simple modification to the 4-state redox model to account for this mixture was used to investigate whether this detail in the model would improve the fit to the data (supplemental Figs. S11 and S12). We were unable to improve the fit accounting for this fact in the model, however. A discussion of this model and results can be found in the supplemental material under “Lipoamide Stereochemistry.”

Calculation of Mammalian E3 4-State Redox Steady-state Distribution and Physiological Flux Surface—The 4-state redox-dependent $K_d$ model, using best-fit parameters (Table 1), was used to calculate a physiological mam-E3 4-state redox steady-state distribution and flux surface (Figs. 7 and 8). These calculations used a physiologically relevant total NAD concentration of 3 mM (54) and lipoamide concentration of 10 mM (1). NAD$^+$/NADH ratios were also chosen based on a range that spans conditions that may represent normal, abnormal, or diseased redox states (58), and were constrained by total NAD and lipoamide concentrations using Equations 8 and 9.

The calculated mam-E3 4-state redox steady-state distribution (Fig. 7) has a similar profile to a previously calculated distribution (41), parameterized by a much smaller data set using human liver E3 data (33, 41). The model is validated by experimental observations using stopped-flow technology (46, 61) that show the redox state-3 ($S_3$), or the FAD reduced state, is present in very low fractions relative to the remaining enzyme redox states.

We calculated the mam-E3 physiological flux surface by both treating the flux as a function of NAD$^+$/NADH and pH, with fixed Lipo/DHL (Fig. 8A), and as a function of NAD$^+$/NADH and Lipo/DHL at pH 7.2 (Fig. 8C). The flux surface was calculated to gain insight into conditions that either produce forward (positive) or reverse (negative) E3 flux. These calculations can then predict the point at which the readily reversible mam-E3 switches direction and magnitude in flux.

Cross-sections from the multidimensional mam-E3 flux surface at different fixed pH (Fig. 8B) and Lipo/DHL (Fig. 8D) show NAD$^+$/NADH ratios that cross a zero flux (equilibrium) threshold, demonstrating inflection points of E3 forward and reverse flux. For example, the calculated mam-E3 flux is near zero at NAD$^+$/NADH ratios of 2.5, 1.5, and 1, at fixed pH values of 7, 7.2, and 7.5, respectively, using a fixed Lipo/DHL ratio of 1.

FIGURE 9. Calculated E. coli E3 NAD$^+$/NADH, Lipo/DHL, and pH-dependent flux surface. The globally fitted parameters obtained from Moxley et al. (41), to the 3-state redox-dependent $K_d$ model, were used to calculate the E. coli E3 NAD$^+$/NADH, Lipo/DHL, and pH-dependent flux surfaces. A, E. coli E3 flux as a function of NAD$^+$/NADH and pH, at a constant Lipo/DHL ratio of 1, was used to calculate the E. coli E3 flux surface. B, E. coli E3 flux (NAD$^+$/NADH, pH, Lipo/DHL = 1) cross-sections at pH 5, 6, 7, 7.5, and 8. C, E. coli E3 flux as a function of NAD$^+$/NADH and Lipo/DHL, at a constant pH of 7.2, was used to calculate the E. coli E3 flux surface. D, E. coli E3 flux (NAD$^+$/NADH, pH = 7.2, Lipo/DHL) cross-sections at Lipo/DHL ratios of 0.1, 1, and 10. The black dashed line is a reference for zero flux. In all panels, the forward and reverse fluxes are defined as being positive and negative, respectively. The forward flux is defined from left to right in Reaction 1 in the text.
A function of NAD between mammalian and E. coli. These calculations illustrate a stark difference in parameter values with the previously described (41) 3-state model, this calculation was also carried out with Ec-E3 (Fig. 12, B). The enzyme state fractions corresponding to each of these limits are also calculated (Fig. 11, A and B, top). Some of these results are included in Table 2. This calculation reveals that the reverse flux as enzyme substrates approach infinity. To illustrate this point, we calculated the $k_{cat}$ for mam-E3 as a function of pH using the 4-state redox model (using best-fit parameters in Table 1) in both the forward and reverse direction (Fig. 11, A and B, top). Some of these results are included in Table 2. This calculation reveals that the reverse $k_{cat}$ produces rates less than the observed turnover (Fig. 6, G–L) at a given pH. The enzyme state fractions corresponding to each of these limits were also calculated (Fig. 11, A and B, bottom). In comparison, this calculation was also carried out with Ec-E3 (Fig. 12, A and B) using the 3-state redox model (41), producing a similar result that we have mentioned elsewhere (41).

Despite $k_{cat}$ producing a submaximal flux in the reverse direction for both mammalian (Fig. 11B) and E. coli E3 (Fig. 12B), the forward $k_{cat}$ (Figs. 11A and 12A) does not suffer from

\[ \text{Mammalian E3 pH-dependent Activation/Inhibition} \]

**FIGURE 10. Calculated E. coli E3 NAD$^+$/NADH, Lipo/DHL, and pH-dependent flux surface.** The globally fitted parameters obtained from Maxley et al. (41), to the 3-state redox-dependent $K_r$ model, were used to calculate the E. coli E3 NAD$^+$/NADH, Lipo/DHL, and pH-dependent flux surfaces. A, E. coli E3 flux as a function of NAD$^+$/NADH and pH, at a constant Lipo/DHL ratio of 5, was used to calculate the E. coli E3 flux surface. B, E. coli E3 flux (NAD$^+$/NADH, pH, Lipo/DHL = 5) cross-sections at pH 7, 7.5, and 8. C, E. coli E3 flux as a function of NAD$^+$/NADH and Lipo/DHL, at a constant pH of 7.5, was used to calculate the E. coli E3 flux surface. D, E. coli E3 flux (NAD$^+$/NADH, pH 7.5, and Lipo/DHL) cross-sections at a Lipo/DHL ratio of 1. The black dashed line is a reference for zero flux. In all panels, the forward and reverse fluxes are defined as being positive and negative, respectively. The forward flux is defined from left to right in Reaction 1 in the text.

(Fig. 8, B and D). Therefore, mam-E3 has the potential to catalyze its reaction in both directions with similar magnitude at or near physiological pH, NAD$^+$/NADH, and reasonable steady-state NAD and lipoamide pools. The steady-state value of the Lipo/DHL ratio is, of course, also an important dictator of E3 flux direction and magnitude (Fig. 8D).

To explore how the error and uncertainty in model fits to data affect these model predictions, we randomly perturbed adjustable parameter values to obtain 1000 new parameter sets within a 10% boundary of the best-fit values from Table 1 (see supplemental material for parameter perturbation). The maximum and minimum model output from the parameter sets shows a wider span of the model simulation, but the qualitative behavior of the model is unchanged. (Compare supplemental Fig. S8 with Fig. 6.) The effects of this parameter perturbation on the predictions shown in Fig. 8 are illustrated in supplemental Fig. S9, revealing that these predictions are largely insensitive to the uncertainty in the model parameterization.

In comparison of the calculated mam-E3 flux surface (Fig. 8), we also computed the Ec-E3 flux surface (Fig. 9) using best-fit parameter values with the previously described (41) 3-state redox model. These calculations illustrate a stark difference between mammalian and E. coli E3 enzymes in their net flux as a function of NAD$^+$/NADH, Lipo/DHL, and pH. Mam-E3 easily toggles between forward and reverse net flux (Fig. 8A), whereas Ec-E3 catalytically favors the forward reaction (Fig. 9A), requiring higher NAD$^+$/NADH and Lipo/DHL ratios for net reverse catalysis at pH 7.2 (Fig. 9D). Despite this observation, we find that after increasing the Lipo/DHL ratio to 5 and/or the pH to 7.5, Ec-E3 is clearly able to switch catalytically direction (Fig. 10) within reasonable physiological conditions. Additional flux surface plots for both mam-E3 (supplemental Fig. S6) and Ec-E3 (supplemental Fig. S7) are given and further illustrate the flux as a multidimensional function of NAD$^+$/NADH, Lipo/DHL, and pH.

\[ \text{Mammalian and E. coli E3 pH-dependent Flux Optimization as a Function of NAD$^+$/NADH and Lipo/DHL—Maximal flux is typically determined by calculating the } k_{cat} \text{ for a specified condition (48, 59, 64). But the } k_{cat} \text{ is not the true maximum (48). It is the limit of the flux as enzyme substrates approach infinity. To illustrate this point, we calculated the } k_{cat} \text{ for mam-E3 as a function of pH using the 4-state redox model (using best-fit parameters in Table 1) in both the forward and reverse direction (Fig. 11, A and B, top); some of these results are included in Table 2. This calculation reveals that the reverse } k_{cat} \text{ produces rates less than the observed turnover (Fig. 6, G–L) at a given pH. The enzyme state fractions corresponding to each of these limits were also calculated (Fig. 11, A and B, bottom). In comparison, this calculation was also carried out with Ec-E3 (Fig. 12, A and B) using the 3-state redox model (41), producing a similar result that we have mentioned elsewhere (41).} \]
this issue. These calculations then illustrate the point that without substrate inhibition or possibly mechanistic randomness (64), this limit will produce the maximum amount of substrate-bound state (i.e. ES) leading to maximal flux. However, mechanisms that have a non-hyperbolic dependence of the rate on the substrate concentration (in this case in the reverse direction) need to be analyzed by other optimization methods rather than the traditional limit of substrate concentration.

To address this issue, mammalian and *E. coli* E3 reverse flux was maximized (Fig. 13, A and B), using the best-fit rate constants and equilibrium constants (Table 1) and by varying NAD$^+$/NADH and Lipo/DHL. (Ec-E3 best-fit rate constants were determined previously (41)). Importantly, NAD$^+$/NADH
Mammalian E3 pH-dependent Activation/Inhibition

TABLE 2
Mammalian and E. coli E3 $k_{cat}$ and flux optimization analysis

Calculations determining $k_{cat}$ as a function of pH and optimization algorithms used to produce the flux maximum ($f_{max}$) are described in “Experimental Procedures.” Flux ($f$) optimizations required to produce $f_{max}$ conserve NAD (54) and lipoamide (1) pool sizes of 3 and 10 msi, respectively.

| Parameter                  | Mammalian E3 | Mammalian E3 pH | Mammalian E3 Reverse | E. coli E3 | E. coli E3 pH | E. coli E3 Reverse |
|---------------------------|--------------|----------------|----------------------|------------|--------------|-------------------|
| $k_{cat}(pH)$             | 2.6 × 10^7 (pH = 8.24) | 369 (pH = 4) | 2.1 × 10^4 (pH = 8) | 9.5 (pH = 5.25) |
| $J_{E3}$                  | 3.5 × 10^4 | 2 × 10^10 | 5.25 × 10^4 | 4.4 × 10^1 |
| NAD$^+/NADH^-$            | 10^-12      | 10^-12      | 9.6 × 10^11 | 22         |
| Lipo/DHL                  | 10^-12      | 10^-12      | 10^-12        | 22 × 10^1 |
| pH                        | 9           | 5.6         | 8.11         | 7.67       |

* Mammalian E3 $k_{cat}$ (pH) and flux maximum ($f_{max}$) were determined using the 4-state redox $K_{eq}$-dependent parameters shown in Table 1.
* E. coli E3 $k_{cat}$ (pH) and flux maximum ($f_{max}$) were determined using the previously described 3-state model and best-fit parameters (41).
* Maximum $k_{cat}$ (pH) values are displayed in units of min$^{-1}$.
* E3 flux maximum ($f_{max}$) are displayed in units of min$^{-1}$.
* NAD$^+/NADH$ and Lipo/DHL ratios were given a boundary between 10^-12 and 10^-12 in this instance to allow for greater flux optimization compared with ratio values that were given tighter bounds that are more consistent with physiological conditions shown in Figs. 13 and 14.

Table 2: Mammalian and E. coli E3 $k_{cat}$ and flux optimization analysis. The table lists the $k_{cat}$ values for Mammalian E3 and E. coli E3 at different pH values, along with the flux maximum ($f_{max}$) and other kinetic parameters. The table also includes the NAD$^+/NADH$ and Lipo/DHL ratios used in the optimizations.

Discussion

Early reports (39, 40) of pig heart E3 reverse reaction kinetics demonstrated a unique NAD$^+$ product activation phenomenon, which is especially prominent at low pH and higher NADH concentrations. However, these earlier reports (39, 40) lacked assay conditions that varied NAD$^+/NADH$ or lipoamide and did not probe physiological pH values.

NAD$^+$ product activation has also been observed in E. coli (30), spinach (32), and human liver (33) E3, in which we previously derived a 3-state redox kinetic model that accurately simulated these data sets (41). By analyzing mammalian data sets from rat (34) and human (33) liver E3, it was determined that the available data were not sufficient to accurately characterize mam-E3 NAD$^+/NADH$ pH-dependent regulation (41).

To address this void, pig heart E3 reverse kinetics were assayed in 162 initial conditions (Fig. 6, G–L) varying NAD$^+/NADH$, α-lipoamide, and pH ranging from 4.5 to 8.5, in physiological ionic strength (170 mM) using KCl as a background salt. We also conducted NAD$^+$ and α-lipoamide equilibrium titrations on oxidized pig heart E3 to more directly identify equilibrium dissociation constants that would be consistent with our kinetic model (Figs. 2 and 3). Other more sophisticated literature-derived (46, 47) active site (FAD) spectroscopic experiments were analyzed by linear algebraic methods (Fig. 4). These data sets (Fig. 4) were also incorporated into our large experimental ensemble (Fig. 6) to maintain consistency with observations that directly probe E3 active site redox states. Furthermore, forward reaction pH-dependent and dihydrolipoamide/NAD$^+$-dependent data sets, from human liver (33) and pig heart (63) E3 (Fig. 6, N and O), were added to accurately model mam-E3 kinetic reversibility.

Our newly collected pig heart E3 reverse kinetic assays have demonstrated a NAD$^+$ inhibitory effect in the physiological pH range (pH 7–7.5), rather than the activation effect traditionally reported at low pH (39, 40). Generally, we find that at a pH near 6, NAD$^+$ switches between an activator and inhibitor (Fig. 5). We have not been able to find this effect demonstrated with any previous E3 kinetic data sets. NAD$^+$ inhibition effects are more physiologically relevant in the grand scheme of PDHC regulation, because it is this type of regulation that manifests near neutral pH. Moreover, product inhibition is ubiquitous among enzyme kinetic characterization studies and is typically expected (59). Although we were unable to find reverse E3 kinetic data demonstrating a NAD$^+$ inhibitory effect, we discovered that our previous 3-state model (41) predicts NAD$^+$ inhibition (supplemental Fig. S1B) at physiological pH by extrapolating [NAD$^+$] > 100 μM using a previously determined (41) best-fit parameter set derived from fitting human liver E3 kinetic data (33, 41). Despite similar 3-state model predictions based on human liver E3 parameters to our pig heart E3 data, we were unable to accurately fit our data using the 3-state model (Fig. 5).
To account for the lack of fit to the data with the 3-state model, we developed a 4-state redox model (Fig. 1A) that accounts for a chemical step that was previously simplified in the 3-state redox model (41). The 4-state redox model accounts for the internal electron transfer between the active site disulfide and the FAD cofactor (Fig. 1, A and B). This added step decomposes the 2e⁻ reduced state in the original 3-state redox model (41), creating an additional state. This mechanism is able...
to simulate both reverse reaction NAD$^+$ activation/inhibition and reaction forward pH dependence (Fig. 5, solid lines).

All model simulations were subjected to thermodynamic constraints (2, 51) and to a global fitting (Fig. 6) approach rather than the more simple (and inconsistent) approach of individual data set fitting, which is ubiquitous in the field of enzyme kinetics (66).

Alternative parameter sets that fit the data (Fig. 6) equally as well from random independent starts are difficult to find. This result is consistent with parameters that are more constrained than our previous result in fitting a small human liver E3 data set (33, 41) that produced numerous best-fit examples with an extremely wide range of parameter values (41). Thus the breadth of the data set reported here facilitates a level of precision in model identification that was not possible based on previously available kinetic data (41).

Beyond data collection, model derivation, and parameterization, we also probed model predictions in flux and enzyme states as a function of NAD$^+$/NADH, Lipo/DHL, and pH to gain insight into E3 kinetics (Figs. 7 and 8). Calculation of E3 redox states as a function of NAD$^+$/NADH, Lipo/DHL, and pH (Fig. 7) reveals an interesting prediction that is consistent with stopped-flow experiments (46). The calculated mam-E3 state-3 (S3), irrespective of most conditions, is present at very low fractions (Fig. 7). This model prediction is consistent with stopped-flow spectroscopic studies, which were unable to observe this state due to its very transient nature using pig heart E3 (46).

To better understand E3 kinetic reversibility, we calculated the mam-E3 steady-state flux surface (Fig. 8) as a function of NAD$^+$/NADH and pH, at Lipo/DHL = 1, which demonstrates a readily reversible catalytic surface. Of particular interest here are the conditions at which this enzyme switches direction in flux because of the kinetic control E3 has on lipoamide (in PDHc L2 domain) redox status (8); the lipoamide redox state is important for PDK binding (22) and thus PDHc phosphorylation status. Therefore, PDHc NAD$^+$/NADH sensing is mediated through E3 and is closely linked to PDHc phosphoryl regulation.

Our 4-state model predicts, using constant NAD and lipoamide pool sizes (see “Experimental Procedures”), at physiological pH that NAD$^+$/NADH values between 1 and 2.5 would begin to change E3 flux direction with a Lipo/DHL ratio set to one (Fig. 8B). In comparison with mam-E3, we also calculated the Ec-E3 flux surface (Fig. 9) and noticed a significant difference in the kinetic reversibility of these homologs in the simulated condi-
Mammalian E3 pH-dependent Activation/Inhibition

FIGURE 14. Maximal mammalian and E. coli E3 forward flux at fixed pH values via NAD/NADH and Lipo/DHL ratio optimization. A–C, 4-state redox model flux expression parameterized (Table 1) by globally fitting the data shown in Fig. 6 was maximized in forward mammalian E3 flux using the NAD+/NADH and Lipo/DHL ratios as adjustable parameters at fixed pH values. D–F, the E. coli E3 forward flux was maximized in the same manner as the mammalian E3 forward flux using a 3-state redox-dependent $k_\text{cat}$ model described and parameterized in Moxley et al. (41). In each case, the total concentration of lipoamide and NAD was fixed to 10 and 3 mM, respectively, according to literature estimates (54). The concentration of lipoamide is based on a previous estimation considering the stoichiometry and volume of the pyruvate dehydrogenase complex (1). The optimized value of the Lipo/DHL ratio was approximately the lower bound of $10^{-12}$ in all cases. Fitted NAD+/NADH values were given tighter boundaries compared with optimizations shown in Figs. 11 and 12, as described under “Experimental Procedures.” A, maximal mammalian E3 forward flux at fixed pH values using NAD+/NADH and lipo/DHL ratios as adjustable parameters with the 4-state redox model flux expression parameterized (Table 1) with the data in Fig. 6. 8, fitted NAD+/NADH ratios at fixed pH values for the mammalian E3 forward flux optimization. C, calculated mammalian fractional enzyme states at fixed pH values, using the fitted NAD+/NADH and Lipo/DHL ratios, in B, D, maximized E. coli E3 forward flux at fixed pH values using NAD+/NADH and lipo/DHL ratios as adjustable parameters with the 3-state redox model flux expression described and parameterized in Moxley et al. (41). E, fitted NAD+/NADH ratios at fixed pH values for the E. coli E3 forward flux optimization. F, calculated E. coli fractional enzyme states at fixed pH values using the fitted NAD+/NADH and Lipo/DHL ratios in E.

These simulations demonstrate the kinetic difficulty of Ec-E3 reverse (Fig. 9) catalysis compared with mam-E3 (Fig. 8). In this respect, Lipo/DHL ratios are very important in dictating flux direction and magnitude, whereas higher Lipo/DHL ratios for Ec-E3 relative to mam-E3 (Figs. 8D, 9, and 10 and supplemental Fig. S7) are more essential for reverse Ec-E3 catalysis. After increasing Lipo/DHL and pH, we found that Ec-E3 also demonstrates a reversible catalytic flux (Fig. 10), simulated using E. coli intracellular pH and NAD+/NADH ratios (55, 67). Sole E3 NAD+/NADH ratio regulation of the E. coli PDHc is expected due to the lack of PDK and PDP enzymes (30, 35).

In order for mam-E3 to kinetically control the lipoamide redox state, and thus PDK binding affinity (22), it needs to effectively catalyze both forward and reverse directions in physiological conditions. It follows then that the demonstration of a readily reversible mam-E3 physiological catalytic surface (Fig. 8) supports the overall mechanism of mam-E3 kinetic regulation of L2 domain redox status, important for PDK binding affinity and subsequent phosphoryl regulation (26).

Although rate laws have been developed for a large number of classical models of enzyme mechanisms (64), our analysis of E3 kinetics required the introduction of a novel mechanism to capture pH-dependent NAD$^+$ product activation (30, 33, 39, 40) and inhibition (shown here in Fig. 5), as well as pH and NAD$^+$-dependent progress curve lag phases (30, 33, 39, 68), substrate inhibition (30, 39), and pH-dependent oligomeric state regulation (44), as discussed elsewhere. In this model, as for many complex enzymatic mechanisms, the conventional method ($k_\text{cat}$) to represent the optimal rate (48) fails to reveal the true catalytic maximum.

For example, in the case of simple substrate inhibition, it is traditional to ignore inhibitory terms to produce a $k_\text{cat}$ value. This value, however, does not reflect the actual mechanism, and thus it is a misrepresentation of the actual limit of enzymatic turnover. In the case of E3, calculating the theoretical reverse maximum flux with pH-dependent substrate inhibition (in NADH), pH-dependent product activation/inhibition (in NAD$^+$), randomness in substrate/product binding, and more than one pathway to product requires solving a multivariate optimization problem to find maximal enzymatic flux.

Our results show that the forward E3 maximal flux with mam-E3 (Fig. 11C, top) and Ec-E3 (Fig. 12C, top) is similar to $k_\text{cat}$ (Figs. 11A and 12A, top), whereas the reverse E3 maximal flux (Figs. 11D and 12D, top), conditions, and enzyme states (Fig. 11D and 12D, bottom) are much different from the reverse $k_\text{cat}$ (Figs. 11B and 12B, top; Table 2). Our optimized conditions are constrained by reasonable physiological NAD and lipoam-
ide pools (see “Experimental Procedures”) and NAD⁺/NADH and Lipo/DHl ratios, where \( k_{\text{cat}} \), does not consider these constraints and produces results that may be submaximal and non-physiological in the extent of extrapolated infinite substrate (48).

We believe that the general numerical approach of optimizing enzyme substrate/product ratios, using a rigorously parameterized enzyme kinetic model, will be useful for understanding enzyme flux optima especially in the context of a mechanism that produces non-Michaelis-Menten behavior (hyperbolic deviation (64)). Furthermore, more physiologically feasible enzyme states and optimal conditions are revealed with the proper constraints. This approach should be of general interest to fields that rely on enzyme optimization such as enzymology and enzyme engineering, as well as more broadly scoped fields including systems biology and bioengineering.

**Author Contributions**—M. A. M. designed and conducted the experiments. M. A. M. and J. N. B. analyzed the experiments and wrote computer code for analysis. M. A. M. wrote the paper. J. N. B., D. A. B., and M. A. M. revised the paper. All authors approved the final manuscript.

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Mammalian E3 pH-dependent Activation/Inhibition

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