

Allostery and Kinetic Proofreading

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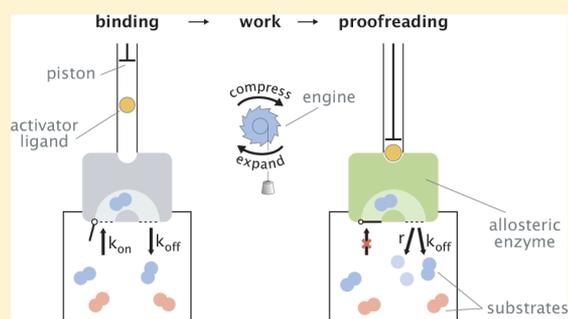
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Supporting Information

ABSTRACT: Kinetic proofreading is an error correction mechanism present in the processes of the central dogma and beyond and typically requires the free energy of nucleotide hydrolysis for its operation. Though the molecular players of many biological proofreading schemes are known, our understanding of how energy consumption is managed to promote fidelity remains incomplete. In our work, we introduce an alternative conceptual scheme called “the piston model of proofreading” in which enzyme activation through hydrolysis is replaced with allosteric activation achieved through mechanical work performed by a piston on regulatory ligands. Inspired by Feynman’s ratchet and pawl mechanism, we consider a mechanical engine designed to drive the piston actions powered by a lowering weight, whose function is analogous to that of ATP synthase in cells. Thanks to its mechanical design, the piston model allows us to tune the “knobs” of the driving engine and probe the graded changes and trade-offs between speed, fidelity, and energy dissipation. It provides an intuitive explanation of the conditions necessary for optimal proofreading and reveals the unexpected capability of allosteric molecules to beat the Hopfield limit of fidelity by leveraging the diversity of states available to them. The framework that we have built for the piston model can also serve as a basis for additional studies of driven biochemical systems.



1. INTRODUCTION

Many enzymatic processes in biology need to operate with very high fidelities in order to ensure the physiological well-being of the cell. Examples include the synthesis of molecules making up Crick’s so-called “two great polymer languages” (i.e., replication,¹ transcription,² and translation³) as well as processes that go beyond those of the central dogma, such as protein ubiquitylation mediated by the anaphase-promoting complex,⁴ signal transduction through MAP kinases,⁵ pathogen recognition by T-cells,^{6,7} or protein degradation by the 26S proteasome.⁸ In all of these cases, the designated enzyme needs to accurately select its correct substrate from a pool of incorrect substrates. Importantly, the fidelity of these processes that one would predict solely on the basis of the free energy difference between correct and incorrect substrate binding is far lower than what is experimentally measured, raising the challenge to explain the high fidelities for which this naive equilibrium thermodynamic thinking fails to account.

The conceptual answer to this challenge was provided more than 40 years ago in the work of John Hopfield⁹ and Jacques Ninio¹⁰ and was coined “kinetic proofreading” in Hopfield’s elegant paper entitled “Kinetic Proofreading: A New Mechanism for Reducing Errors in Biosynthetic Processes Requiring High Specificity”.⁹ The key idea behind kinetic

proofreading is to introduce a delay between the substrate binding and turnover steps, effectively giving the enzyme more than one chance to release the incorrect substrate (hence, the term “proofreading”). The sequential application of substrate filters on the way to product formation gives directionality to the flow of time and is necessarily accompanied by the expenditure of free energy, making kinetic proofreading an intrinsically nonequilibrium phenomenon. In a cell, this free energy is typically supplied to proofreading pathways through the hydrolysis of energy-rich nucleotides, whose chemical potential is maintained at large out-of-equilibrium values through the constant operation of the cell’s metabolic machinery (e.g., ATP synthase).

Since its original formulation by Hopfield and Ninio, the concept of kinetic proofreading has been generalized and employed in explaining many of the high-fidelity processes in the cell.^{8,11–17} However, despite the fact that the molecular players and mechanisms of these processes have largely been identified, we find that an intuitive picture of how energy transduction promotes biological fidelity is still incomplete. To

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complement our understanding of how energy is managed to beat the equilibrium limit of fidelity, we propose a conceptual model called “the piston model of kinetic proofreading” in which chemical hydrolysis is replaced with mechanical work performed by a piston on an allosteric enzyme. Our choice of allostery is motivated by the fact that in proofreading schemes hydrolysis typically triggers a conformational change in the enzyme–substrate complex and activates it for product formation^{18–20}—an effect that our model achieves through the binding of a regulatory ligand to the enzyme. By temporally controlling the concentration of regulatory ligands which determine the catalytic state of the enzyme, the piston sequentially changes the enzyme’s state from inactive to active, creating the delay in product formation that is necessary to increase the fidelity of substrate discrimination. The piston actions are, in turn driven by a Brownian ratchet and pawl engine powered by a lowering weight, whose function is akin to that of ATP synthase. The mechanical design of the piston model allows us to transparently control the energy input into the system by tuning the “knobs” of the engine and examine the graded changes in the model’s performance metrics, intuitively demonstrating the driving conditions required for optimal proofreading.

We begin the presentation of our results by first introducing in section 2 the high-level concept behind the piston model of proofreading while at the same time drawing parallels between its features and those of Hopfield’s original scheme. Then in sections 3.1 and 3.2 we provide a comprehensive description of the two key constituents of the piston model, namely, the Brownian ratchet and pawl engine that drives the piston actions and the allosteric enzyme whose catalytic state is regulated by an activator ligand. This is followed by construction of the full thermodynamically consistent framework of the piston model in section 3.3, where we couple the external driving mechanism to the enzyme and introduce the expressions for key performance metrics of the model. In sections 3.4 and 3.5, we explore how tuning the “knobs” of the engine leads to graded changes and trade-offs between speed, fidelity and energy dissipation, and probe the performance limits of the piston model as a function of a select set of key enzyme parameters.

2. MODEL

The piston model of kinetic proofreading is designed in analogy with Hopfield’s scheme. The main idea there was to give the enzyme a second chance to discard the wrong substrate by introducing an additional kinetic intermediate for the enzyme–substrate complex (Figure 1A). The difference between substrate binding energies in Hopfield’s original formulation was based solely on their unbinding rates (i.e., $k_{\text{off}}^{\text{W}} > k_{\text{off}}^{\text{R}}$ and $k_{\text{on}}^{\text{W}} = k_{\text{on}}^{\text{R}} = k_{\text{on}}$)—a convention we adopt throughout our analysis. The first layer of substrate discrimination in Hopfield’s scheme is achieved during the initial binding event, where the ratio of right and wrong substrate-bound enzymes approaches $k_{\text{off}}^{\text{W}}/k_{\text{off}}^{\text{R}}$. The complex then moves into its catalytically active high-energy state accompanied by the hydrolysis of an NTP molecule, after which the second discrimination layer is realized. Specifically, right and wrong substrates are turned into products with an additional bias given by the ratio of their Michaelis constants, namely, $(k_{\text{off}}^{\text{W}} + r)/(k_{\text{off}}^{\text{R}} + r)$. Importantly, for this second layer to be efficiently realized, the rates of binding directly to the second

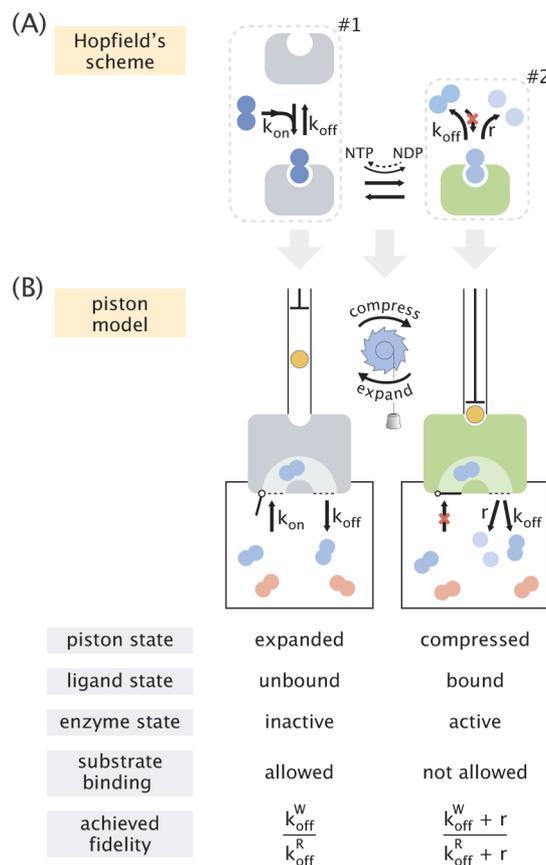


Figure 1. Conceptual introduction to the piston model. (A) Hopfield’s scheme of kinetic proofreading in which two layers of substrate discrimination take place on the driven pathway—the first one during the initial binding of energy-rich substrates (#1 in the diagram) and the second one upon the release of the energy-depleted substrates (#2 in the diagram). Energy consumption takes place during the hydrolysis reaction $\text{NTP} \rightleftharpoons \text{NDP}$ accompanying the transition between the two intermediates. (B) Pedagogically simplified conceptual scheme of the piston model. The orange circle represents the activator ligand. Blue and red colors stand for the right and wrong substrates, respectively. The closed “entrance door” with the red cross on the binding arrow in the active state of the enzyme suggests the vanishingly small rate of substrate binding when in this state. The ratchet with a hanging weight stands for the mechanical engine that drives the piston actions. Various features of the system in the two piston states along with the expressions for the achieved fidelities are listed below the diagram. Transparent arrows between (A) and (B) indicate the analogous parts in Hopfield’s scheme and the piston model.

kinetic intermediate need to be vanishingly small in order to prevent the incorporation of unfiltered substrates.⁹

With this information in mind, consider now the conceptual illustration of the piston model shown in Figure 1B, where we have made several pedagogical simplifications to help verbally convey the model’s intuition, reserving the full thermodynamically consistent treatment to the following sections. The central constituent of the model is an allosteric enzyme, the catalytic activity of which is regulated by activator ligands (the orange circle). The enzyme is inactive when it is not bound to a ligand, and conversely, it is active when bound to a ligand. The volume occupied by ligands and hence their concentration are in turn controlled by a piston. The ligand concentration is very low when the piston is expanded and very high when the

piston is compressed in order to guarantee that in those piston states the ligand is free and bound to the enzyme, respectively.

The active site of the enzyme is exposed to a container filled with right and wrong substrates with concentrations $[R]$ and $[W]$, respectively, which we take to be equal for the rest of our analysis ($[R] = [W]$). Unlike in Hopfield's scheme, where the substrates exist in energy-rich and energy-depleted states (e.g., tRNAs first arrive in the EF-Tu·GTP·tRNA ternary complex and then release EF-Tu and GDP after hydrolysis), in the piston model substrates exist in a single state and do not carry an energy source. In the expanded piston state (Figure 1B, left), substrates can bind and unbind to the inactive enzyme but do not get turned into products. The highest level of discrimination achievable in this state is therefore given by

$$\eta_1 = \frac{k_{\text{off}}^{\text{W}}}{k_{\text{off}}^{\text{R}}} \quad (1)$$

in analogy to that achieved during the initial binding step of Hopfield's scheme.

After the first layer of substrate discrimination is established in the expanded state of the piston, mechanical work is performed to compress it. This increases the ligand concentration, which in turn leads to activation of the enzyme, where catalytic action is now possible. To prevent the incorporation of unfiltered substrates, we assume that in the active enzyme state the rate of substrate binding is vanishingly small, similar to Hopfield's treatment (Figure 1B, right). If the piston is kept compressed long enough, a filtered substrate that got bound earlier when the piston was expanded will experience one of these two outcomes: it will either turn into a product with a rate r (which is taken to be the same for the two kinds of substrates) or it will fall off with a rate k_{off} . The product formation reaction will take place with probability $r/(k_{\text{off}} + r)$. Thus, because of the difference in the falloff rate constants for the right and wrong substrates, the extra fidelity achieved after piston compression becomes

$$\eta_2 = \frac{k_{\text{off}}^{\text{W}} + r}{k_{\text{off}}^{\text{R}} + r} \quad (2)$$

Once this extra fidelity is established, the piston is expanded back, repeating the cycle (the detailed derivation of the results for η_1 and η_2 is provided in Supporting Information (SI) section A). Notably, the total fidelity achieved during the piston expansion and compression cycle, namely,

$$\eta = \eta_1 \eta_2 = \left(\frac{k_{\text{off}}^{\text{W}}}{k_{\text{off}}^{\text{R}}} \right) \left(\frac{k_{\text{off}}^{\text{W}} + r}{k_{\text{off}}^{\text{R}} + r} \right) \quad (3)$$

exceeds the Michaelis–Menten fidelity (η_2) by the factor $\eta_1 = k_{\text{off}}^{\text{W}}/k_{\text{off}}^{\text{R}}$, demonstrating the attainment of efficient proofreading.

The cyclic compressions and expansions of the piston in our model also stand in direct analogy to the hydrolysis-involving transitions between the two enzyme–substrate intermediates in Hopfield's scheme. In particular, they need to be externally driven for the mechanism to do proofreading. We perform this driving using a mechanical ratchet and pawl engine powered by a lowering weight. In our pedagogical description of the model's operation, we have implicitly assumed that this weight is very large in order to enable the mechanism to proofread, similar to how the hydrolysis energy needs to be large for Hopfield's scheme to operate effectively.⁹ In the full treatment of the model in section 3, however, we will demonstrate how

tuning of the weight can give us graded levels of fidelity enhancement and show that in the absence of this weight the equilibrium fluctuations of the piston alone cannot lead to proofreading.

In our model introduction, we have also made several simplifying assumptions for clarity of presentation that do not conform with the principle of microscopic reversibility, and it is important that we relax them in the full treatment of the model to make it thermodynamically consistent. In particular, we have assumed that the ligand is necessarily unbound and that the enzyme is necessarily inactive when the piston is expanded, with the reverse assumptions made when the piston is compressed. We have also assumed that substrate binding is prohibited in the active state of the enzyme. These assumptions allowed us to claim that no premature product formation takes place in the expanded piston state and that no unfiltered substrates bind to the activated enzyme in the compressed piston state, which in turn justifies the use of the long waiting times between the piston actions that are necessary to establish high levels of fidelity in each piston state. In the detailed analysis of our model presented in section 3, we relax these assumptions and consider the full diversity of enzyme states at each piston position with reversible transitions between them. As we will demonstrate, this will not only ensure the thermodynamic consistency of our treatment but also reveal the possibility of doing proofreading more than once by leveraging the presence of multiple inactive intermediates between enzyme's substrate-unbound and production states, which were not taken into account in our conceptual introduction of the model.

3. RESULTS

3.1. Ratchet and Pawl Engine Enables Tunable Control of Piston Actions. To drive the cyclic compressions and expansions of the piston necessary for achieving proofreading, we use a ratchet and pawl engine whose design is inspired by Feynman's original work.²¹ In his celebrated lectures, Feynman presented two implementations of the ratchet and pawl engine—one operating on the temperature difference between two thermal baths and the other driven by a weight that moves down as a result of gravity. In the piston model we adopt the second scheme, as it involves fewer parameters and illustrates the process of energy transduction more transparently.

The ratchet and pawl engine coupled to the piston is shown in Figure 2A. The engine is powered by a weight of mass m that is hanging from an axle connected to the ratchet. The free rotational motion of the ratchet is rectified by a pawl; when the pawl sits on a ratchet tooth, it prevents the ratchet from rotating in the clockwise (backward) direction. The mechanical coupling between the engine and the piston is achieved through a crankshaft mechanism that translates each discrete ratchet step into a full compression (up (u) → down (d)) or a full expansion (d → u) of the piston. We assume that the volume regulated by the piston contains a single ligand—a choice motivated by Szilard's thermodynamic interpretation of information, where a piston compressing a single gas molecule was considered.²²

The clockwise (backward) and counterclockwise (forward) steps of the microscopic ratchet are enabled through environmental fluctuations. Specifically, a backward step is taken whenever the pawl acquires sufficient energy from the environment to lift itself over the ratchet tooth on which it is

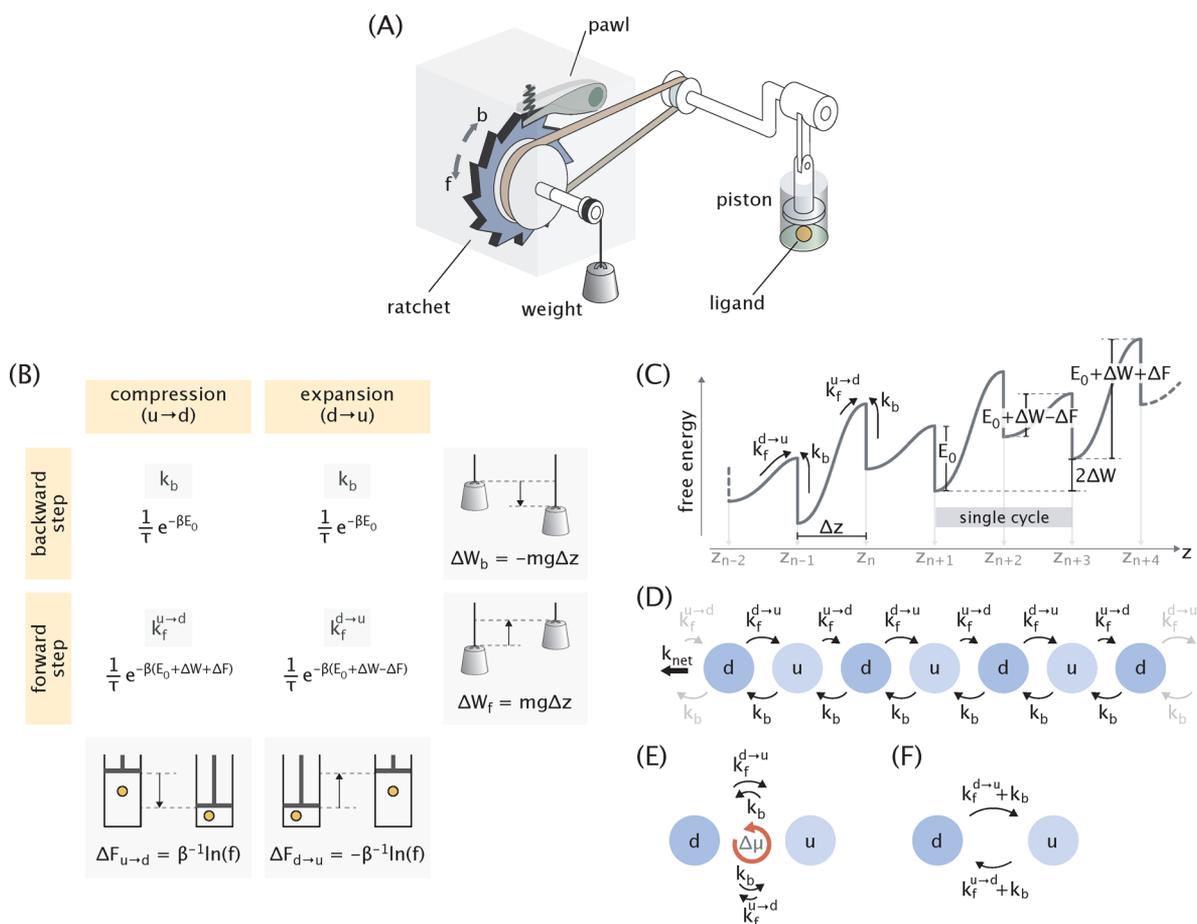


Figure 2. Ratchet and pawl mechanism coupled to the piston. (A) Schematic representation of the mechanism. The different radii of the ratchet wheel and the axle of the crankshaft ensure that a single ratchet step translates into a full compression or a full expansion of the piston (i.e., a 180° rotation of the crankshaft). Arrows with symbols “b” and “f” indicate the directions of backward and forward ratchet rotation, respectively. (B) Rates of the four kinds of transitions (symbols in shaded boxes with explicit expressions below) along with the accompanying changes in the potential energy of the weight and the free energy of the ligand. (C) Free energy landscape corresponding to the nonequilibrium dynamics of the system in the presence of a nonzero weight. Discrete positions of the weight (z_n) corresponding to energy minima of the landscape are marked on the reaction coordinate. (D) Infinite-chain representation of the dynamics of discrete system states. k_{net} stands for the net rate at which the weight goes down. (E) Equivalent two-state representation of the engine dynamics, where the driving force $\Delta\mu = 2\Delta W$ breaks the detailed balance in the diagram. (F) Collapsed representation of the diagram in (E) shown with the net transition rates from the two pathways.

sitting, allowing the tooth to slip under it (hence, the name “backward”). Following Feynman’s treatment,²¹ we write the rate of such steps as

$$k_b = \tau^{-1} e^{-\beta E_0} \quad (4)$$

where τ^{-1} is the attempt frequency, E_0 is the amount of energy needed to lift the pawl over a ratchet tooth, and $\beta = 1/k_B T$ is the inverse of the thermal energy scale (see SI section B.1 for a detailed discussion of the ratchet and pawl mechanism). Every backward step of the ratchet is accompanied by either a full compression or a full expansion of the piston as well as a lowering of the weight by an amount Δz , which reduces its potential energy by $\Delta W = mg\Delta z$.

Unlike in backward stepping, for a forward step to take place, the rotational energy acquired by the ratchet through fluctuations should be sufficient not only to overcome the resistance of the spring pressing the pawl onto the ratchet but also to lift the weight and to alter the state of the piston. This is a pure consequence of the geometric design of the ratchet and the positioning of the pawl. We assume that piston actions take place isothermally and in a quasistatic way, and therefore, we

write the changes in ligand free energy upon compression ($u \rightarrow d$) and expansion ($d \rightarrow u$) as

$$\Delta F_{u \rightarrow d} = \beta^{-1} \ln f \quad (5)$$

and

$$\Delta F_{d \rightarrow u} = -\beta^{-1} \ln f \quad (6)$$

respectively, where $f = V_u/V_d \geq 1$ is the compression factor. The signs of the free energy differences suggest that piston compressions slow down the forward steps, while expansions speed them up. These features are reflected in the two kinds of forward stepping rates, which are given by

$$k_f^{u \rightarrow d} = \tau^{-1} e^{-\beta(E_0 + \Delta W + \Delta F)} = f^{-1} k_b e^{-\beta \Delta W} \quad (7)$$

$$k_f^{d \rightarrow u} = \tau^{-1} e^{-\beta(E_0 + \Delta W - \Delta F)} = f k_b e^{-\beta \Delta W} \quad (8)$$

where $\Delta F = \beta^{-1} \ln f$ was used with a “+” and “−” sign in the place of $\Delta F_{u \rightarrow d}$ and $\Delta F_{d \rightarrow u}$ respectively. The rates of all four kinds of transitions, namely, forward or backward ratchet steps accompanied by either a compression or an expansion of the piston, are summarized in Figure 2B.

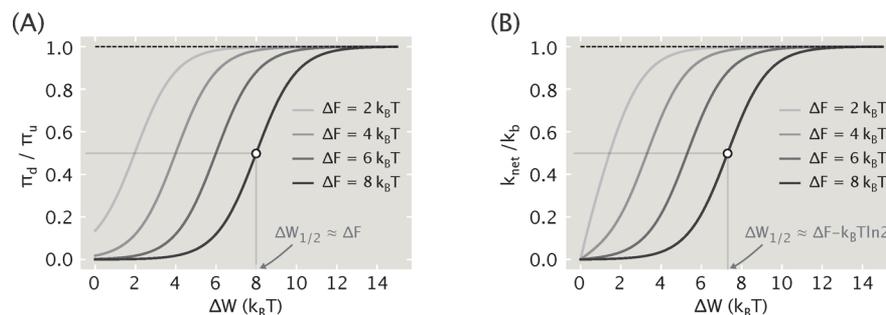


Figure 3. Nonequilibrium features of the engine–piston coupling. (A) Steady-state probability ratio of compressed (“d”) and expanded (“u”) piston states and (B) normalized net rate of backward stepping (k_{net}/k_b) as a function of the work per step (ΔW) for different choices of the ligand compression energy (ΔF). The $\Delta W_{1/2}$ expressions stand for the values of ΔW for which the corresponding value on the y axis is 0.5 (see SI section B.2). Negative ΔW values are not considered, as they further increase the undesired bias in piston state occupancies.

In the presence of a nonzero weight ($\Delta W > 0$), the ratchet will on average rotate backward—a feature reflected in the tilted free energy landscape shown in Figure 2C. As can be seen, the average dissipation per step is ΔW , and it is independent of ΔF . In addition, the work performed on the ligand upon compression is fully returned upon expansion, which, as we will demonstrate in section 3.4, will generally not be the case when we introduce the enzyme coupling. To further study the nonequilibrium dynamics of the driving mechanism, we map the local minima of the energy landscape corresponding to discrete vertical positions of the weight (or, equivalently, discrete ratchet angles) into an infinite chain of transitions, as shown in Figure 2D. There “d” and “u” stand for the compressed and expanded states of the piston, respectively. The net stepping rate k_{net} at which the weight goes down can be written as

$$k_{\text{net}} = (k_b - k_f^{d \rightarrow u})\pi_d + (k_b - k_f^{u \rightarrow d})\pi_u \quad (9)$$

where π_d and π_u are the steady-state probabilities of the compressed and expanded piston states, respectively. These probabilities can be obtained by considering the equivalent two-state diagram in Figure 2E, where the vertical position of the weight has been eliminated and the nonequilibrium nature of the dynamics is instead captured via the cycle through two alternative pathways connecting the piston states. The driving force $\Delta\mu$ in this cycle is given by²³

$$\Delta\mu = \beta^{-1} \ln \left(\frac{k_b^2}{k_f^{d \rightarrow u} k_f^{u \rightarrow d}} \right) = 2\Delta W \quad (10)$$

demonstrating the broken detailed balance in the presence of a nonzero weight and confirming the dissipation of $2\Delta W$ per cycle observed in the energy landscape (Figure 2C). We note that this procedure of mapping a linear network onto a cyclic one has also been used to model the processivity of molecular motors, where the linear coordinate corresponds to the position of the motor while the alternating states correspond to different motor conformations.^{24,25}

At steady state, the net incoming and outgoing fluxes at each piston state in Figure 2E should cancel each other (as can be seen more vividly in the collapsed diagram in Figure 2F):

$$(k_f^{d \rightarrow u} + k_b)\pi_d = (k_f^{u \rightarrow d} + k_b)\pi_u \quad (11)$$

Substituting the expressions for forward stepping rates (eqs 7 and 8) into eq 11 and additionally imposing the probability normalization constraint ($\pi_d + \pi_u = 1$), we can solve for π_d and π_u to obtain

$$\pi_d = \frac{1 + e^{-\beta(\Delta W + \Delta F)}}{2[1 + \cosh(\beta\Delta F)e^{-\beta\Delta W}]} \quad (12)$$

$$\pi_u = \frac{1 + e^{-\beta(\Delta W - \Delta F)}}{2[1 + \cosh(\beta\Delta F)e^{-\beta\Delta W}]} \quad (13)$$

Notably, in the absence of an external drive ($\Delta W = 0$), the piston state occupancies follow the Boltzmann distribution, that is, $(\pi_d/\pi_u)_{\text{eq}} = e^{-\beta\Delta F} = f^{-1}$, suggesting that at equilibrium the piston will predominantly dwell in the expanded state. Conversely, as can be seen in Figure 3A, when the work per step exceeds ΔF by several $k_B T$, the occupancies of the two piston states become equal to each other. This happens because at large ΔW values forward ratchet stepping becomes very unlikely and the dynamics proceeds only through backward steps with a rate k_b that is identical for both compressive and expansive steps. As will be shown in section 3.4, suppressing this equilibrium bias set by ΔF is essential for achieving efficient proofreading, analogous to the need for driving the transitions between the two enzyme–substrate intermediates in Hopfield’s scheme (Figure 1A).⁹

With the steady-state probabilities known, we can now substitute them into eq 9 to find the net rate at which the weight goes down:

$$k_{\text{net}} = \frac{(1 - e^{-2\beta\Delta W})k_b}{1 + \cosh(\Delta F)e^{-\beta\Delta W}} \quad (14)$$

As expected, k_{net} vanishes at equilibrium ($\Delta W = 0$), and asymptotes to k_b at large ΔW values, as shown in Figure 3B. The knowledge of k_{net} allows us to calculate the power (P) dissipated for the maintenance of the nonequilibrium steady state. Specifically, since k_{net} is the rate at which the weight goes down and ΔW is the dissipation per step, the power P is given by their product:

$$P = k_{\text{net}}\Delta W \quad (15)$$

The formalism developed in this section for characterizing the steady-state behavior of the system will be used as a basis for defining the different performance metrics of the model in section 3.3.

3.2. Thermodynamic Constraints Make Fidelity Enhancement Unattainable in the Absence of External Driving. In order to implement a thermodynamically consistent coupling between the engine and the allosteric enzyme, we need to consider the full diversity of possible enzyme states²⁶ and not just the dominant ones depicted in

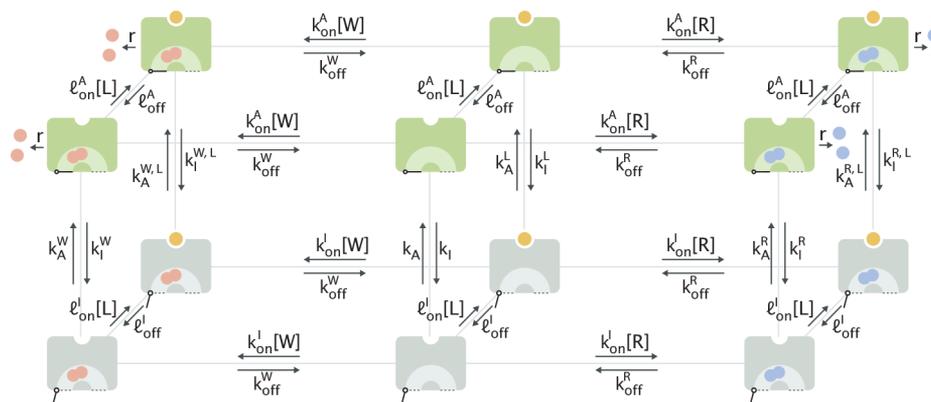


Figure 4. Network diagram of enzyme states and transitions between them. Right (“R”) and wrong (“W”) substrates are depicted in blue and red, respectively. The orange circle represents the ligand (“L”). Active (“A”) and inactive (“I”) enzymes are shown in green and gray, respectively.

Figure 1B. Therefore, in this section we provide a comprehensive discussion of the enzyme in an equilibrium setting before introducing its coupling to the engine.

The network diagram of all possible enzyme states is depicted in Figure 4. As can be seen, each of the 12 states is defined by the enzyme’s catalytic activity and whether a ligand and/or a right/wrong substrate is bound to the enzyme. Following the principle of microscopic reversibility,²⁷ we assign nonzero rate constants to the transitions between enzyme states. Only product formation (with rate r) is taken to be an irreversible reaction, under the assumption that the system is open where the formed products are taken out and an influx of new substrates is maintained. Since in our model neither the enzyme nor the substrates carry an energy source, the choice of the different rate constants cannot be arbitrary. Specifically, the cycle condition needs to be satisfied for each closed loop of the diagram, requiring the product of rate constants in the clockwise direction to equal the product in the counter-clockwise direction (see SI section C.1).²³

With these equilibrium restrictions imposed on the rate constants, we can show that when the ligand concentration is held fixed ($[L](t) = \text{constant}$), the fidelity of the enzyme cannot exceed that defined by the ratio of the off rates, namely, $k_{\text{off}}^W/k_{\text{off}}^R$ (see SI section C.2). What allows the enzyme to beat this equilibrium limit of fidelity without direct coupling to hydrolysis is the cyclic alteration of the ligand concentration between low and high values (thus, $[L](t) \neq \text{constant}$). In our model, we achieve this cyclic alteration through the ratchet and pawl engine driving the piston actions—a choice motivated by our objective to provide an explicit treatment of energy management. We note, however, that fidelity enhancement can be achieved irrespective of the driving agency as long as the cyclic alteration of ligand concentration is maintained at a certain “resonance” frequency, the presence of which we demonstrate in section 3.4.

3.3. Coupling the Engine to the Enzyme Gives the Full Description of the Piston Model. Having separately introduced the driving mechanism in section 3.1 and the allosteric enzyme with the full diversity of its states in section 3.2, we now couple the two together to obtain the full driven version of the piston model, as shown in Figure 5A. The coupling is achieved by exposing the ligand binding site of the enzyme to the piston compartment where the activator ligand is present. The enzyme can therefore “sense” the state of the piston (and thus the effects of driving) through the induced periodic changes in the ligand concentration.

In the absence of enzyme coupling, the network diagram capturing the nonequilibrium dynamics of the system was an infinite one-dimensional chain (Figure 2D) in which each discrete state was defined by the vertical position of the weight (z_n) and the state of the piston (“u” or “d”). In the layout where the engine and the enzyme are coupled, the full specification of the system state now requires three items: the position of the weight (z_n), the piston state (“u” or “d”), and the state of the enzyme (one of the 12 possibilities). By converting the three-dimensional view of the enzyme state network (Figure 4) into its planar equivalent, we represent the nonequilibrium dynamics of this coupled layout again through an infinite chain, but this time each slice at a fixed weight position (z_n) corresponds to the planar view of the enzyme state network (Figure 5B). The slices alternate between the compressed and expanded piston states (dark- and light-blue circles, respectively), with high and low ligand concentrations used in the transition network inside each slice.

Arrows between the slices (for clarity, not all of them are shown) represent the forward and backward steps of the ratchet. Crucially, as a consequence of coupling, the rates of forward stepping now depend on the state of the enzyme. In particular, when the ligand is bound to the enzyme, it no longer exerts pressure on the piston, and therefore, in those cases the forward stepping rates become simply

$$k_f^{u \rightarrow d, L} = k_f^{d \rightarrow u, L} = k_b e^{-\beta \Delta W} \quad (16)$$

where the superscript “L” indicates that the ligand is bound (orange circles in Figure 5B). We note that in the general case with N ligands, the pressure would decrease to that of $(N-1)$ ligands upon ligand binding, correspondingly altering the rates of forward stepping (see SI section D.1 for details). This adjustment of forward rates is essential for thermodynamic consistency of coupling the engine to the enzyme. Specifically, it ensures that any cycle of transitions that brings the enzyme and the weight back into their original states is not accompanied by dissipation, consistent with the fact that in the piston model energy is spent only when there is a net lowering of the weight. As a demonstration of this feature, consider the cycle in Figure 5C, which is extracted from the larger network. Using the expressions of forward stepping rates in eqs 7 and 16, we can write the cycle condition for this subnetwork as

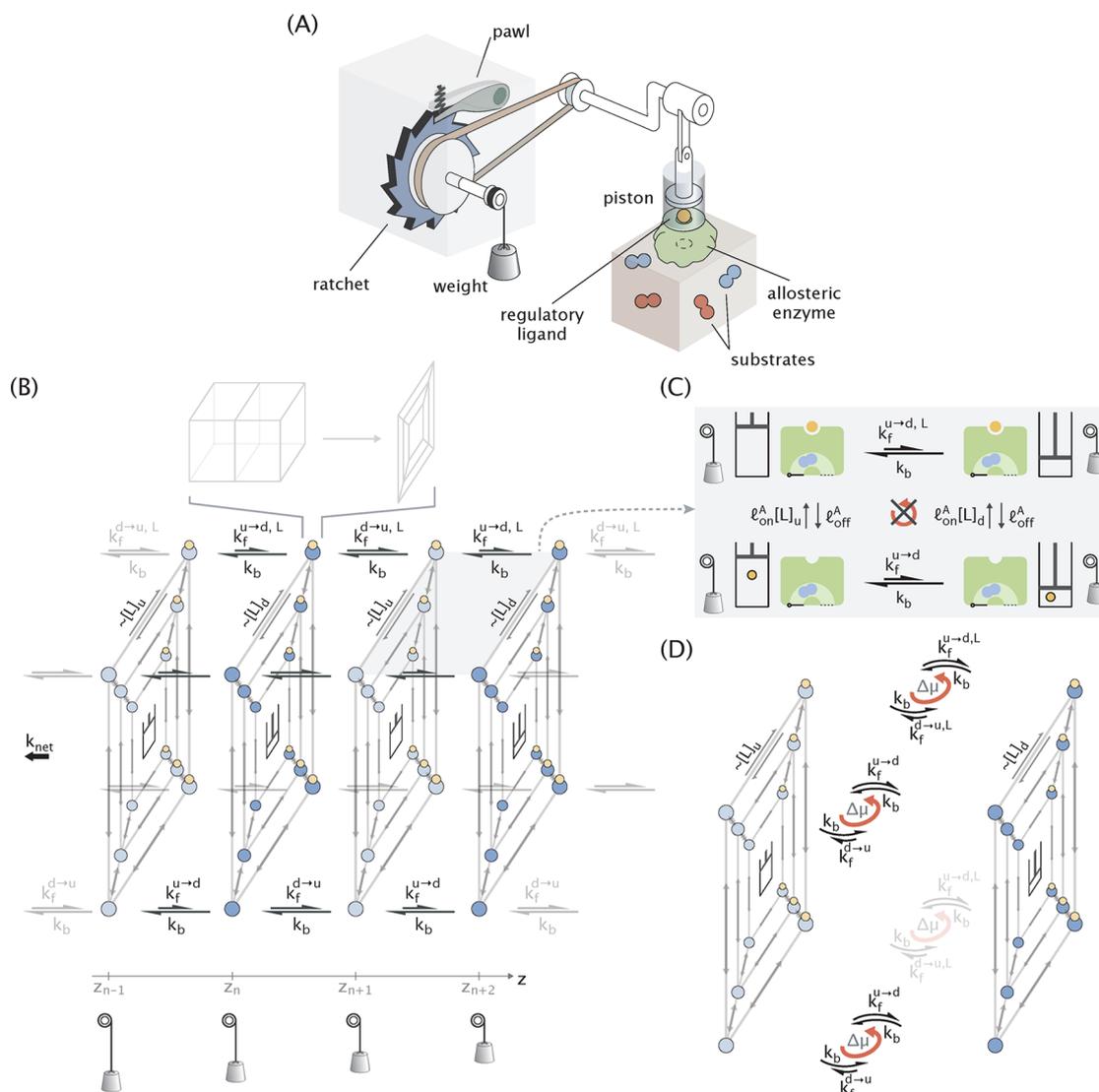


Figure 5. Full and thermodynamically consistent treatment of the piston model of proofreading. (A) Schematic representation of the full model, with the ratchet and pawl engine coupled to the enzyme. (B) Network diagram of the full model. Each slice of the diagram represents the planar view of the enzyme state network, with the alternating colors corresponding to the compressed (dark blue) and expanded (light blue) states of the piston. Ligand-bound enzyme states are marked with an orange circle. The horizontal arrows connecting the slices stand for forward and backward ratchet steps. Only those at the outer edges are shown for clarity; however, transitions are present between all horizontally neighboring enzyme states. Also for clarity, the stepping rate constants are shown only at two of the outer edges, where the ligand is either unbound (bottom edge) or bound to the enzyme (top edge). The hanging weight at different vertical positions is displayed below the diagram to symbolize energy expenditure as it gets lowered with a net rate k_{net} . (C) Subnetwork of the full diagram in (B) in which the state of the system is unchanged after a cycle is traversed. The red arrow with a cross on top indicates that the cycle condition holds in the subnetwork. (D) Finite-state equivalent of the full network in (B) with the weight position (z_n) eliminated. Red arrows indicate driving with a force $\Delta\mu = 2\Delta W$.

$$\frac{k_f^{u \rightarrow d, L} \times I_{off}^A \times k_b \times I_{on}^A[L]_u}{k_b \times I_{off}^A \times k_f^{u \rightarrow d} \times I_{on}^A[L]_d} = \frac{[L]_u f}{[L]_d} = 1 \quad (17)$$

where the equality $f = V_u/V_d = [L]_d/[L]_u$ was used. The fact that the products of rate constants in clockwise and counterclockwise directions are identical shows that no dissipation occurs when the cycle is traversed.

Now, to study how driving affects the proofreading performance of the piston model, we need to obtain the steady-state probabilities of the different enzyme states. To that end, we convert the full network diagram into the equivalent form shown in Figure 5D, where we have eliminated the position of the weight (z_n), akin to the earlier treatment of the uncoupled engine in Figure 2E. It should be noted that the

transitions between the two slices again represent piston compression and expansion events driven by a force $\Delta\mu = 2\Delta W$, as in eq 10. The steady-state probabilities π_i of the 24 different states in Figure 5D (12 enzyme states \times 2 piston states) can be obtained from the set of all rate constants, the details of which we discuss in SI section D.2. With these probabilities known, we calculate the rate of energy dissipation (P), speed of forming right products (ν_R), and fidelity (η) as

$$P = \sum_{i=1}^{24} \frac{(k_b - k_f^{(i)})\pi_i}{k_{net}} \times \Delta W \quad (18)$$

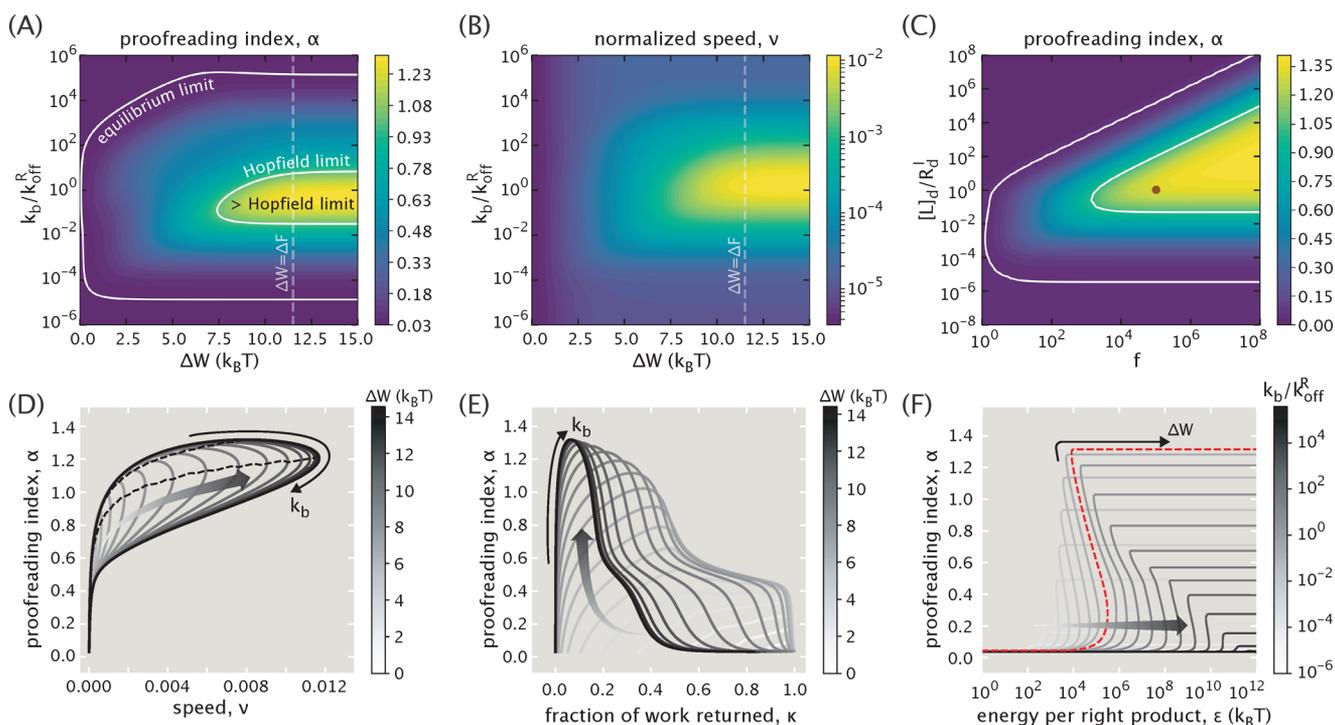


Figure 6. Parametric studies of the changes in the performance of the piston model in response to tuning the “knobs” of the engine. (A, B) Variations in (A) the proofreading index and (B) the speed as the rate of backward stepping (k_b) and the work per step (ΔW) are tuned. The dotted line corresponds to the value of ΔW equal to the ligand free energy change upon compression (ΔF). (C) Variations in the proofreading index when the high ligand concentration ($[L]_d$) and the compression factor (f) are tuned. R_d^1 represents the ligand dissociation constant in the inactive enzyme state. The red dot indicates the pair of $[L]_d$ and f values used in the studies shown in the other panels. (D) Fidelity–speed trade-off as k_b is continuously varied for different choices of ΔW (the gradient arrow shows the direction of increase). The dotted black lines connect the highest fidelity and speed values as ΔW is tuned. Between these dotted lines the fidelity and speed are negatively correlated. (E) Relation between fidelity and fraction of returned work for discrete choices of ΔW and continuously tuned k_b values (the gradient arrow indicates increasing ΔW). (F) Fidelity–dissipation trade-off obtained by continuously tuning ΔW for discrete choices of the hopping rate (k_b). The gradient arrow indicates the direction of increasing k_b . The red dotted curve corresponds to the case with resonance k_b .

$$v_R = \sum_{i \in S_R^A} \pi_i \times r \quad (19)$$

$$\eta = \frac{v_R}{v_W} = \frac{\sum_{i \in S_R^A} \pi_i}{\sum_{i \in S_W^A} \pi_i} \quad (20)$$

where $k_i^{(i)}$ is the rate constant for making a forward step from the i th state ($1 \leq i \leq 24$), and S_R^A and S_W^A are the sets of catalytically active enzyme states with a right and wrong substrate bound, respectively.

One significant downside of using these “raw” metrics in the numerical evaluation of the model performance is their high sensitivity to the particular choices of parameter values. We therefore introduce their scaled alternatives, which we will use for the numerical studies in sections 3.4 and 3.5. Specifically, as a measure of energetic efficiency, we use the dissipation per right product formed, defined as

$$\varepsilon = \frac{P}{v_R} \quad (21)$$

This way, the metric of energetics has units of $k_B T$ and is independent of the choice of absolute time scale. Next, as a dimensionless metric of speed, we introduce the normalized quantity

$$\nu = \frac{v_R}{v_R^{MM}} \quad (22)$$

which represents the fraction of the rate of forming right products in the proofreading setting (v_R) relative to that in the simple Michaelis–Menten scheme where the allosteric effects are absent (v_R^{MM}). This normalizing Michaelis–Menten speed is given in terms of the model parameters as follows:

$$v_R^{MM} = \frac{\frac{k_{on}^1[R]}{k_{off}^R + r}}{1 + \frac{k_{on}^1[R]}{k_{off}^R + r} + \frac{k_{on}^1[W]}{k_{off}^W + r}} \times r \quad (23)$$

We then define the proofreading index α as a fidelity metric that represents the degree to which the fidelity is amplified in multiples of k_{off}^W/k_{off}^R over its Michaelis–Menten value (η_{MM}), that is,

$$\eta = \frac{\left(\frac{k_{off}^W + r}{k_{off}^R + r}\right) \left(\frac{k_{off}^W}{k_{off}^R}\right)^\alpha}{\eta_{MM}} \quad (24)$$

which rearranges to

$$\alpha = \frac{\ln \eta - \ln \eta_{MM}}{\ln \left(\frac{k_{off}^W}{k_{off}^R}\right)} \quad (25)$$

It should be noted that the proofreading index of Hopfield’s scheme is $\alpha_{\text{Hopfield}} = 1$, as it involves a single proofreading realization. Also, since in the absence of external driving the

highest fidelity is $\eta_{\text{eq}}^{\text{max}} = k_{\text{off}}^{\text{W}}/k_{\text{off}}^{\text{R}}$, the corresponding upper limit in the proofreading index becomes $\alpha_{\text{eq}} = 1 - (\ln \eta_{\text{MM}})/(\ln \eta_{\text{eq}}^{\text{max}})$.

As a final descriptor of the piston model's nonequilibrium behavior, we introduce the fraction of returned work (κ), defined as the ratio of the rate at which the ligand performs work on the piston upon expansion to the rate at which the piston performs work on the ligand upon compression. We calculate κ via

$$\kappa = - \frac{\sum_{i \in S_d} (k_b + k_f^{(i)}) \pi_i \Delta F_{d \rightarrow u}^{(i)}}{\sum_{i \in S_u} (k_b + k_f^{(i)}) \pi_i \Delta F_{u \rightarrow d}^{(i)}} \quad (26)$$

where S_d and S_u are the sets of states where the piston is compressed and expanded, respectively. The negative sign is introduced to account for the fact that the ligand free energy decreases upon piston expansion (i.e., the system gets the work back). In the absence of enzyme coupling (section 3.1), this ratio was 1 because the ligand constantly exerted pressure on the piston. With enzyme coupling, however, the work performed on the ligand upon compression may not be fully returned since with some probability the ligand will be bound to the enzyme and exert no pressure on the piston during expansion. We therefore expect κ to be generally less than 1, indicating a net rate of performing work on the ligand in the nonequilibrium setting.

Having defined analytical expressions for the key model performance metrics, we now proceed to studying their graded changes and the trade-offs between them numerically.

3.4. Energy–Speed–Fidelity Trade-Off in the Piston Model. Because of its mechanical construction, the piston model of proofreading has a distinguishing feature: in it the external driving mechanism is physically separated from the allosteric enzyme. This feature allows us to independently examine how tuning the “knobs” of the engine and varying the kinetic parameters of the enzyme alter the performance of the model.

We begin our numerical analysis by first exploring the effects of external driving, where the tuning “knobs” include the rate of backward stepping (k_b), the work per step (ΔW), the ligand concentration in the compressed piston state ($[L]_d$), and the compression factor ($f = [L]_d/[L]_u$). Choosing a set of enzyme kinetic parameter values that make proofreading possible (see SI section D.3 for the full list of parameters), we keep them fixed for the rest of the analysis. We conduct the first parametric study by tuning k_b and ΔW and evaluating the proofreading index (Figure 6A). As anticipated, the proofreading index does not exceed its equilibrium limit in the absence of driving ($\Delta W = 0$). This expected feature can be paralleled by Brownian motors, for which purely equilibrium fluctuations of the motor's energy landscape are unable to generate directed motion.²⁸ In addition, the proofreading index achieves its highest value when ΔW is comparable to or larger than the ligand compression energy ΔF and when the backward hopping rate k_b is at its “resonance” value. The presence of a “resonance” hopping rate is intuitive: if piston actions take place very slowly, then the fidelity will be reduced because of the small but nonzero rate of forming unfiltered products (i.e., “leakiness”) in the quasi-equilibrated enzyme states; conversely, if piston actions take place too rapidly, then the activator ligand will almost always be bound to the enzyme, preventing the realization of multiple substrate discrimination

layers through sequential enzyme activation and inactivation. We note that analogous resonance responses were also identified for Brownian particles, which attain their highest nonequilibrium drift velocity in a ratchetlike potential landscape when the temperature²⁹ or the landscape profile³⁰ is temporally modulated at specific resonance frequencies. A similar feature is present in Hopfield's model as well: optimal proofreading is attained only when the rate of hydrolysis is neither too low nor too high.¹² Interestingly, when the driving is hard enough ($\Delta W \gtrsim \Delta F$) and the backward hopping rate is close to its resonance value, the fidelity of the piston model beats the Hopfield limit ($\alpha = 1$) and raises the question of the largest attainable fidelity, which we discuss in the next section.

Trends similar to those for the proofreading index are also observed for the speed of forming right products (Figure 6B). Specifically, product formation is very slow in the absence of driving and increases monotonically with ΔW until it plateaus for $\Delta W \gtrsim \Delta F$. Also, the highest speed is achieved at a resonance k_b value different from that of the proofreading index. The existence of such a resonance frequency is again intuitive, since at high rates of piston action the enzyme is predominantly active and unable to bind new substrates, while at low rates activation of the enzyme for catalysis via piston compression happens very rarely. Notably, since the enzyme parameters were chosen in such a way as to yield high fidelities, the highest speed value is substantially lower than the corresponding speed for a single-step Michaelis–Menten enzyme ($v_{\text{max}} \approx 10^{-2}$).

In the last parametric study, we explore how the choice of the high and low ligand concentrations affects the performance of the model. To that end, we tune the high ligand concentration ($[L]_d$) and the compression factor ($f = [L]_d/[L]_u$) and evaluate the highest proofreading index at the resonant k_b value with $\Delta W > \Delta F$. As can be seen, large fidelity enhancements are achieved when $[L]_d$ is comparable to or larger than the ligand dissociation constant in the inactive enzyme state (R_d^1), which is necessary to activate the enzyme upon piston compression. In addition, the compression factor must be large enough (or equivalently, the ligand concentration in the expanded piston state must be low enough) to inactivate the enzyme when the piston enters its expanded state. This requirement of a large free energy difference between the compressed and expanded piston states ($\beta \Delta F = \ln f \gg 1$) can be paralleled with a similar condition in Hopfield's model where for optimal proofreading the energy of the activated enzyme–substrate complex needs to be much larger than that of the inactive complex.

Knowing separately how tuning the engine “knobs” affects the fidelity and speed, we now explore the trade-offs between the model's performance metrics as we vary the driving parameters k_b and ΔW while holding the high and low ligand concentrations at fixed values (the red dot in Figure 6C). We start with the trade-off between fidelity and speed, depicted in Figure 6D, where we continuously tune the hopping rate k_b for different choices of the driving force ΔW . As expected from the results of the individual parametric studies in Figure 6A,B, both the fidelity and speed increase monotonically with ΔW . Also, since the values of the hopping rate k_b that maximize the fidelity and speed are not identical, these two performance metrics are negatively correlated in the range of k_b values defined by the two different resonance rates (the region between the dotted lines in Figure 6D) but are positively correlated otherwise. Variations in the metrics in the region of

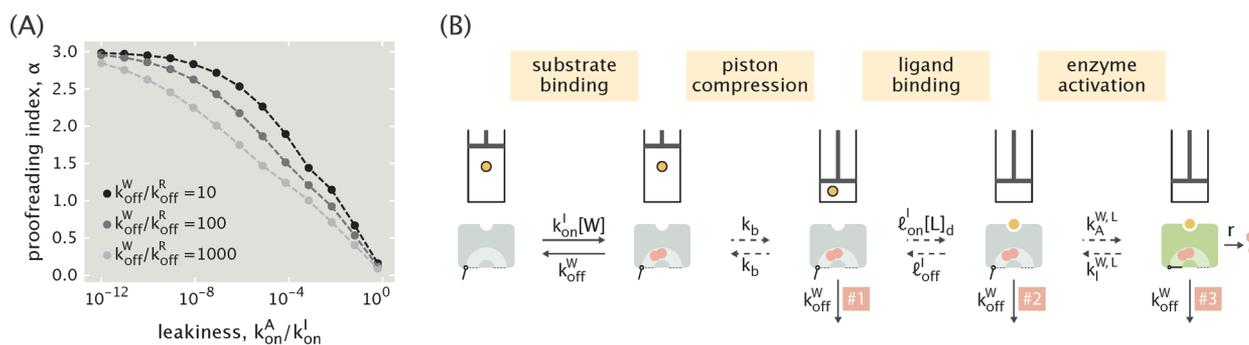


Figure 7. Proofreading performance of the piston model under optimized enzyme parameters and external driving conditions. (A) Highest proofreading index (α) available to the piston model as a function of leakiness (k_{on}^A/k_{on}^I) for different choices of k_{off}^W . (B) Dominant trajectory that the system takes to form a wrong product in the case where $k_{on}^A/k_{on}^I = 10^{-12}$. Numbers 1, 2, and 3 stand for the different proofreading filters along the trajectory. The dotted arrows indicate that the respective rates are much lower than the substrate unbinding rate k_{off}^W (see SI section D.5 for their numerical values for the case of $k_{off}^W/k_{off}^R = 100$).

their negative correlation, however, are moderate, suggesting that for an allosteric enzyme that has been optimized for doing proofreading, the highest speed and fidelity could be achieved under similar external driving conditions.

Next, we consider the relation between fidelity and fraction of work returned, shown in Figure 6E. As can be seen, no fidelity enhancement is achieved when κ is close to 1, which happens either in the absence of driving (lighter curves) or in the presence of driving when the hopping rate is very high. On the other hand, κ is much less than 1 at the peak fidelity, which is achieved when the hopping rate is at its resonance value and driving is large ($\Delta W \gtrsim \Delta F$). Overall, this trade-off study demonstrates that irreversible work performed on the ligand is a required feature for attaining fidelity enhancement in the piston model.

Lastly, we look at how the fidelity varies with the energy dissipation, with the latter characterized through the energy expended per right product (ϵ). The results of the trade-off study are shown in Figure 6F, where the driving force is continuously tuned for different choices of the hopping rate. As can be seen, there is a minimum dissipation per product required to attain the given level of performance. This minimum dissipation (the first intercept at a given y level) is achieved when the hopping rates are less than the corresponding resonant values (the lighter curves on the left side of the dotted red curve). Additionally, for a given hopping rate, increasing the driving force (ΔW) could lead to increased proofreading performance and decreased dissipation per product up to a critical point where the performance metric reaches its saturating value (horizontal region), demonstrating how increasing the driving force could in fact improve the energetic efficiency of proofreading. We note here that the minimum ϵ values needed for significant proofreading are $\sim 10^3$ – $10^4 k_B T$ in Figure 6F, which are ~ 2 orders of magnitude higher than what is calculated for translation by the ribosome.¹² This low energetic efficiency can be a consequence of our particular parameter choice for the study as well as the performance limitations of our engine design, the investigation of which we leave to future work.

3.5. Up to Three Proofreading Realizations Are Available to the Piston Model. In the previous section, we chose a set of kinetic rate constants for the enzyme and, keeping them fixed, explored the effects of tuning the external driving conditions on the performance of the model. In this section, we explore the parameter space from a different angle,

namely, how tuning the enzyme's kinetic parameters changes the model performance under optimal driving conditions. Since there are more than a dozen rates defining the kinetic behavior of the enzyme, it is impractical to probe their individual effects. Instead, we choose to vary two representative parameters about the effects of which we have a prejudice. These include the rate of substrate binding to the active enzyme (k_{on}^A) and the unbinding rate of wrong substrates (k_{off}^W). We know already from Hopfield's analysis that for efficient proofreading the direct binding of substrates to the active enzyme state should be very slow. Therefore, we expect the proofreading performance to improve as k_{on}^A is reduced. We also expect the minimum requirement for k_{on}^A to be lower for larger k_{off}^W values in order to ensure that wrong substrates do not enter through the unfiltered pathway.⁹

With these expectations in mind, we performed a parametric study to find the highest fidelity, the results of which are summarized in Figure 7A. There we varied k_{on}^A for several choices of k_{off}^W and for each pair numerically optimized over the enzyme's remaining kinetic rates and external driving conditions to get the maximum fidelity (see SI section D.4 for implementation details). As expected, the highest attainable fidelity decreases monotonically with increasing "leakiness" (k_{on}^A/k_{on}^I), and the minimum requirement on k_{on}^A decreases with increasing k_{off}^W .

Interestingly, we also see that for small enough leakiness, the piston model manages to perform proofreading (i.e., to enhance the fidelity by a factor of k_{off}^W/k_{off}^R) up to three times, as $\alpha_{max} \approx 3$ (Figure 7A). To understand this unexpected feature, we identified the dominant trajectory that the system would take to form a wrong product for the case where $k_{on}^A/k_{on}^I = 10^{-12}$ (Figure 7B; see SI section D.5 for details). As can be seen, after initial binding the wrong substrate indeed passes through three different proofreading filters, and these are realized efficiently because the transitions between intermediate states are much slower than the rate of substrate unbinding. The first filter occurs right after piston compression, while the enzyme is waiting for the activator ligand to bind (#1). We note that this particular filter is made possible by the presence of alternative piston states (equivalently, alternative environments that the enzyme could "sense"). The remaining two filters (#2 and #3) take place while the ligand-bound enzyme is waiting to get activated and while the active enzyme is waiting to turn the wrong substrate into a product, respectively. The presence of these two filters is purely a consequence of

allostery. Importantly, the $\alpha_{\max} \approx 3$ result in Figure 7 represents the theoretical upper limit of the model's proofreading index—a feature that we justify analytically in SI section D.5.

In light of this analysis, we can now explain why the pedagogically simplified version of the model introduced in section 2 achieved only a single proofreading realization. There we made the implicit assumption that ligand binding after piston compression and enzyme activation after ligand binding took place instantly. Because of this, proofreading filters #1 and #2 were not realized, leaving filter #3 as the only available one, which we showed in Figure 1B.

4. DISCUSSION AND CONCLUSIONS

A distinctive feature of kinetic proofreading is that it is a nonequilibrium mechanism, by virtue of which its operation needs to involve energy expenditure.^{9,10} Mechanical work, as an intuitive representation of energy expenditure, has been used in the past to elucidate important physical concepts such as information-to-energy conversion in the thought experiment by Szilard²² and the mechanical equivalence of heat in Joule's apparatus.³¹ Yet, a similar demonstration of how mechanical work could be harnessed in a graded fashion to beat the equilibrium limit in substrate discrimination fidelity has been lacking. Our aim in this work was to offer such a demonstration through the mechanically designed piston model of proofreading.

We started off by providing the conceptual picture of the piston model, with its constituents having direct parallels with Hopfield's original proofreading scheme⁹ (Figure 1). The key idea of the model was to replace the nucleotide hydrolysis step present in Hopfield's scheme with piston compression, which played an identical role of activating the enzyme, although in our case this was achieved through allostery and mechanical work. Just as in the case of biological proofreading, where hydrolysis itself cannot lead to fidelity enhancement unless the nucleotide triphosphates are held at fixed out-of-equilibrium chemical potentials, in the case of the piston model the compressive and expansive actions of the piston cannot result in proofreading unless they are driven by an energy-consuming engine. Motivated by Feynman's ratchet and pawl mechanism,²¹ we then proposed a dissipative mechanical engine to drive the cyclic piston actions, which maintained the nonequilibrium distribution of enzyme states necessary to achieve proofreading. The function of this engine can be paralleled to that of ATP synthase in the cell, whose constant operation maintains a finite ATP chemical potential which different biochemical pathways can then take advantage of.

To study how the cyclic variations in ligand concentration generated by the engine alter the occupancies of enzyme states, we performed a thermodynamically consistent coupling between the engine and the enzyme (Figure 5). There we considered the full diversity of states that the enzyme could take and, importantly, the feedback mechanism for the engine to "sense" the state of the enzyme. The accounting of this latter feature, which makes the piston model an example of a bipartite system,^{32,33} was motivated by our aim to propose a framework in which we could consistently calculate the total dissipation as opposed to only the minimum dissipation needed for maintaining the nonequilibrium steady state of the enzyme (without considering the driving engine).^{12,34,35} Although the dissection of different contributions to dissipation and their interconnectedness was not among the

objectives of our work, the framework proposed in our model can serve as a basis for additional studies of periodically driven molecular systems (e.g., Brownian clocks or artificial molecular motors), where the driving protocol and thermodynamics are of importance.^{36–38} As noted earlier, however, in the presence of a periodically changing ligand concentration, the allosteric enzyme could perform proofreading irrespective of the driving agency, which suggests a possible biochemical mechanism of fidelity enhancement without direct coupling of the enzyme state transitions to hydrolysis.

Having explicit control over the "knobs" of the mechanical engine, we then probed the performance of the model under different driving conditions. We found that both the speed and fidelity increased as we tuned up the mass of the hanging weight until they plateaued at a point where the free energy bias of the expanded piston state was fully overcome ($\Delta W \gtrsim \Delta F$), beyond which increasing the weight only increased the dissipation without improving the model performance (Figure 6A,B). This result can be paralleled with the presence of a minimum threshold for the strength of driving in Hopfield's model past which the highest fidelity becomes attainable.⁹ In addition, we found that in the piston model there is a "resonance" rate of piston actions that maximizes the fidelity, analogous to the similar feature in Hopfield's scheme where both very high and very low rates of hydrolysis reduce the quality of proofreading.¹²

The tunable control over the driving parameters also allowed us to study the trade-off between fidelity, speed, and energy spent per right product. These studies revealed that the correlation between speed and fidelity could be either positive or negative as the rate of driving is varied. Notably, theoretical investigations of translation by the *Escherichia coli* ribosome under Hopfield's scheme identified a similar behavior for the fidelity–speed correlation in response to tuning of the GTP hydrolysis rate, with the experimentally measured values being in the negative correlation (i.e., trade-off) region.¹⁷ In contrast to the ribosome study, however, where the two metrics vary by several orders of magnitude in the trade-off region, in the piston model the variations in fidelity and speed in the negative correlation region are moderate (Figure 6D), calling for additional investigations of the underlying reasons for this difference and a search for the realization of the latter advantageous behavior in biological proofreading systems. Furthermore, our studies showed that the minimum dissipation required to reach the given level of fidelity was achieved for hopping rates necessarily lower than their resonance values and that increasing the work performed per step (or analogously, the chemical potential of ATP) could actually improve the energetic efficiency of the model—features that again motivate the identification of their realization in biochemical systems.

At the end, we explored the limits on the proofreading performance of the piston model for various choices of the allosteric enzyme's "leakiness" ($k_{\text{on}}^{\text{A}}/k_{\text{on}}^{\text{I}}$) and the ratio of the wrong and right substrate off rates ($k_{\text{off}}^{\text{W}}/k_{\text{off}}^{\text{R}}$). We found that the trends for the highest available fidelity matched analogously with the features of Hopfield's original scheme, suggesting their possible ubiquity for general proofreading networks. More importantly, our analysis revealed that the piston model could do proofreading not just once but up to three times in the limit of very low leakiness, despite the fact that energy consumption takes place during a single piston compression. This is in contrast to the typical involvement of

several energy consumption instances in multistep proofreading schemes that manage to beat the Hopfield limit of fidelity, as, for example, in the cases of the T-cell or MAPK activation pathways, which require multiple phosphorylation reactions.^{5,6,34} Our finding therefore suggests the possibility of achieving several proofreading realizations with a single energy-consuming step by leveraging the presence of multiple inactive intermediates intrinsically available to allosteric molecules. We would like to mention here that the presence of a similar feature was also experimentally demonstrated recently for the ribosome, which was shown to use the free energy of a single GTP hydrolysis to perform proofreading twice after the initial tRNA selection, first at the EF-Tu-GDP-bound inactive state and second at the EF-Tu-free active state.³⁹

In the presentation of the piston model, we focused on the thermodynamic consistency of the framework for managing the energy dissipation and did not consider strategies for improving the performance of the mechanism. One such possibility that can be considered in future work is to use a more elaborate design for the ratchet and pawl engine with alternating activation barriers for pawl hopping, which would allow the use of different rates of piston compression and expansion, analogous to how hydrolysis and condensation reactions generally occur with different rates in biological proofreading.^{17,40} Another avenue would be to consider alternative ways of allocating the mechanical energy dissipation across the different ratchet transition steps, similar to how optimization schemes for allocating the free energy of ATP hydrolysis were studied for molecular machine cycles.⁴¹ Incorporating these additional features would allow us to probe the performance limits of the piston model and compare them with the fundamental limits set by thermodynamics.⁴²

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcc.9b08380>.

Python scripts and Jupyter notebooks that can be used to reproduce the results of the different numerical studies (ZIP)

Details of the discrimination steps in the conceptual scheme of the piston model, in-depth discussion of the operation of the ratchet and pawl engine, equilibrium constraints on the enzyme's transition rates, details of the engine–enzyme coupling; procedure for implementing the numerical studies, and investigation of the highest discriminatory capacity of the model (PDF)

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Notes

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