Universal free-energy landscape produces efficient and reversible electron bifurcation

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For decades, it was unknown how electron-bifurcating systems in nature prevented energy-wasting short-circuiting reactions that have large driving forces, so synthetic electron-bifurcating molecular machines could not be designed and built. The underpinning free-energy landscapes for electron bifurcation were also enigmatic. We predict that a simple and universal free-energy landscape enables electron bifurcation, and we show that it enables high-efficiency bifurcation with limited short-circuiting (the EB scheme). The landscape relies on steep free-energy slopes in the two redox branches to insulate against short-circuiting using an electron occupancy blockade effect, without relying on nuanced changes in the microscopic rate constants for the short-circuiting reactions. The EB scheme thus unifies a body of observations on biological catalysis and energy conversion, and the scheme provides a blueprint to guide future campaigns to establish synthetic electron bifurcation machines.

Significance

Electron bifurcation is an efficient and reversible redox reaction at the heart of key bioenergetic and biocatalytic reaction pathways used in nature. Electron bifurcation oxidizes a two-electron donor, using the electrons to reduce cofactors on two separate electron-transfer redox chains. The coupling of these redox reactions allows one of the electrons to move thermodynamically uphill, leveraging the downhill flow of the other electron. Thus, electron bifurcation may generate strong oxidants or reductants with minimal free-energy loss (i.e., reversibly). Not surprisingly, life harnesses electron bifurcation in biochemical pathways that perform challenging chemical reactions, including proton translocation across membranes, nitrogen fixation, and CO2 reduction. We predict that there is one universal free-energy landscape that supports efficient electron bifurcation reactions.

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enzymes (~20 Å or more) (21, 22), substantially slowing this short-circuit reaction.

The Q cycle was the first electron-bifurcation reaction that was found to be reversible on relevant physiological timescales (20). Since the tunneling distances for short-circuit transfers (Fig. 1C) are the same as for productive transfers, the rate constants for the productive electron transfers are expected to be similar to those for the short-circuit electron transfers (21). To prevent short-circuiting, “gating mechanisms” were proposed to suppress short-circuiting reactions, including concerted two-electron transfer (21), conformational gating (5, 23), “spring loading” of the Rieske iron–sulfur protein (24), Coulombic interactions (25), and other possible mechanisms termed double-redox gating (20, 21). However, after almost 20 years of searching, no experimental “smoking gun” in support of these gating mechanisms has been found. For example, it is understood that conformational motion of the Rieske iron–sulfur protein is required to explain how electrons tunnel through the high-potential branch. But, this conformational motion does not itself serve as a gating mechanism (to suppress short-circuiting electron transfer rate constants) because the reactions operate under near-reversible conditions (5, 6, 21). Indeed, there is no consensus on how the Q cycle accomplishes reversible operation with such high efficiency.

In addition to the quinone-based Q-cycle complexes, other novel flavin-based electron-bifurcating enzymes were discovered in the last decade (4, 5, 9, 26, 27). Many (if not all) of these flavin-based electron-bifurcating enzymes are also reversible (4), and many are not membrane bound (19, 22); others seem to lack significant conformational flexibility (3, 5, 19). Short-circuiting electron transfer also creates a challenge to flavin-based electron-bifurcating enzymes (5), and how these bifurcating flavoenzymes avoid short-circuiting, while maintaining reversibility, is unknown.

**A Thermodynamic Landscape to Enable Efficient Electron Bifurcation**

The analysis presented here indicates that a universal mechanism of high-efficiency bifurcation is used by all electron-bifurcating enzymes. We find that the secret to avoiding slippage (short-circuiting electron transfer) in electron-bifurcation reactions lies in the steep free-energy (reduction potential) landscapes of the spatially separated high- and low-potential branches, which is considered to be an enigmatic (but conserved) feature of electron-bifurcating enzymes (4, 5, 28). This landscape has a form similar to the redox potential landscapes in photosynthesis (29), although the mechanism for electron bifurcation is drastically different from that of photosynthesis.

In nature, steep free-energy landscapes are not unique to electron bifurcation. Photosynthesis uses steep landscapes to prevent charge recombination and to induce high-yield electron transfer following photoexcitation (29). Fig. 2 shows nine possible free-energy landscapes for electron bifurcation, discussed in detail below. Only one landscape, indicated in Fig. 2G supports efficient electron bifurcation by suppressing short-circuiting (vide
intra). Without the EB-scheme design principle, successful synthetic electron bifurcation (i.e., the equal and reversible yield of the high- and low-potential redox products) seems tremendously difficult to accomplish. This free-energy design principle, described and analyzed in detail below, explains how nature elegantly skirts a major obstacle (short-circuiting reactions) to producing high-value redox species.

Candidate Free-Energy Landscapes for Electron Bifurcation

There are three main ways that the thermodynamic landscape may influence electron transfer rates in an oxidoreductase. First, electron transfer rate constants in proteins are determined by tunneling pathways and distances between cofactors, reorganization energies, and thermodynamic driving forces (30). Thus, the reduction potential landscapes of the electron bifurcation branches, the cofactor placement, and the protein structure (31) determine the productive and short-circuit electron transfer rate constants. Second, the thermodynamic landscape establishes steady-state populations for each possible redox state. Indeed, these steady-state populations determine the effective activation free energies for short-circuiting electron transfer (vide infra). Third, the free-energy difference between initial and final catalytic states determines the catalytic driving force (and hence whether the reaction runs in the forward or reverse direction). The overall driving force for electron bifurcation is

$$\Delta G_{\text{bifur}} = 2FE_D - FE_{AH} - FE_{AL},$$

[1]

where $E_D$, $E_{AL}$, and $E_{AH}$ are the (midpoint) reduction potentials of the D, AL, and AH substrates, respectively, and $F$ is Faraday’s constant. For electron bifurcation to be spontaneous, $\Delta G_{\text{bifur}} < 0$.

Nine possible free-energy landscapes for electron bifurcation are categorized in Fig. 2. Landscapes A, B, and C have $\Delta G_{\text{bifur}} \ll 0$ and hence are not reversible, only operating in the electron bifurcation direction. Landscapes D, E, and F have $\Delta G_{\text{bifur}} \gg 0$ and only operate in the electron confurcating direction. Thus, only landscapes G, H, and I, with $\Delta G_{\text{bifur}} \approx 0$, are suited for reversible electron bifurcation/confurcation. To drive catalysis in the electron bifurcation (confurcating) direction with these landscapes, one would simply tune the reduction potentials of the terminal substrates to tilt the free-energy balance slightly (Eq. 1) (via reactant concentrations or the transmembrane potential for membrane-bound proteins). The reversibility of electron bifurcation is the source of its energetic efficiency (3, 5, 32).

The EB Scheme

Now, we describe how the EB scheme shown in Fig. 2G insulates the kinetic network from short circuits, while producing high-efficiency (reversible) electron bifurcation, and we prove this claim numerically in the next section (the other two energy-conserving landscapes, illustrated in Fig. 2H and I, lead to copious short-circuiting and are not viable). The slopes of the H and L redox branches in Fig. 2G cause electrons to pile up near B in the low-energy branch (blue), and holes in the high-energy branch (red) near B. Since the one-electron cofactors cannot accept a second electron at relevant potentials and must be in the reduced state to donate an electron, the EB scheme insulates the enzyme against short-circuiting by an electron occupancy blockade effect, despite having large short-circuiting rate constants. For an energy-wasting short-circuiting reaction to occur, a hole must occupy the low-energy branch (blue) and an electron must occupy the high-energy branch. Taken together, these processes create a very large free-energy barrier for short-circuiting. That is, the EB scheme is protected against short circuits by Boltzmann occupancy factors, so the enzyme will rarely enter a state where short circuits can occur. For productive electron bifurcation (confurcation) to occur, only a hole (electron) must move down (up) the low- (high-) energy branch, so the productive transfers have a much smaller free energy of activation to overcome. This occupancy effect, arising from the EB-scheme landscape, can lead to highly efficient partitioning of electrons into the high- and low-potential branches. The viable EB scheme (Fig. 2G), examined in detail here, uses crossed potentials at the bifurcating site B, but we have not examined whether crossed potentials are a requirement for effective electron bifurcation; the role of crossed potentials in electron bifurcation was discussed recently (3, 5, 12, 32). Next, we show how these principles emerge quantitatively from a kinetic model for electron bifurcation that describes the electron flux, notably including cofactor occupancy effects.

Many-State, Many-Electron Kinetics of Electron Bifurcation

Attempts were made in earlier studies to model the kinetics of electron bifurcation, and those studies succeed in describing many features of the kinetics. However, some of the previous models are not reversible (3, 21) and, as such, are inconsistent with the known reversibility of biological electron bifurcation. Other models restrict the number of tunneling electrons in the enzyme to just two (33) (inconsistent with access to pools of one- and two-electron redox substrates) or use rate constants that are physically unmotivated (34, 35), including ad hoc turning off of short-circuit reactions (36, 37). The scheme described here avoids these unnatural constraints and treats productive electron transfers (Fig. 1B) on the same footing as short circuits (Fig. 1C), allowing electrons to tunnel freely with rate constants estimated using nonadiabatic electron transfer theories with appropriate Marcus factors (30, 38), but only when a mobile electron resides on the donor, and a hole on the acceptor (i.e., we explicitly track the occupancies of all redox-active species). The substrates D+, AHI, and AL were modeled as electron reservoirs, which release and accept electrons at the reduction potential of the substrate, with adjustable rate constants that were tuned so that they are not rate limiting (that is, the intrinsic kinetics of the electron bifurcation enzyme are assumed rate limiting). Two electrons move together in one kinetic step into B. For the Q cycle, this describes quinone diffusion into the Q0 site. Details of the kinetics model appear in SI Appendix.

For each of the three free-energy conserving schemes (Fig. 2G–I) for electron bifurcation, we implemented a minimalistic kinetic model, mutatis mutandis, for electron bifurcation enzymes. The model (Fig. 3A), and the resulting kinetics at steady state (Fig. 3B–F), are shown in Fig. 3. The B/B* and B*/B+ standard reduction potentials were set to $-400$ and $400$ mV, respectively, and the nearest-neighbor distance between cofactors was set to 10 Å (next-to-nearest distance of 20 Å, etc.). Nature’s electron bifurcation systems vary these parameters, but the chosen values are typical (4). While the efficiency and turnover time can be tuned by changing these parameters (SI Appendix, Fig. S1), energy-dissipating rapid short-circuiting ($\sim 10^5$ s) as in Fig. 3B and C is never observed when the EB scheme is present. Nearly perfect one-to-one partitioning of electrons to the high- and low-potential substrates with full reversibility can be accomplished without requiring a gating mechanism (Fig. 3E and F).

We explored the short-circuit behavior of the landscapes in Fig. 2 G–I as a function of the driving force $\Delta G_{\text{bifur}}$ with this kinetic model. For the landscape of Fig. 2I, the electron flux away from A and into AH is large ($\sim 10^5$ electrons/s), reflecting short-circuit–dominated kinetics. For landscape H, the short-circuiting flux is still large ($\sim 10^3$ electrons per second). Only when the slope of the branches follows landscape G (the EB scheme) do the electron fluxes into AH and A have the same sign, reflecting electron bifurcation (confurcation)-dominated
kinetics when the overall driving force \( \Delta G_{\text{slope}} \) is negative (positive). Any difference between the \( \Delta G_L \) and \( \Delta G_H \) oxidation/reduction rates (separation between the red and blue curves) reflects short-circuiting behavior, so the near-supersposition of the curves in Fig. 3F indicates very low short-circuit currents.

The EB Scheme Suppresses Electron Short-Circuiting

When the magnitude of the energetic slopes of the two EB-scheme redox pathways is increased (Fig. 3F), the short-circuiting flux shrinks compared to the electron bifurcating/confurcating turnover rates, as reflected in the negligible difference between the electron fluxes into/out of the \( A_L \) and \( A_H \) reservoirs. Using the EB scheme, electron bifurcation can achieve high efficiency (equal partitioning of electrons into the \( A_L \) and \( A_H \) reservoirs), at the cost of turnover speed and reducing power of the low-potential acceptor \( A_L \). Presumably, electron bifurcation enzymes in nature evolved to balance these tradeoffs, insulating against short circuits while enabling catalysis to proceed with sufficient speed to meet physiological demands. Importantly, alternate gating mechanisms are not required for reversible and efficient electron bifurcation in the EB scheme. In fact, electron bifurcation and confurcating emerge naturally from the kinetic network (Fig. 2G and 3A) at steady state, but only when the EB scheme is employed. Our model does not unnaturally privilege productive electron transfers over short circuits in any way. Indeed, short-circuit electron transfers are successfully insulated in the EB scheme, even when the short-circuit rate constants are set orders of magnitude faster than the productive electron transfers, due to the cofactor occupancy blockade effects (SI Appendix, Fig. S1D).

When short-circuit fluxes are small (i.e., as occurs in the EB scheme), the high- and low-potential redox branches quickly reach approximate chemical equilibrium with themselves, despite being out of equilibrium with the other branch (6) (i.e., quasi-equilibrium). Thus, the short-circuit fluxes are thermally activated. Fig. 3D shows the short-circuit flux into the high-potential \( A_H \) reservoir when \( \Delta G_{\text{bifurc}} = 0 \) (the electron bifurcation enzyme is “idling”) as a function of temperature, where two distinct linear regimes are observed at low and high temperature, which indicates a thermally activated tunneling mechanism for the short circuits (this linear behavior is analyzed in detail in SI Appendix). The high-temperature regime is dominated by \( B^- \)-mediated short circuits, which are fast but have a large thermal activation energy. The low-temperature regime is dominated by the \( L_1 \) to \( H_1 \) short circuit, which is slower but has a smaller thermal activation energy, allowing this short circuit to dominate at low temperatures.

The energetic landscapes of electron bifurcation have been proposed to be important many times before (e.g., see refs. 3–5, 28, 32, and 36.), but the special and universal nature of the EB scheme to nearly eliminate short circuits and remain fully reversible has not been shown previously. This is because a minimalistic model must include the potent combination of: 1) reversibility (20, 21), 2) explicit tracking of the entire enzyme’s redox state (not just the average state of each cofactor) (35, 37), 3) three explicit electron reservoirs that are each free to exchange electrons in the branches at each reservoir’s chemical potential, and 4) the explicit modeling of the energetic slopes along the entire length of the high- and low-potential branches, not just the cofactors near the bifurcating site (6). The model described here explicitly shows a reverse electron flux with negligible short-circuiting when the driving force, \( \Delta G_{\text{bifurc}} \), is reversed, unlike many previous models.

While reversibility, electron blockading, and explicit reservoirs are crucial to capture efficient electron bifurcation, combining all three into a tractable kinetic model is not simple because the number of differential equations governing the kinetics grows...
exponentially with the number of cofactors (35, 37). To construct the very large model that underpins Fig. 3, we procedurally generated the equations governing the dynamics (SI Appendix). In fact, our model is similar to that found in refs. 35 and 37, except that we answer quantitatively the apparently central question, namely why electron bifurcation enzymes never use any of the landscapes in Fig. 2, aside from landscape G. Understanding precisely how landscape G insulates against short circuits allows us to make the strong prediction that landscape G of Fig. 2 (the EB scheme) is universal in electron bifurcation, and that this scheme is key for the design of synthetic electron bifurcation systems (see the final section below).

Interestingly, the privileged EB-scheme landscape follows a free-energy profile that is similar to the steep slopes in reduction potentials that are found in the Z scheme of photosynthesis (29). However, the mechanism of electron flow in bifurcating enzymes is drastically different, as a consequence of the reversibility of electron bifurcation reactions, in contrast to strongly driven photosynthetic reactions.

Electron bifurcation enzymes can surely exhibit complexities that are not captured in our model. For example, proton-coupled electron transfer (5, 6), two-electron cofactors (flavins or quinones) in the H and L branches (22), conformational changes (23, 39), and electron transfer between electron bifurcation monomers (40) may all add kinetic richness. In fact, conformational motion in the Q cycle is understood to be required for electrons to reach the high-potential cytochrome $c_1$, which is too far away for direct electron tunneling from the electron bifurcation $Q_o$ site (33). However, none of these specific features interfere with the essential short-circuit–insulating nature of the conserved and predicted universal EB scheme.

**Fig. 4.** Short-circuit insulation in the Q cycle (complex III) arising from the EB scheme. Using our multielectron kinetic model (SI Appendix), we built a simplified model (A) for the Q cycle using previously published reduction potentials and tunneling distances (28, 33). Despite these simplifications, the EB scheme observed in the measured reduction potentials seems (B) effective at insulating against short circuits. With minor changes to the reduction potentials (designed to increase $\Delta G_{\text{elec}}$ and $\Delta G_{\text{red}}$ of the activation process shown in SI Appendix, Fig. S2D) that are likely within the range of experimental uncertainty (C), the EB scheme of the Q cycle provides the preponderance of insulation against short-circuiting. In B and C, no confurcation appears for the values of $\Delta G_Q$ shown since the reduction potentials are measured in the absence of the membrane potential (28), by which energy is ultimately conserved in the Q cycle. The influence of the membrane potential on all of the cofactor reduction potentials (and hence the EB scheme) is unknown. We present two possible cases, chosen to reflect the range of possible impacts of the membrane potential on the L-branch cofactor reduction potentials. In D the reduction potentials of the low-potential branch all decrease by 150 mV. In E the reduction potential of the Q site alone decreases by 150 mV. In the case of D, the electron bifurcation landscape may be sufficiently preserved to insulate from short circuits. In E, the electron bifurcation landscape is significantly disrupted, as the energy required to move an electron to cytochrome $b_L$ (in SI Appendix, Eq. 58) in order to initiate short-circuiting is negligible. This disruption of the landscape turns on short-circuiting, but may not reflect the reality of cytochrome $bL$ in the presence of a membrane potential.

Short-Circuiting in the Q Cycle
A fully detailed kinetic model of the Q cycle is beyond the scope of this study, but a simplified model is sufficient to account for the primary cause behind the short-circuit insulation in the Q cycle. Our model (Fig. 4A) uses distances and energetics suggested by experiment and indicates that the electron bifurcation enzyme landscape explains most of the short-circuit insulation in the Q cycle (see also SI Appendix, Supplementary Text). Cofactor reduction potentials were measured previously (28), and the tunneling distance values were used in previous studies (33). The first electron transfer from $Q_o$ to ISP (ISP = iron–sulfur protein) is proton-coupled and rate limiting (6). This was modeled by setting an effective electron tunneling distance, which was tuned until the overall steady-state turnover was ~50/s, placing the model in quantitative agreement with experimental steady-state turnover rates (6, 40). This fitting procedure forced the $SO \rightarrow$ ISP (SO = semiquinone) short-circuit rate constant to be favored over the productive HQ $\rightarrow$ ISP (HQ = hydroquinone) rate constant by several orders of magnitude. Even with this preference for a short-circuit rate constant over a productive one, short circuits were still successfully insulated (Fig. 4B and C). The motion of the ISP was not explicitly modeled, but was assumed to
be sufficiently fast so that electrons can tunnel directly to cytochrome $c_1$ once ISP is reduced. The Q site was modeled as a one-electron reservoir, since the two one-electron reduction potentials of ubiquinone at the Q$_i$ site are similar (41).

A few specific experiments have been interpreted as indicating a need for gating mechanisms in the Q cycle to assure its efficient function. For instance, in the Q cycle of cytochrome bc$_1$, the inhibitor antimycin A (which prevents electrons from leaving the low-potential branch) is known to decrease the overall steady-state turnover by a factor of about 30 (6, 40). Gating mechanisms were proposed to explain this slowdown of the redox flux with a compromised L branch (20, 21, 33, 37). Our simplified model of the Q cycle using experimental parameters (Fig. 4) shows that the EB scheme insulates against short circuits in that system. Not surprisingly, slower turnover is not observed in our simplified scheme insulates against short circuits without the EB scheme) and a compromised L branch as compared to the uninhibited case (SI Appendix, Fig. S3), which suggests that additional features not captured in our model play a role in the Q cycle.

Molecular features behind the observed difference between uninhibited and L-branch inhibited kinetics in the Q cycle may include subtle structural changes resulting in tunneling distance changes of about 3 Å or less between the Q$_o$ site and its iron-sulfur cofactor partner (SI Appendix, Fig. S3), or subtle electrostatic interactions between the low-potential branch and the Q$_o$ site (SI Appendix; these are likely not the only possible explanations), rather than by a gating mechanism per se. Because the measured change in steady-state turnover in the presence of inhibitors and L-branch cofactor knockouts (20, 40) is so subtle [about a factor of 30 less (40)], these and other mechanisms will be difficult to identify uniquely (See SI Appendix for extended discussion).

The effect of the EB scheme in preventing short circuits is orders of magnitude larger than the observed difference between the L-branch inhibited and noninhibited turnover. Specifically, the rate constants for short-circuit electron transfers are $\sim10^7$/s (35), which must be defeated. In the absence of additional assistance from protein gating, the EB scheme will reduce the flux to $\sim10^7$/s, and these values will be further reduced if the L branch is not inhibited (Figs. 3 and 4), supporting the central role played by the electron bifurcation landscape in defeating short circuits. Since additional order of magnitude is needed to bring this turnover rate to the observed L-branch inhibited turnover rates ($\sim10^7$/s) (40), which indicates that the efficiency gained by such a mechanism is less than 1% of the gain produced by the EB scheme (measuring efficiency with short-circuit rates). Thus, the EB scheme explains most of the short-circuit insulation in the Q cycle of cytochrome bc$_1$.

**Natural Electron Bifurcation: Exploiting the EB Scheme**

The important lesson learned is that any additional mechanisms in natural electron bifurcation enzymes, beyond the EB scheme, are not the key features that underpin the short-circuit insulation in electron bifurcation systems, including the Q cycle. There is a tremendous difference between a gating mechanism that changes a rate constant by nine orders of magnitude (which is required to insulate against short circuits without the EB scheme) and a mechanism that intrinsically prevents that kinetic pathway from ever being accessed by the system under normal operating conditions (this is how the EB scheme works; see SI Appendix, Fig. S2). Other subtle features and mechanisms might shelve off the last order of magnitude or two of short-circuiting flux when the L branch is inhibited, or even permit some short-circuiting and serve as “release valves” that can reroute electrons to add robustness to biochemical pathways. For instance, certain photosynthetic bacteria were found to be able to grow with an inhibited Q cycle (42). These organisms required a short-circuit flux across a Q cycle to grow. Importantly, our analysis does not disentangle subtle features of electron bifurcation enzymes, which likely differ from system to system. We do, however, propose that the EB scheme is sufficient to accomplish robust electron bifurcation, explains the lion’s share of short-circuit insulation in known electron bifurcating systems, and may serve as a core design framework for synthetic electron bifurcation systems.

**Synthetic Electron Bifurcation: Exploiting the EB Scheme**

The EB scheme enables reversible electron bifurcation, insulates against wasteful short-circuit reactions, and thus appears to remove the two primary roadblocks that prevent the design and synthesis of electron bifurcation molecular machines (5). A robust and general scheme to prevent short circuits suggests that synthetic electron bifurcation is not a distant dream.

We envision that the EB scheme (Fig. 2G) may be realized with several kinds of molecular architectures. For instance, covalently linked molecular redox species, DNA origami motifs (43), tailored linked quantum dots (44), or even semiconductor nanostructures may serve as possible frameworks in which to realize electron bifurcation. For example, the EB-scheme landscape is found in the band bending of n-p semiconductor junctions (45), which suggests that semiconductors may play a role in synthetic electron bifurcation.

In the EB scheme, each redox site other than the bifurcating site must be made to accommodate only one mobile electron at a time and must not be allowed to interact further than its nearest neighbors. For example, if L$_2$ could donate an electron to H$_2$ (Fig. 1), or if H$_1$ could receive several electrons from B$^-$, the EB scheme would no longer insulate against short circuits (these processes were included in our model, but since the distance between nonneighbor cofactors is at least 20 Å in the model, the corresponding tunneling rate constant is negligibly small). In addition, the terminal electron acceptors, D, A$_4$, and A$_5$, must not exchange electrons directly with each other, or with any of the redox active sites in the scaffold, other than with the terminal branch sites. This level of microscopic control is challenging to realize, and anchoring the A$_4$ and A$_5$ acceptors to the ends of the branches may be acceptable for proof-of-concept experiments. Care must also be taken to avoid short-circuit channels during the D$^-$ to B electron refilling process (short-circuiting during refilling).

Electron bifurcation in nature allows the reversible reduction of compounds with low reduction potentials, using compounds with much higher (midpoint) reduction potentials, analogous to the function of a voltage amplifier. Understanding the manner of this redox conversion in the warm, wet environment of biology provides inspiration for novel synthetic redox catalysts.

**Data Availability**

Python data have been deposited in GitHub (https://github.com/JYuly/EB_kinetics). All other study data are included in the article and SI Appendix.

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