The maximum entropy production requirement for proton transfers enhances catalytic efficiency for β-lactamases

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HIGHLIGHTS

• Catalytic efficiency and entropy production increases for more evolved lactamases.
• Proton transfer steps contribute most to overall entropy production.
• Maximal entropy production requirement can augment performance parameters.
• Natural upper limits are similar for dissipation-driven efficiency of β-lactamases.

ABSTRACT

Movement of charges during enzyme catalytic cycle may be due to conformational changes, or to fast electron or proton transfer, or to both events. In each case, entropy production can be calculated using Terrel L. Hill’s method, if relevant microscopic rate constants are known. When ranked by their evolutionary distance from putative common ancestor, three β-lactamases considered in this study show correspondingly increased catalytic constant, catalytic efficiency, and overall entropy production. The acylation and deacylation steps with concomitant proton shuttles are the most important contributors to overall entropy production. The maximal entropy production requirement for the ES↔EP or EP↔E + P step leads to optimal rate constants, performance parameters, and entropy production values, which are close to those extracted from experiments and also rank in accordance with evolutionary distances. Concurrent maximization of entropy productions for both proton transfer steps revealed that evolvability potential of different β-lactamases is similarly high. These results may have implications in particular for latent potential of β-lactamases to evolve further and in general for selection of optimized enzymes through natural or directed evolution.
1. Introduction

Evolution of enzymes, such as β-lactamases, is not just of academic interest. The resistance mechanism toward β-lactam antibiotics is mostly due to the expression of β-lactamases, which can hydrolyze β-lactams [1]. This serious clinical problem, connected to rapid evolution of bacterial β-lactamases during past decades, raises the question of whether there are any fundamental thermodynamic and kinetic limits to additional increase in catalytic efficiency of these enzymes. Lactamases were produced by Gram-negative and Gram-positive bacteria for millions of years before they caught the attention of biochemists after introduction of benzylpenicillin in clinical trials about 70 years ago [2]. High efficiency and specificity of β-lactam chemotherapy was soon challenged by evolution of β-lactamases from pathogenic bacterial strains. Despite including β-lactamases in the class of almost perfect catalysts [3,4], the end of evolutionary development was not reached for them.

We can ask the question, how it is known that an additional increase in enzyme catalytic constant $k_{cat}$ (the turnover number) and specificity constant $k_{cat}/K_M$ is not possible? These kinetic constants, sometimes named together as catalytic efficiency, are specific for a kinetic scheme of considered enzyme, which can exist in a number of discrete states connected with transitions including substrates and products. In the case of a reversible kinetic scheme, an entropy production can be associated with each transition between enzyme functional states [5]. The maximal value of entropy production can always be found for any chosen transition in a steady-state, and corresponding optimal values for kinetic constants are sometimes similar to measured values [6,7]. This opened the question, which physical principles are the most relevant for natural or directed evolution of enzymes [8]. For instance, what is more important for amazing catalytic efficiency of enzymes – dynamics or electrostatics of enzyme catalysis [9]? Also, it opened the possibility to use entropy productions due to transitions between functional enzyme states to identify microscopic fluxes which are rate-limiting for catalytic efficiency.

Our first goal in this study was to answer the question, does catalytic efficiency of an enzyme increased together with increased distance from its common ancestor and is it accompanied with higher overall entropy production as well? The positive answer to this question may provide an additional kinetic and thermodynamic handle for better understanding eons-long acceleration of enzyme reaction rates up to the factor of $10^{19}$-fold rate enhancement [10]. It may be also useful in predicting the evolution potential for present day enzymes, such as β-lactamases, forced by human use and misuse of β-lactams to undergo additional accelerated evolution.

We assume that the evolution of enzyme kinetics is guided by both physical and biological selection principles. For three non-homologous β-lactamases we show that increased catalytic efficiency and overall entropy production is in accord with their evolutionary distance from common ancestor. Entropy production associated with any chosen transition between enzyme states also increased during evolution. Separate or simultaneous maximization of entropy production in the acylation and deacylation proton transfer steps predicted an additional increase in catalytic efficiency and overall entropy production. Reconstructing past and predicting future evolution of enzyme kinetics for enzymes under selection pressure, or engineered enzymes, can therefore profit from recognition that physical selection principles too left their imprint in evolutionary development of biological sequences and performance parameters of these bionanomachines.

2. Methods

2.1. The phylogenetic tree construction

The evolutionary history of S. aureus PC1, E. coli RTEM, and B. cereus β-lactamase 1 [3] was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [11]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved 36 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 169 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [12].

2.2. Kinetic modeling

We use the data from Christensen et al. 1990 [3] for three different types of β-lactamase enzymes: β-lactamase 1, PC1 β-lactamase and RTEM β-lactamase, which are assumed to obey Michaelis-Menten kinetics. We use the superscript “+” or “−” to denote forward and backward rate constants respectively. The superscript “**” is used to distinguish second-order rate constants. The rate constants for acylation $k_2^+$ and deacylation $k_3^+$ of substrate with lactamases were associated by Christensen et al. to vanishing backward rate constants $k_2^- = k_3^+ = 0$, as an irreversible representation of the Michaelis-Menten scheme, allowing only for substrate hydrolysis. Remaining rate constants $k_1^+$, $k_1^-$, $k_2^+$ and $k_3^+$ were measured in a closed reaction system by using stopped-flow experiments in a quasi-steady state established 0.12 s after the initiation of a reaction.

For the kinetic modeling we used the reversible Michaelis-Menten three-state cyclic scheme with three forward and three backward rate constants (Fig. 1).

We calculated the time evolution of system variables, that is, the concentrations of substrate (S), product (P), free enzyme (E), enzyme in complex with substrate (ES) and enzyme in complex with product (EP) concentrations were determined with the system of differential equations:

\[
\begin{align*}
\frac{d[S]}{dt} &= -v_1 \\
\frac{d[P]}{dt} &= v_3 \\
\frac{d[E]}{dt} &= -v_1 + v_3 \\
\frac{d[ES]}{dt} &= v_1 - v_2 \\
\frac{d[EP]}{dt} &= v_2 - v_3
\end{align*}
\]

where $v_1$, $v_2$ and $v_3$ are net reaction fluxes in first, second and third transition of the reaction, respectively, and are given as:

\[
v_1 = k_1^+ [S][E] - k_1^- [ES]
\]

Fig. 1. Three-state scheme for considered β-lactamases. The predominant manner of enzyme cycling is counterclockwise, but this reversible scheme allows for rare turnovers from product to substrate too.
In a quasi-steady state, it must hold that all three net reaction fluxes are approximately equal \( v_1 \approx v_2 \approx v_3 \). By using kinetic Eqs. \((1a) - (1e), (2a) - (2c)\) and experimental data [3], we first calculated the values of fluxes \( v_1, v_2 \) and \( v_3 \) at the moment \( t = 0.12 \) s after the initiation of the reaction for irreversible kinetic scheme, i.e. with \( k_2 = k_3 = 0 \) and with \([S]_0 = 1500 \) µM and \([E]_0 = [E]_{tot} = 1.0 \) µM [3]. Then, by assuming that values \( k_2^- \) and \( k_3^- \) are very low in comparison to \( k_2^+ \) and \( k_3^+ \) and by using the condition \( v_1 = v_2 = v_3 \) we calculated the missing values of \( k_2^- \) from Eq. \((2b)\) and the missing values of \( k_3^- \) from Eq. \((2c)\) for all three types of enzymes. In this way we were able to analyze the enzyme reaction of β-lactamase enzymes as reversible.

Estimated values of \( k_2^- \) and \( k_3^- \) were further used in calculations of catalytic constants (\(k_{cat}\)), Michaelis-Menten constants (\(K_M\)) and specificity constants (\(k_{cat}/K_M\)) for all three enzymes by using reversible enzyme model. The values of \(k_{cat}\) and \(K_M\) for reversible three state enzyme reaction are defined as [13]:

\[
\frac{k_{cat}}{K_M} = \frac{k_{cat}^+}{k_{cat}^- + k_{cat}^+ + k_{cat}^-} = \frac{k_{cat}^+}{k_{cat}^-} + \frac{k_{cat}^-}{k_{cat}^+}
\] (3a)

and

\[
k_{cat}^+ \approx \frac{k_2^+ k_3^+ + k_3^+ k_4^+ + k_4^+ k_2^-}{k_2^+ [k_2^+ + k_3^- + k_4^-]}
\] (3b)

respectively. The expression \((3a)\) is the maximal enzyme activity, which can be measured when product formation is considered.

### 2.3. Shannon information entropy

Our model considers statistical ensemble of enzyme molecules, which can be found in three different functional states — \(E, ES \) and \(EP\)— with corresponding steady-state probabilities \(p_1, p_2\) and \(p_3\), respectively. These probabilities are defined as:

\[
p_i = \frac{k_i}{[E]_{tot}}, \text{ for } i = 1, 2, 3
\] (4)

where \(x_i = E \) for \( i = 1 \), \(x_i = ES \) for \( i = 2\) and \(x_i = EP \) for \( i = 3\). It holds that . Shannon information entropy of probabilities \(p_i\) is given as:

\[
H = -\sum_{i=1}^{3} p_i \ln(p_i)
\] (5)

### 2.4. Entropy production

Entropy production for an enzyme reaction is given with equation:

\[
\sigma = \frac{\nu X}{T}
\] (6)

where \(\nu\) is the steady state overall reaction flux of a reaction, \(X\) is overall steady state thermodynamic force and \(T\) is the absolute temperature. The overall steady state reaction flux \(\nu\) can be calculated either with kinetic modeling according to Eqs. \((1a) - (1e)\) and \((2a) - (2c)\) or with the Michaelis-Menten rate equation for three state enzyme reaction. The later is given as [7,13]:

\[
\nu = \frac{k_2^+ k_3^+ [S] - k_3^+ k_4^+ [P]}{N} = k_2^+ k_3^+ + k_3^+ k_4^+ + (k_1^+ k_2^- + k_2^+ k_3^- + k_1^+ k_2^- + k_3^+ k_4^-) [S] + (k_2^- k_3^- + k_2^- k_4^- + k_2^- k_3^-)[P]
\] (7)

and describes the rate of a reaction per total enzyme concentration measured in units (s\(^{-1}\)).

The overall thermodynamic force of a reaction is:

\[
X = k_B T \ln \left( \frac{[S][P]_{eq}}{[P][S]_{eq}} \right)
\] (8)

where \(k_B\) is the Boltzmann constant, while \([P]_{eq}/[S]_{eq}\) is the equilibrium constant \(K_{eq}\).

### 2.5. Optimizations using maximization of entropy production for transitions between enzyme functional states

We used our published proof (named ‘theorem’ henceforth) that maximal entropy production can be found for any chosen transition between enzyme functional states [7,14]. All optimizations were performed in a quasi-steady state reached 0.12 s after the experiments were started [3]. All equilibrium constants for enzyme functional states were kept at their constant values determined in experiments and kinetic modeling. The constancy of equilibrium values ensured that we are dealing with the same quasi-steady state and the same set of free-energy values. These values are characteristic and different for each of three β-lactamases. Overall thermodynamic forces were also constant as calculated from Eq. \((8)\) taking into account equilibrium constants and constant concentrations of substrate and product at considered steady state. The optimization procedure for individual transitions \(i (i = 1, 2, 3)\) was the same as in [14]. Briefly, we used the MaxEP condition in each transition:

\[
\frac{d\sigma}{dk^+} = 0
\] (9)

to find maximal \(\sigma_i\) and optimal \(k_i^+\) in a chosen transition \(i\). All other rate constants were held fixed at their experimental or inferred values (from kinetic modeling), except the \(k_i^-\), which was calculated from corresponding equilibrium constant \(K_i = (k_i^-)^{eq}/k_i^+\).

Iterative optimizations \(\sigma_i \sigma_f\) were calculated by finding optimal kinetic constants in such a way that entropy productions for two transitions are maximized at the same time until corresponding optimal forward kinetic constants were found for chosen transitions. For brevity, the omission of the superscript symbol for kinetic constants in the following text would always mean that we are dealing with forward rate constants. From assumed constancy of known equilibrium constants for transition states, the backward rate constants can always be calculated for considered transitions. For instance, optimal \(k_2\) was found from maximal entropy production \(\sigma_1\) in the second transition \(ES \rightarrow EP\), optimal \(k_3\) was found from maximal entropy production in the third transition \(EP \rightarrow E + P\) to find \(\sigma_2 \sigma_3\) optimization results after iterations when optimal \(k_2\) and \(k_3\) did not change any more. We also used the equations and procedure described in [14], but due to the complexity of simultaneous optimizations, the conditions

\[
\frac{d\sigma_1}{dk_2} = \frac{d\sigma_2}{dk_2} = \frac{d\sigma_3}{dk_2} = \frac{d\sigma_1}{dk_3} = \frac{d\sigma_2}{dk_3} = 0
\] (10)

were not solved analytically, but numerically, by choosing appropriate step values and range for each variable in order to perform iterations until maximal values for transition entropy productions and optimal values for forward constants did not change any more. The \(k_1\) and reverse kinetic constant in the first transition were fixed at their measured values. The iterative procedure for all optimizations was repeated 50 times, but optimal values were mostly found already after 10-th iteration.

A similar procedure was applied to double mixed optimizations of the type \(H \sigma_1, \sigma_2, \sigma_3\) and to double optimizations of the type \(H \sigma_1, H \sigma_2\). The Shannon entropy \(H\) for three enzymatic states was maximized, when it was possible to find maximal \(H\), first by finding optimal \(k_1\) in the \(i\)-th transition, then by finding maximal \(\sigma_i\) or maximal \(H\) for optimal \(k_i\) in the \(j\)-th transition. This procedure was also repeated until optimal kinetic constants did not change any more.

The \(\sigma_1 \sigma_2 \sigma_3\) optimization and \(\sigma_{tot}\) maximization used the additional constraint that product of all forward rate constants \(k_1^- k_2^- k_3^-\) is equal...
to the experimental value for that product. For the σ1σ2σ3 optimization the k1 optimal value was found by using this constraint after optimal k2 and k3 were found from the maximal entropy production requirement in the second and third transition. Instead of using maximums in partial entropy productions to find optimal kinetic parameters, it is also possible to find optimal k2, k3, and k4 after the σopt maximization by using the same additional constraint k1 k2 k3 k4 = const1, which ensures that maximum in overall entropy production exists. Due to constancy of external driving force, the k1 k2 k3 k4 = const1 constraint implies the k1 k2 k3 = const2 constraint as well. Together, these two constraints can be represented as the constancy of the equilibrium constants product: K1 K2 K3 = const, which is the chemical analogy of the Kirchhoff’s loop law for electrical circuits [15].

2.6. Limitations of the study

After initial in silico modeling to find missing backward rate constants, we used the steady state restriction for all optimization results. In addition to fixed external force due to maintenance of chosen stationary nonequilibrium substrate, product, and enzyme concentrations, we assumed that the same substrate (benzylpenicillin) was used for all lactamases and constancy of all three equilibrium constants for corresponding catalytic steps. Not using some restrictions in this study is just as important as using other steady state restrictions. We did not use the restriction that some kinetic constants are not subject to evolutionary change due to diffusion limit as we did in our previous publication [7], and we did not use the restriction that product of all kinetic constant in a chosen direction must be the constant determined in experiments [16], except to show that it leads to decreased performance parameters and overall entropy production.

FORTRAN programs used to calculate optimized or maximal values are freely available at: https://sites.google.com/site/lactamase990777programs/. Three optimizations σ2σ3 (PC1), σ2σ4 (RTEM) and σ2σ3 (Lac 1) are included in this program package, as representative of all other iterative optimizations.

3. Results

3.1. Evolutionary relationship among β-lactamases

The phylogenetic tree (Fig. 2) illustrates the relationship between different members of Ambler class A β-lactamases. Our choice to compare S. aureus PCI, E. coli RTEM, and B. cereus β-lactamase 1 was inspired with extensive studies of rate constants for these enzymes performed by Christensen and coauthors [3]. The tree with the highest log likelihood (~7175.90) is shown in Fig. 2. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. In this tree (Fig. 2) it is apparent that S. aureus PCI β-lactamase splits from the common ancestor before E. coli RTEM lactamase, and that RTEM lactamase splits before B. cereus β-lactamase 1 divergence. Several trees constructed by other methods also support the conclusion that the distance from common ancestor is the greatest for the B. cereus β-lactamase 1 and the smallest for the S. aureus PCI β-lactamase (data not shown). When expressed as branch lengths, the evolutionary distances from common ancestor are: 1.07 for the PCI, 1.34 for the RTEM, and 1.63 for the lactamase 1.

3.2. Kinetic and thermodynamic parameters extracted from kinetic modeling of experimental data

The steady-state values for kinetic constants are found after all time derivatives from eqs. (1) are set up to zero values, while all of three transition fluxes from eqs. (2) are equated. The results of these calculations are presented in Table 1. The estimated values of k2 and k3 for all three enzymes are two to three orders of magnitude lower than corresponding forward rate constants k2 and k3. Estimated values of kcat and KM (Eqs. (3a) and (3b) respectively), as well as their ratio (kcat/KM), and steady state reaction flux (Eq. (7)), are also given in Table 1 and are very close to their measured values [3].

In the Table 2, we present the values of [S], [P], X/kT, H, p1, p2, p3, σ1, σ2, σ3, and σtot, which are calculated for all three types of β-lactamase enzymes using the experimental values and kinetic modeling in the same quasi-steady state (eqs. 4, 5, 6, and 8). State probabilities p1 and entropy productions σ1/kT (s−1) are associated with three enzyme functional states (i = 1,2,3) and transitions between these states, respectively.

For easier comparison of kinetic modeling results (Tables 1 and 2) and phylogenetic tree analysis for three chosen β-lactamases (Fig. 2), we have depicted in the bold type all these parameters, which are ranked in the same order as are enzymes ranked according to their evolutionary distance from a common ancestor. S. aureus PCI β-lactamase has the smallest, while B. cereus β-lactamase 1 has the greatest distance from a common ancestor. The reaction flux v, catalytic constant kcat, catalytic efficiency kcat/KM, Shannon’s information entropy H, transitional entropy productions σi, and total entropy production σtot, are all increased with greater evolutionary distance. Going from the least evolved (S. aureus PCI β-lactamase) to the most evolved lactamase (B. cereus β-lactamase 1) total entropy production increased for a factor of 20 and catalytic constant increased for a factor of 30.

We verified for all three β-lactamases that at the chosen time of t = 0.12 s there are very little changes in information entropy, transition entropy productions and overall entropy production. The time-dependence of these parameters is shown only for the β-lactamase 1 (Fig. 3). Significant changes in state probabilities (reflected in information entropy H changes) and other parameters occurred only during first several milliseconds after the reaction start.

3.3. Optimizations for the highest entropy production and/or highest information entropy

Calculations up to this point did not use any optimization procedure. This section will be devoted to exploring kinetic and thermodynamic limits for additional evolution of lactamases, starting from the assumption that under given constraints either overall maximal entropy production, or maximal entropy production due to particular transitions between enzyme functional states, or maximal Shannon information entropy are good indicators for evolution potential of these enzymes. Optimization constraints observed in this section are the same as the constraints used in kinetic modeling (see Methods).

Recent publications [6,7,14,16–21] suggest that looking for maximal or as high as possible entropy production under given constraints is a valid approach for finding optimal patterns in different applications of nonequilibrium thermodynamics in biology. We shall here explore in the case of evolutionary connected enzymes if optimization for maximal entropy production values leads to a significant increase in catalytic efficiency as well. Two of us (DJ and ZBL) proved that maximal entropy production associated with any chosen enzyme transition can be always found [7,14]. This theorem can be used as an alternative tool for examining the limits for evolution potential and optimal allocation of dissipation to optimize flux in each particular case [22].

We shall also examine if more equitable distribution of state probabilities and consequently higher Shannon information entropy, consistent with given constraints, is likely to accelerate enzyme turnover rate and increase related kinetic and thermodynamic parameters. Maximizing the Gibbs-Shannon entropy (5) was proposed as a basis for understanding the MaxEP selection principle [23], and it led to rough agreement with observed rate constants in kinetic schemes for some free-energy transducing biological systems [17,18].

The second column in Tables 3, 4 and 5 reproduces kinetic modeling and experimental results already presented in Tables 1 and 2. Columns 3 to 5 with the MaxEP title contain optimization results obtained when maximal entropy production is found for the first (E + S→ ES), second
(ES↔EP), or third transition (EP↔E + P) respectively. For easier grasp of many numbers presented in Tables 3 to 5, the increased composite kinetic and thermodynamic parameters for all of three lactamases are depicted in inverse type, as white symbols against black background. Decreased parameters with respect to values extracted from experiments and kinetic modeling are outlined with light grey background. Surprisingly so, maximizing entropy production in the substrate binding transition E + S↔ES led to significant decrease in catalytic efficiency $k_{\text{cat}}/K_M$ to less than 2%, less than 14% and less than 16% from wild-type values for, respectively, PC1, RTEM, and lactamase 1. It also leads to entropy production decrease in subsequent proton-transfer steps, but as expected (see Eq. (3a)) the catalytic constant is not changed. The maximization of entropy production associated with the formation of enzyme-product complex (second transition) or with product release (third transition) led to increase in catalytic efficiency for all of three β-lactamases. If we are looking for limits to evolvability of A-class β-lactamases, it obviously makes sense to keep everything as it is in wild type enzymes undergoing the first catalytic step (substrate binding), and to simultaneously optimize remaining two steps of reversible Michaelis-Menten scheme for β-lactamase catalytic cycle.

![Fig. 2. Molecular phylogenetic analysis by maximum likelihood method for 36 β-lactamases with bold font used to put abbreviations for considered three lactamases next to branch length lines and values leading to them. The tree is drawn to scale, with branch lengths (number above each branch) measured as the number of substitutions per site (scale bar, 0.5 substitutions per site). Summing all relevant branch lengths leads to following results in evolutionary distances: 1.07 for the PC1, 1.34 for the RTEM, and 1.63 for the lactamase 1.](image)

**Table 1**

Kinetic parameters of three different types of β-lactamase enzymes arranged from top to bottom row according to increasing evolutionary distance.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{1}$ ($\mu M s$)$^{-1}$</th>
<th>$k_{-}$ ($s$)$^{-1}$</th>
<th>$k_{2}$ ($\mu M s$)$^{-1}$</th>
<th>$k_{3}$ ($s$)$^{-1}$</th>
<th>$k_{\text{cat}}$</th>
<th>$k_{M}$</th>
<th>$v$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>22</td>
<td>196</td>
<td>173</td>
<td>4</td>
<td>96</td>
<td>61</td>
<td>6</td>
</tr>
<tr>
<td>RTEM</td>
<td>123</td>
<td>11,800</td>
<td>2800</td>
<td>6</td>
<td>1500</td>
<td>975</td>
<td>41</td>
</tr>
<tr>
<td>Lac 1</td>
<td>41</td>
<td>2320</td>
<td>4090</td>
<td>50</td>
<td>3610</td>
<td>1905</td>
<td>73</td>
</tr>
</tbody>
</table>

Parameters $k_{1}$ ($\mu M s$)$^{-1}$, $k_{-}$ ($s$)$^{-1}$, $k_{2}$ ($\mu M s$)$^{-1}$, $k_{3}$ ($s$)$^{-1}$ are taken from experimental data [3]. Parameters $k_{2}$ ($s$)$^{-1}$, $k_{3}$ ($\mu M s$)$^{-1}$, catalytic constant $k_{\text{cat}}$($s$)$^{-1}$, Michaelis-Menten constant $k_{M}$($\mu M$), specificity constant ($k_{\text{cat}}/k_{M}$($\mu M s$)$^{-1}$, and reaction flux $v$($s$)$^{-1}$) are theoretically estimated with kinetic modelling in a quasi-steady state at the time 0.12 s after initiation of a reaction. Initial concentrations of substrate and enzyme used in simulations are $[S]_0$ = 1500 $\mu M$ and $[E]_0$ = 1.0 $\mu M$, respectively. Bold type parameters increased with the evolutionary distance.
Simultaneous maximization of entropy production in first and second, or first and third transition, produced a weaker performance of lactamases when compared with observed values. This is the reason that we did not present optimization results for iterative procedure involving the first transition, except for the RTEM lactamase (Table 4).

Out of six \( \sigma_2 \sigma_3c \) and \( \sigma_{\text{out}} \) optimizations (Tables 3 to 5), only the \( \sigma_2 \sigma_3c \) optimization for RTEM lactamase resulted in slightly increased specificity constant (Table 4). All calculated parameters after \( \sigma_2 \sigma_3c \) optimization for RTEM lactamase and \( \beta \)-lactamase 1 are the same order of magnitude as observed values, but the same claim can be made in the case of all three lactamases for \( \sigma_2 \) or \( \sigma_3 \) maximizations, which do not use the \( k_1^-k_2^+k_3^+ \) constancy condition. In effect, the constraints to experimental values of rate constants in two transitions or constraints restricting the product of forward kinetic constants to experimental values serve the same purpose to stay as close as possible to measured values.

When optimal enzyme performance parameters are found and compared for all three lactamases, the best optimization results, producing the greatest \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_{\text{M}} \), are obtained from the \( \sigma_2 \sigma_3c \) maximization. With the exception of information entropy, which decreased for the RTEM lactamase, other kinetic and thermodynamic parameters increased for up to two orders of magnitude (highlighted white numbers with black background in Tables 3 to 5) in comparison with values extracted from experiments. The highest increase occurred for the PCI lactamase bringing this least evolved enzyme to the same order of magnitude with predicted limits for performance parameters of more evolved RTEM and Lac 1 \( \beta \)-lactamases. In the case of RTEM lactamase, mixed double optimizations of the type \( H_0 \), \( \sigma_1 H_1 \), \( H_2 \), \( H_3 \) are not included in the Table 4, because the maximal value for Shannon's information entropy \( H \) does not exist for the range of kinetic constant values where we found maximums for transition entropy productions.

### 3.4. Connecting sequence evolution with performance parameters

From the phylogenetic tree (Fig. 2), it appears that lactamases PC1, RTEM, and Lac 1 can be ranked in that order (from minimal to maximal) with respect to their evolutionary distance from a common ancestor. Evolutionary distances from a common ancestor can be calculated from the phylogenetic tree as the sum over all branch lengths leading to the PC1, RTEM and Lac 1 enzymes (Fig. 2). Since sequence differences are numerous, with less than 40% identity for any sequence pair, it is likely that postulated common ancestor existed a long time ago, may be more than billion years ago. Its divergence produced such diversity that some descendants evolved faster than other. Are more evolved \( \beta \)-lactamases more efficient as well in their catalytic activity? The additional question we asked is whether thermodynamic, kinetic, and evolutionary data can be all connected in a meaningful pattern.

Observed enzyme turnover numbers \( (k_{\text{cat}}) \) and their optimization by the \( \sigma_1 \), \( \sigma_2 \), or \( \sigma_3 \) maximizations exhibit nearly linear increase with the evolutionary distance (Fig. 4). The decrease in the activation energy for the transition ES\( \leftrightarrow \)EP state and EP\( \leftrightarrow \)E + P can be inferred when corresponding proton transfers are optimized with maximum entropy production requirement for these transitions (the \( P_2 \) and \( P_3 \) symbols respectively from Fig. 4). Obtained optimal values for microscopic rate constants are the same order of magnitude as measured values. Beneficial mutations can increase enzyme performance parameters. However, one or two mutations can produce only a minor change in the evolutionary distances found for wild-type enzymes even after causing a significant change in the catalytic constant. Accordingly, maintaining the same evolutionary distance for optimized as for corresponding wild-type enzymes (Fig. 4) is an adequate approximation leading to linear-like increase in performance and entropy production for more evolved enzymes.

The double optimization procedure \( \sigma_2 \sigma_3 \), i.e. looking for the simultaneous maximum in entropy productions for both proton shuttle steps, produces one to two order of magnitude increase in performance parameters and in overall entropy production. Optimal catalytic constant reaches the same order of magnitude for all of three lactamases, which is very close if not inside the diffusion-limit range [24], and may represent thermodynamic and kinetic limit for the evolution of these enzymes. This limit is, however, a way above catalytic constant values for wild-type \( \beta \)-lactamases, so that the description of some \( \beta \)-lactamases as perfect enzymes [3] is open to challenge. To sum this section, presented results connect increases in turnover number \( k_{\text{cat}} \) and in overall entropy production to increased evolutionary distance for more evolved A-class \( \beta \)-lactamases, equally well as inferred from experiments or as calculated from maximal entropy production requirement for proton transfers. Shannon's information entropy \( H \) of discrete enzyme states also increased in accord with increased evolutionary distance (Tables 2-5). However, unlike entropy production for transitions between enzyme functional states, the maximal \( H \) cannot be always found if we do not apply additional restrictions.
Table 3
PCI lactamase kinetic and thermodynamic parameters derived from experiments and kinetic modeling (Exp&Kin), optimizations for MaxEP (σi), or double optimizations MaxENT&MaxEP (Hσj, σiHj, H_j, σiσj).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Exp&amp;Kin</th>
<th>MaxEP</th>
<th>MaxENT&amp;MaxEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>σ_i</td>
<td>σ_j</td>
<td>σ_t</td>
<td>σ_σ</td>
</tr>
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<td>0.17</td>
<td>22.0</td>
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<td>196.5</td>
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<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Values of parameters with bold symbols are white against black background, or outlined with light grey background, when increased or decreased respectively with respect to Exp&Kin values.

Values extracted from experiments and kinetic modeling. We used in all columns the same substrate S = 1492 μM and product P = 8 μM concentrations, with corresponding external force X/(k_B T) = 11.3 and equilibrium constants K_{1} = 167.47, K_{2} = 43.25, and K_{3} = 12.00.

Optimizations with maximal entropy production found respectively in transitions 1, 2 and 3.

See the main text for the description of iterative optimizations σ_iσ_j, H_jσ_i, σ_iH_j, H_jσ_j, or σ_ent. The last two of listed optimizations use the experimental value for the product of kinetic constants in forward direction for the PCI lactamase: k_{1} k_{2} k_{3} = 86,917 s^{-3} as the constraint.

Table 4
RTEM lactamase kinetic and thermodynamic parameters derived from experiments and kinetic modeling (Exp & Kin), optimizations for MaxEP (σi), or double optimizations MaxEP&MaxEP (σiσj).

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<tbody>
<tr>
<td>σ_i</td>
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<tr>
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<tr>
<td>k_{2}^{+}(μs^{-1})</td>
<td>40</td>
<td>40</td>
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</table>

Values extracted from experiments and kinetic modeling. We used in all columns the same substrate S = 1390 μM and product P = 110 μM concentrations, with corresponding external force X/(k_B T) = 7.74 and equilibrium constants K_{1} = 14.99, K_{2} = 466.67, and K_{3} = 0.34.

Optimizations with maximal entropy production found respectively in transitions 1, 2 and 3.

See the main text for the description of iterative optimizations σ_iσ_j, σ_iσ_j, and σ_ent. The last two of listed optimizations use the constraint k_{1} k_{2} k_{3} = 7.18 × 10^{11} s^{-3}, i.e. the experimental value for the product of kinetic constants in forward direction for the RTEM lactamase.
4. Discussion

4.1. Enzyme catalysis and relevant physical principles

The relevance of physical principles to enzyme evolution remains uncertain. Different principles of biological function and fitness have often been evoked in attempts to understand enzyme evolution. We suggest in this work that entropy production associated with enzyme catalysis can help to illuminate both dynamics and electrostatics of enzyme catalysis. The optimal values of kinetic constants derived from the maximum entropy production theorem [7,14] are the same order of magnitude as their measured values, the observation which supports the importance of dynamics and of associated entropy production for enzyme evolution and catalysis. Entropy production is always connected with some fluxes in the presence of driving forces, which ensures that dynamics and rate limiting steps can be examined with simple mathematical tools when all microscopic rate constants are known for cyclic conversion between different enzyme functional states. Furthermore, if rate constants are left as free variables for some transitions between enzyme functional states, it is possible to single out those transitions that are the most important for enzyme catalysis, evolution, and increase in entropy production. This study illustrates that the catalytic steps in the catalytic cycle are very different with respect to their contribution to turnover number $k_{\text{cat}}$, catalytic efficiency $k_{\text{cat}}/K_M$, entropy production, evolvability potential, and rate-limiting role in evolutionary related enzymes having non-homologous sequences. The MaxEP optimization of each catalytic step separately reveals these differences, but not the reason or reasons for these differences.

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Table 5

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Exp &amp; Kin</th>
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<th>MaxENT &amp; MaxEP</th>
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</thead>
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</tr>
<tr>
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</tr>
<tr>
<td>$k^- [\mu M^{-1}]$</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

| $p_{\text{in}} [s^{-1}]$ | 1760 | 1246 | 2244 | 2203 | 5943 | 21805 | 6890 | 2622 | 1631 | 16789 |
| $p_{\text{out}} [s^{-1}]$ | 0.05 | 0.24 | 0.98 | 0.07 | 0.13 | 0.42 | 0.19 | 0.24 | 0.06 | 0.33 |
| $s_{\text{in}} [s^{-1}]$ | 0.43 | 0.31 | 0.27 | 0.54 | 0.39 | 0.23 | 0.26 | 0.32 | 0.32 | 0.33 |
| $s_{\text{out}} [s^{-1}]$ | 0.52 | 0.46 | 0.68 | 0.39 | 0.48 | 0.35 | 0.55 | 0.44 | 0.62 | 0.34 |

aSee Table 3 legend.

bValues extracted from experiments and kinetic modeling. We used in all columns the same substrate $S = 1285 \mu M$ and product $P = 215 \mu M$ concentrations, with corresponding external force $X/(k_B T) = 8.3$ and equilibrium constants $K_1 = 22.71$, $K_2 = 81.80$, and $K_3 = 2.10$.

Optimizations with maximal entropy production found respectively in transitions 1, 2 and 3.

See the main text for the description of iterative optimizations $a_\alpha H_\beta$, $a_\alpha H_\beta$, $a_\alpha H_\beta$, $a_\alpha H_\beta$, $a_\alpha H_\beta$, or $a_\alpha H_\beta$. The last two optimizations use the constraint $k_1 + k_2 + k_3 = 7.8 \times 10^{11} s^{-3}$, i.e. the experimental value for the product of kinetic constants in forward direction for the $\beta$-lactamase 1.

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Fig. 4. Ranking $\beta$-lactamases by their evolutionary distance from putative common ancestor, by catalytic constant $k_{\text{cat}}$, and by overall entropy production $\sigma_{\text{tot}}/k_B$. Black empty squares, blue empty circles, pink full circles, and red full squares represent values respectively obtained from experiments and kinetic modeling, after optimization for maximal entropy production in the first proton transfer step leading to the formation of enzyme-product complex (the P2 symbol), after optimization for maximal entropy production in the second proton transfer step leading to the formation of free enzyme and product (the P3 symbol), and after optimization for maximal entropy production due to substrate binding and formation of enzyme-substrate complex (the P1 symbol).
4.2. Fluxes connected to enzyme dynamics

Fluxes connected to enzyme dynamics can be associated with directional movements of electrons, protons, atoms, amino acid residues or whole sequence segments. One example is the triosephosphate isomerase, which catalyses proton transfer coupled with the loop-6 opening and product release during the catalytic cycle [25]. The proton transport associated with product release is the rate-limiting step for the triosephosphate isomerase, which is also associated with the highest increase in overall entropy production [14]. In β-lactamas, fluxes are associated with proton shuttles and proton micro-circuits inside enzyme active domain, which by itself can be more rigid than the rest of enzyme when stabilized with appropriate transition state analogue [26]. The rigidity of the active site domain facilitates the maintenance of substrate-activated proton movements among critical residues serving as proton donors and acceptors in a dense hydrogen-bonding network within the active state. The acylation and deacylation steps with concomitant proton shuttles are the most important contributors to overall entropy production of β-lactamas catalytic cycle.

4.3. Catalytic constant, catalytic efficiency, overall entropy production, and evolutionary distance

We have shown in this paper that there is a linear-like relationship between increase in the catalytic constant, the increase in overall entropy production, and the increase in the evolutionary distance of three wild-type lactamas from common ancestor. The data from four decades ago, which we used in this paper, are more likely to represent natural then accelerated evolution of lactamas under the influence of β-lactam antibiotics. The turnover number $k_{cat}$ together with the specificity constant $K_{M}$, are often regarded as the best indicators of the catalytic efficiency [2]. Increase in catalytic efficiency is expected and observed during biological evolution of enzymes. We did not propose any specific biological mechanism underlying how increased performance parameters can be reached together with increased entropy production, but it is hard to imagine how this can happen without mutations exploring the evolutionary space and selection restricting the pathway for preservation of beneficial mutations.

4.4. Dissecting entropy production for each transition between enzyme states

Maximal entropy production requirement for either acylation or deacylation step (or for both proton transfer steps) leads to increase of entropy production in all catalytic steps and to increase of enzyme performance parameters with respect to their measured values. This is a common feature of the catalytic cycle for evolutionary distant β-lactamase, that is, that there are two rate-limiting steps both involving proton nanocurents.

The comparison with similar dissection of entropy production contributions for four catalytic steps of triosephosphate isomerase [14] also reveals MaxEP importance for finding and optimizing those catalytic steps that are critical and rate-limiting for increasing enzyme performance parameters. Maximal entropy production requirement for all steps preceding the product release step decrease overall entropy production and triosephosphate isomerase performance parameters. Only the maximal entropy production requirement for the proton transfers and loop motion coupled to product release $E_{prod}$ increases enzyme performance parameters.

4.5. Maximizing entropy production for proton shuttling and product release

The requirement for maximal entropy production, both in the acylation and in the deacylation step, takes into account the most important electrodynamics and nonequilibrium thermodynamics of enzyme cycle through proton movements. After MaxEP optimization for the proton shuffle steps, the least evolved RTEM β-lactamase exhibits two orders of magnitude higher optimal turnover number $k_{cat}$ and overall entropy production in comparison to its wild-type “parent”. The RTEM and lactamase 1 have about one order of magnitude higher optimal catalytic constant and overall entropy production than their wild-type “parents”. However, the optimization results are roughly similar for all of three optimized lactamas. It appears that all of considered A-class β-lactamas have similar latent evolutionary potential and limits to evolvability.

4.6. Unrealized potential of transition state mutations

If optimization only in the one transition between functional states represents the transition state (TS) mutation lowering activation energy for that step [27], then the optimization in two transitions opens at least a thousand time larger search space for double mutations [28] lowering activation energy for all proton shuttles in β-lactamas acylation and deacylation steps. The present analysis puts a natural upper limit for evolution of β-lactamas, derived from physical principles, which may or may not be reached by natural or human-directed evolution [29]. We can conclude that wild-type lactamas, often considered to belong to an exclusive group of near-perfect enzymes [24], have surprised us in practice with amino acid substitutions enabling them to inactivate a broad substrate profile [30], and may surprise us in future with TS mutations accelerating greatly their turnover and catalytic efficiency with the best substrates, positioning such “super-lactamas” close to or even inside the diffusion-limit zone. The restrictions we used (constancy of equilibrium constants for all catalytic steps), nevertheless allowed decreases in activation energies due to TS mutations, the main driving engine for evolution of enzymes.

4.7. Information entropy change during optimizations

When maximal $H$ can be found (Tables 3 and 5 for PCI and β-lactamase 1 respectively) it reflects almost equal probability for all three functional states, which also corresponds to equally high specificity constant and even higher entropy production than those obtained from MaxEP requirement for two last transitions. In the case of the RTEM lactamase, the information entropy decreased, while all other of considered parameters increased after optimal values for forward kinetic constants $k_{2}$ and $k_{3}$ were found from requirement that entropy production is maximal both for the second and for the third enzymatic transition.

4.8. Maximum entropy production principles and evolution of enzymes

Maximum entropy production principles are currently strongly defended [23,31] or disputed [32], but entropy production increase toward some asymptotic maximal value during evolution of metabolic networks is less controversial [20]. Our present and past results in this field [6,7,14,17,18] confirm that maximal entropy production principle can be applied in a limited way as maximal entropy production requirement for enzyme transitions between most important functional states, and also that it is much more relevant for the evolution of enzymes, and generally in bioenergetics, than Prigogine’s minimal entropy production theorem [33]. If MaxEP can be regarded as physical selection principle connected to fundamental MaxENT principle from statistical mechanics [23], it should be also relevant for biological selection and evolution.

Interestingly, the direct application of maximum entropy production principle to enzyme catalysis, that is, the requirement that overall entropy production during catalytic cycle is maximal, requires assumed conservation of activation free energy changes during cycling in either direction [16] and leads to lower overall entropy production (see, for
instance, the $\delta_{\text{mut}}$ row and the $\sigma_{\text{mut}}$ last column for the PC1 lactamase from Table 3) then maximizations of entropy productions in proton transfer steps. The search for unique maximal flux is in our case equivalent to the search for maximal overall entropy production and leads to the same disappointing result of decreased performance parameters. In addition, maximal flux search is generally equivalent to the search for maximal catalytic efficiency [4] or maximal catalytic constant [34] and does not satisfy our goal of finding optimal enzyme performance parameters from deeper physical principle. Furthermore, although acylation and deacylation reactions are better balanced after $P_{\text{mut}}$, optimization for all of three considered $\beta$-lactamases, this did not lead to increased but to decreased catalytic efficiency with respect to values extracted from experiments.

Many other optimization principles have been used to study the evolution of enzymes [13], most of them focused upon one or several biological functions of the system in question with pessimistic conclusion that multitude of biological functions should be used. The fundamental difference of our central hypothesis is that we should first try to study this problem by using simple universal physical principle as appropriated for the biochemical reaction system, which is examined together with all of required constraints.

4.9. Evolution coupling hypothesis

Somewhat contrary to common sense, high efficiency of free-energy transduction and high entropy production are tightly connected for biological systems open to exchange of energy and matter with external environment. In a quasi-steady state, that can be far from equilibrium state due to external forcing, living cells and organisms change very little with time (biological evolution is mostly slow process taking many generations to produce significant differences), but exert a major influence on their environment in the form of increased entropy production. This is in accord with the evolution coupling hypothesis we postulated earlier [35]: biological evolution is not opposed to thermodynamic evolution but, on the contrary, it accelerates it. Order from disorder, or order created by free-energy gradients and promoting disorder, is another name for evolution of biological complexity [36]. Our results describe a specific example of entropy-production-driven reaction network capable of increasing catalytic efficiency. Increases in entropy production for one order of magnitude together with increases in catalytic efficiency is surprising for those who regard dissipation as the very opposite of efficiency. We should take into account, however, that nothing in our calculations restricted us to near equilibrium situations or to static head steady state when entropy production is minimal, while efficiency of free-energy transduction can be low, minimal, or even vanish [18].

The results for $\beta$-lactamase can be easily tested and verified or falsified for other enzymes when similar research program is adapted: Put chosen enzymes on an evolutionary landscape in the cases when most or all rate constants are known and when phylogenetic trees can be constructed for large enough collection of evolutionary related sequences. Then examine which catalytic steps can increase enzyme performance parameters after being optimized for maximal entropy production and focus on mechanistic details of these crucial enzymatic transitions to answer the question are they connected to electron or proton transfer, or to movements of whole amino acid segments. Maybe we can then learn from enzymes how and why those nanocurrents develop which are at the same time the most efficient in dissipating free-energy gradients and in performing catalysis. A substrate binding for some enzymes is the likely trigger for releasing electron/proton current and substrate to product conversion flux, which is essential for catalysis, but also inseparably associated with entropy production. The guiding physical principle of this research program can also serve as an additional guide for de novo computational and experimental design of catalytically efficient enzymes [37]. In a nutshell – we propose that mutations improving catalytic activity are often those that increase entropy production as well.

Acknowledgements

We would like to thank Prof. Dan Tawfič and Dr. Mahendra Mariadassou for useful comments. A.D. gratefully acknowledges the financial support of the Slovenian Research Agency (ARRS) under the contract No. P1-0055. The authors acknowledge Prof. George M. Slavich help with language editing.

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