Is the catalytic activity of triosephosphate isomerase fully optimized? An investigation based on maximization of entropy production

Željana Bonačić Lošić¹ · Tomislav Dondović² ·
Davor Juretić³

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Abstract Triosephosphate isomerase (TIM) is often described as a fully evolved housekeeping enzyme with near-maximal possible reaction rate. The assumption that an enzyme is perfectly evolved has not been easy to confirm or refute. In this paper, we use maximization of entropy production within known constraints to examine this assumption by calculating steady-state cyclic flux, corresponding entropy production, and catalytic activity in a reversible four-state scheme of TIM functional states. The maximal entropy production (MaxEP) requirement for any of the first three transitions between TIM functional states leads to decreased total entropy production. Only the MaxEP requirement for the product (R-glyceraldehyde-3-phosphate) release step led to a 30% increase in enzyme activity, specificity constant k_{cat}/K_M , and overall entropy production. The product release step, due to the TIM molecular machine working in the physiological direction of glycolysis, has not been identified before as the rate-limiting step by using irreversible thermodynamics. Together with structural studies, our results open the possibility for finding amino acid substitutions leading to an increased frequency of loop six opening and product release.

Keywords Enzyme kinetic scheme · Triosephosphate isomerase · Maximum entropy production · Kinetic constants

✉ Davor Juretić
davor.juretic@gmail.com

¹ Faculty of Science, University of Split, Ruđera Boškovića 33, 21000 Split, Croatia

² Medical High School, Šibenik, Ante Šupuka bb, 22000 Šibenik, Croatia

³ Mediterranean Institute for Life Sciences, Šetalište Ivana Meštrovića 45, 21000 Split, Croatia

Abbreviations

TIM	Triosephosphate isomerase
DHAP	Dihydroxyacetone phosphate
GAP	D-glyceraldehyde 3-phosphate
MaxEP	Maximum entropy production

1 Introduction

Energy flow through open biological systems, such as a living cell, for a biologist leads to maintenance and reproduction of beautiful and complex macromolecular systems, but for a physicist it is apparent that a majority of the free-energy flow is dissipated, so that the most striking consequence of being alive is increased entropy production in the universe. Enzymes are macromolecules with a high turnover rate, costly in terms of the free energy dissipated during their synthesis and degradation, but they are essential to speed up chemical reactions in a living cell [1], and at constant temperature the reaction speed is determined by how their structure affects their function. This structure is an outcome of biological evolution, which through the eons has increased the reaction rate by many orders of magnitude.

Different optimization principles have been proposed to study the evolution of enzymes [2, 3]. In each case, the objective optimization function is supplemented with more or less subjective constraints. For instance, for reaction-rate maximization, upper bounds for kinetic constants must be set, a procedure that leads to many complications and uncertainties.

Since both biological evolution and the maintenance of macromolecules in an active cell are highly dissipative processes, it is natural to ask if some physical principle from the field of irreversible thermodynamics is relevant for life and its evolution [4]. Also, how we can judge if some enzymes have become fully evolved, as has been claimed [5, 6]? Presumably, fully evolved proteins with enzymatic function leave no space for any additional optimization of rate constants in the kinetic scheme used to describe their catalysis, in overall reaction rate or in the specificity constant (catalytic activity).

Free-energy transduction is the central concept studied by bioenergetics [7]. In physics, restriction to small forces, a linear response, and a situation close to equilibrium, leads to Prigogine's theorem of "minimum entropy production" [8]. We should stress that Prigogine's MinEP theorem is not a selection principle, but simply a property of the unique near-equilibrium steady state (the static head state) when it is kept away from equilibrium due to some constant driving force. The efficiency of free energy transduction becomes equal to zero in the MinEP state, whereas free-energy transduction is essential for the evolution of life. Prigogine's theorem is not relevant for nonlinear relationships between forces (affinities) and corresponding fluxes (reaction rates) and the high dissipation rates typical for biological processes, but the principle of the "maximum entropy production" (the MaxEP principle) may still be applicable [9, 10] within known restrictions of the Ziegler or generalized MaxEP principle [11]. In contrast to MinEP, the MaxEP principle selects among multiple steady states, which obey the same constraints, and predicts optimal parameters for the selected state. A caveat is that it is still an open question whether the MaxEP principle can be falsified, or established as more than a conjecture and it is not always clear which EP should be chosen for maximization. Nevertheless, the ever-growing number of studies using MaxEP to obtain results which can be verified in experiments, testify to the potential importance and generality of MaxEP [11].

Previously, maximization of entropy production was used for enzymes which can be found in three functional states (Michaelis–Menten reversible kinetic scheme), as a test to find out if they are fully evolved or not [12]. It was shown that the entropy production reaches a maximal value for an optimal value of kinetic constant in a chosen transition. The optimal values of kinetic constants, in all three transitions, did not differ much from experimentally measured values for beta-lactamases [12]. However, modeling of enzyme kinetics in this and earlier attempts of MaxEP application [13, 14] resulted in different degrees of agreement with experimental results, possibly because different mathematical procedures were used. Also, different set of transitions were considered in the calculation of EP – the internal transition in the case of beta-lactamases [12], the open-state transition coupled to ATP synthesis/hydrolysis in the case of ATP-synthase [14] and four transitions leading to charge separation out of seven transitions in the case of two simple models for bacterial photosynthesis [13]. Common to these results is that the total entropy production was not considered in relationship to detailed distribution of entropy productions for optimized transitions between functional states. In each case, only a subset of transitions was considered so that a clear conclusion could not be derived as to which transition is rate-limiting, with the greatest possible contribution to catalytic activity and to total entropy production.

In this paper, we have chosen to examine the steady state of the triosephosphate isomerase (the TIM enzyme), whose catalysis is represented as a reversible cyclic kinetic scheme, with four transitions among four functional states. The goal of this work is to compare the prediction of optimal kinetic constants, optimal reaction rate, the specificity constant k_{cat}/K_M , and calculated total MaxEP in all transitions between functional states, with the experimental observations for all these transitions [15]. Fixed substrate and product concentrations are assumed, as well as fixed equilibrium constants for all transitions, while forward kinetic constants are allowed to vary from their known experimental values. MaxEP is applied to each of the four transitions between TIM functional states and compared with the detailed distribution of entropy productions and with total EP. Also, a different set of transitions was considered simultaneously in the calculations of EP – the internal transitions and the open-state transitions.

The TIM enzyme is one of the most ancient housekeeping enzymes present in almost all living cells. It catalyses the isomerization of dihydroxyacetone phosphate (DHAP) to D-glyceraldehyde 3-phosphate (GAP), an essential process in the glycolytic pathway, because only GAP can be subsequently used for glycolysis-derived ATP synthesis. The TIM enzyme also has a prominent role in gluconeogenesis, lipid metabolism, and the pentose phosphate pathway. For human TIM, even a conservative change in a single amino acid residue (e.g., Glu-104 into Asp-104) leads to premature death at an early age [16]. While impairments of TIM catalytic activity are easily achieved, it is in principle possible to find improvements too, which would be useful in agro-food biotechnology. For example, efforts to improve TIM design by using site-directed mutagenesis benefited from careful comparison of conserved active site residues among many different species and resulted in the creation of a superstable enzyme, which also exhibited a slight improvement in its catalytic power [17]. Increasing interest in designing enzymes with industrial applications would profit from any rational method leading to increased specificity constant k_{cat}/K_M in addition to increased stability.

Our main objective here is to use the MaxEP principle to gauge if TIM is fully optimized, and to identify the rate-limiting step which can produce a significant increase both in total entropy production and in catalytic activity. Our starting point here was that increased total entropy production should also lead to increased enzymatic activity, due to the direct proportionality between reaction rate and entropy production for a one-cycle reaction scheme with

constant external forcing. Therefore, we explored different ways of increasing entropy production within known constraints, without making any prior assumptions, such as that TIM evolves according to MaxEP, that optimized rate constants should be identical to empirical rate constants, or that some contributions to entropy production are more important than other contributions.

We found that optimization of entropy production in the fourth transition, when product is released, allows for significant additional increase in the reaction rate, specificity constant k_{cat}/K_M , and total entropy production. This suggests that the TIM enzyme is not as perfect and fully evolved as previously supposed [5, 6]. Our results are in accord with studies suggesting that there is still room for improvement of the TIM enzyme [3].

In Section 2, we describe Hill's diagram method for calculating EP [18], and provide a short proof that maximum entropy production can be found for each of the enzyme transitions between functional states; In Section 3, we give the numerical results for optimal kinetic constants and optimal transition fluxes [7] and compare them with the corresponding experimental data for the TIM enzyme kinetic scheme [15] to find a key transition able to achieve the highest increase in total entropy production and in overall catalytic flux. Our results are summarized and discussed in Section 4.

2 Maximum entropy production in enzyme internal transitions

Our analysis proceeds by considering entropy production in enzyme transitions between functional states using Hill's diagram method [7, 18]. Each transition between discrete steady states for an enzyme is associated with entropy production, which can be calculated starting from known or assumed forward and backward rate constants. It is possible to find the optimal forward kinetic constant associated with the maximal entropy production in any given transition, by applying a general approach [12] to the case of transitions for triosephosphate isomerase (TIM). This can be found in four functional states [5]: 1 is the free enzyme (E), 2 is the enzyme-substrate complex (ES), 3 is a transition state intermediate (EZ) and 4 is the enzyme-product bound complex (EP) as shown in Fig. 1. Let us consider two states i and j , where a transition flux connecting these states is nonzero. The entropy production associated

Fig. 1 The four-state kinetic diagram, where k_{ij} and k_{ji} are transition kinetic constants ($i = 1, 2, 3, 4$). Functional states are: 1 the enzyme (E), 2 the enzyme-substrate complex (ES), 3 an intermediate (EZ), and 4 the enzyme-product bound complex (EP)

with the transition $i \rightarrow j$ is defined as the product of the flux and the corresponding affinity,

$$\sigma_{ij}T = X_{ij}J_{ij}[Js^{-1}], \quad (1)$$

where T is the temperature. Here, the affinity and the flux of the transition $i \rightarrow j$ are

$$X_{ij} = RT \ln \frac{k_{ij}p_i}{k_{ji}p_j} [Jmol^{-1}], \quad (2)$$

$$J_{ij} = n(k_{ij}p_i - k_{ji}p_j) [mol s^{-1}], \quad (3)$$

respectively, where R is the gas constant, n is the number of moles of enzyme molecules and

$$p_i = \Sigma_i / \Sigma, \quad (4)$$

is the stationary probability of the i -th macromolecular state with Σ_i and Σ denoting respectively the sum of the directional diagrams toward state i and the sum of the directional diagrams of all states. The directional diagrams for state i can be subdivided into diagrams $a_{ji}k_{ji}$, including the rate constant k_{ji} , and diagrams b_{ij} , excluding the rate constant k_{ji} . Analogously, the directional diagrams for state j can be subdivided into diagrams $a_{ij}k_{ij}$, including the kinetic constant k_{ij} , and diagrams b_{ji} , excluding the kinetic constant k_{ij} . Thus, we can write

$$\begin{aligned} \Sigma_i &= a_{ji}k_{ji} + b_{ij} \\ \Sigma_j &= a_{ij}k_{ij} + b_{ji}. \end{aligned} \quad (5)$$

Using expressions (4) and (5) we get the affinity

$$X_{ij} = RT \ln \frac{K_{ij}b_{ij} + k_{ij}a_{ij}}{b_{ji} + k_{ij}a_{ij}}, \quad (6)$$

where $K_{ij} = k_{ij}/k_{ji}$, is the equilibrium constant for the ij transition, and the flux

$$J_{ij} = n \frac{b_{ij} - b_{ji}/K_{ij}}{\Sigma/k_{ij}}. \quad (7)$$

Finally, inserting expressions (6) and (7) into (1) we obtain the entropy production

$$\sigma_{ij}T = RT \ln \frac{K_{ij}b_{ij} + k_{ij}a_{ij}}{b_{ji} + k_{ij}a_{ij}} n \frac{b_{ij} - b_{ji}/K_{ij}}{\Sigma/k_{ij}}. \quad (8)$$

As before, the entropy production and the equilibrium constant in the expression (8) are associated with the transition $i \rightarrow j$ in both directions, forward and reverse. The optimal value for the forward kinetic constant associated with the extreme value of entropy production (8) is obtained from the condition

$$\frac{d\sigma_{ij}}{dk_{ij}} = 0. \quad (9)$$

We assume that equilibrium constant K_{ij} does not change but that coordinated change in corresponding forward and backward kinetic constants is still possible. This condition gives the equation for the optimal value of kinetic constant k_{ij} [12]

$$\ln \frac{K_{ij}b_{ij} + k_{ij}a_{ij}}{b_{ji} + k_{ij}a_{ij}} = \frac{a_{ij}(K_{ij}b_{ij} - b_{ji})k_{ij}(\sum_a + \sum_b)}{\sum_b(k_{ij}a_{ij} + K_{ij}b_{ij})(k_{ij}a_{ij} + b_{ji})} \quad (10)$$

where $k_{ij}\sum_a$ and $\sum_b$ are the sums of all state directional diagrams, respectively, with or without a line describing the transition $i \rightleftharpoons j$, and

$$\sum = k_{ij}\sum_a + \sum_b. \quad (11)$$

Equation (10) has a solution corresponding to the optimal forward kinetic constant (k_{ij}).

The extreme value of the entropy production for the optimal value of the forward rate constant, denoted k_{ij}^0 , is then given by the expression

$$\sigma(k_{ij}^0) = \frac{nR}{K_{ij}} \frac{a_{ij}(k_{ij}^0)^2 (K_{ij}b_{ij} - b_{ji})^2}{\sum_b(k_{ij}^0 a_{ij} + K_{ij}b_{ij})(k_{ij}^0 a_{ij} + b_{ji})}. \quad (12)$$

This is the maximal value of the entropy production (8) as its second derivative is negative for the optimal value of the forward kinetic constant.

As an illustrative example, we can find the entropy production (8) in the transition $2 \rightarrow 3$, the optimal value of the forward kinetic constant k_{23} from expression (10) and the maximum entropy production (12). The general result derived above guarantees that a maximum of entropy production does exist in this transition, as in any other transition between functional states in a kinetic scheme for enzyme catalysis. Figure 2 shows the directional diagrams for the kinetic scheme in Fig. 1 that contribute to the sum $\sum_i$. Each directional diagram in Fig. 2 contributes a product of three kinetic constants represented by corresponding transition lines. The corresponding expressions for the sums in Fig. 2 are:

$$\begin{aligned} \sum_1 &= k_{43}k_{32}k_{21} + k_{21}k_{34}k_{41} + k_{41}k_{32}k_{21} + k_{23}k_{34}k_{41} \\ \sum_2 &= k_{34}k_{41}k_{12} + k_{14}k_{43}k_{32} + k_{12}k_{43}k_{32} + k_{32}k_{41}k_{12} \\ \sum_3 &= k_{41}k_{12}k_{23} + k_{21}k_{14}k_{43} + k_{14}k_{43}k_{23} + k_{12}k_{23}k_{43} \\ \sum_4 &= k_{32}k_{21}k_{14} + k_{12}k_{23}k_{34} + k_{14}k_{23}k_{34} + k_{21}k_{14}k_{34}. \end{aligned} \quad (13)$$

Fig. 2 The sums of directional diagrams for states 1, 2, 3 and 4

$$\begin{aligned}
\Sigma_1 &= \begin{array}{c} \uparrow \\ \square \end{array} + \begin{array}{c} \nearrow \\ \square \end{array} + \begin{array}{c} \nwarrow \\ \square \end{array} + \begin{array}{c} \leftarrow \\ \square \end{array} \\
\Sigma_2 &= \begin{array}{c} \downarrow \\ \square \end{array} + \begin{array}{c} \square \\ \leftarrow \end{array} + \begin{array}{c} \searrow \\ \square \end{array} + \begin{array}{c} \square \\ \nwarrow \end{array} \\
\Sigma_3 &= \begin{array}{c} \square \\ \rightarrow \end{array} + \begin{array}{c} \square \\ \downarrow \end{array} + \begin{array}{c} \square \\ \searrow \end{array} + \begin{array}{c} \square \\ \swarrow \end{array} \\
\Sigma_4 &= \begin{array}{c} \square \\ \rightarrow \end{array} + \begin{array}{c} \square \\ \uparrow \end{array} + \begin{array}{c} \square \\ \nearrow \end{array} + \begin{array}{c} \square \\ \nwarrow \end{array}
\end{aligned}$$

Using expression (13), we obtain the sum

$$\Sigma = \Sigma_1 + \Sigma_2 + \Sigma_3 + \Sigma_4 \quad (14)$$

entering expression (8). In the case of transition from the state 2 into the state 3 we have two kinetic constants, k_{23} and k_{32} . We take into account all constants besides the one that we want to predict (in this case k_{23}) while calculating the values of a_{23} , b_{23} , and b_{32} entering expressions (8), (10), and (12), and Σ_a and Σ_b entering expressions (10) and (12). Then, using expression (5) we obtain the sum

$$\Sigma_2 = a_{32}k_{32} + b_{23}$$

and by comparison with the corresponding sum in expression (13)

$$\Sigma_2 = k_{32}(k_{14}k_{43} + k_{12}k_{43} + k_{41}k_{12}) + k_{34}k_{41}k_{12}$$

we find the expressions for a_{32} and b_{23} , respectively,

$$\begin{aligned}
a_{32} &= k_{14}k_{43} + k_{12}k_{43} + k_{41}k_{12} \\
b_{23} &= k_{34}k_{41}k_{12}.
\end{aligned} \quad (15)$$

Analogously, we proceed to find a_{23} and b_{32}

$$\begin{aligned}
\Sigma_3 &= a_{23}k_{23} + b_{32} \\
\Sigma_3 &= k_{23}(k_{14}k_{43} + k_{12}k_{43} + k_{41}k_{12}) + k_{21}k_{14}k_{43} \\
a_{23} &= k_{14}k_{43} + k_{12}k_{43} + k_{41}k_{12} \\
b_{32} &= k_{21}k_{14}k_{43}
\end{aligned} \quad (16)$$

and from expression for the sum (14) and using expression (11) we find the values for Σ_a and Σ_b

$$\Sigma_a = k_{34}k_{41} + k_{41}k_{12} + k_{14}k_{43} + k_{12}k_{43} + k_{12}k_{34} + k_{14}k_{34} + (k_{43}k_{21} + k_{41}k_{21} + k_{14}k_{43} + k_{12}k_{43} + k_{41}k_{12} + k_{21}k_{14})/K_{23} \quad (17)$$

$$\Sigma_b = k_{21}k_{34}k_{41} + k_{34}k_{41}k_{12} + k_{21}k_{14}k_{43} + k_{21}k_{14}k_{34}. \quad (18)$$

In the next section, we find numerically the optimal kinetic constants and the corresponding maximum entropy productions for four transitions of the TIM enzyme.

3 Numerical results for optimal kinetic constants and comparison with experimental results

In calculating the optimal values of the kinetic constants, the experimental values of the rate constants are taken from reference [15] for the triosephosphate isomerase (Table 1). The assumed reference steady state [5] is such that the concentration of substrate is $[S] = 40 \mu\text{M}$, while the concentration of product is $[P] = 0.064 \mu\text{M}$. The values of the kinetic constants k_{12} and k_{14} in Table 1 for the assumed reference steady state are obtained respectively from expressions $k_{12} = k_{12}^*[S]$ and $k_{14} = k_{14}^*[P]$, where second-order rate constants k_{12}^* , k_{14}^* are measured in $(\mu\text{Ms})^{-1}$. We also calculate the equilibrium constants $K_{ij} = k_{ij}/k_{ji}$ using experimental data [15] shown in Table 1. The stability of this steady state, i.e., system's ability to return to it after small perturbations, has been verified previously [3].

3.1 MaxEP applied to single transitions

The procedure developed in the previous section can be used to calculate optimal values of the kinetic constants for all four transitions. For instance, the optimal value of the kinetic constant k_{23} is obtained by solving numerically Eq. (10) with $ij = 23$, while inserting experimental values from the reference [15] for all other kinetic constants. Analogously, the optimal values of the kinetic constants k_{12} , k_{34} , and k_{41} are found. Table 1 compares measured [15] and calculated optimal values of all kinetic constants. Backward optimal constants are calculated from the requirement that equilibrium constants do not change with respect to their measured values. The closest agreement (within a factor of two) with the corresponding experimental values is obtained in the fourth transition (GAP release or binding, i.e., $ij = 41$ or 14). This last transition is the only one that speeds up after MaxEP optimization.

As an example, the dependence of entropy production (8) for the transition from state 2 to state 3 on the kinetic constant k_{23} is shown in Fig. 3. It is seen that entropy production σ_{23}/nR increases from zero to the maximal value, and then decreases as k_{23} further increases. It has a

Table 1 Experimental values of kinetic constants and equilibrium constants for the enzyme triosephosphate isomerase [15] and numerically calculated optimal values of kinetic constants. The substrate and product concentration were respectively $[S] = 40 \mu\text{M}$ and $[P] = 0.064 \mu\text{M}$ for results presented in this table and in Tables 2, 3, and 4

Kinetic constant	Experimental value $[\text{s}^{-1}]$	Optimal value from MaxEP applied to corresponding transition $[\text{s}^{-1}]$	Equilibrium constant experimental value
k_{12}	400	28.54	$K_{12} = 0.057$
k_{21}	7000	499.43	
k_{23}	2000	661.30	$K_{23} = 0.333$
k_{32}	6000	1983.91	
k_{34}	60,000	1597.20	$K_{34} = 0.667$
k_{43}	90,000	2395.80	
k_{41}	4000	7847.53	$K_{41} = 156.25$
k_{14}	25.60	50.22	

maximal value of 2.95 s^{-1} for $k_{23} = 661.30 \text{ s}^{-1}$ (Table 1). This optimal value is three times lower than the measured value, and corresponds to a slower reaction rate. Therefore, optimizing the internal transition $2 \rightarrow 3$ (second transition) for a maximal EP in that transition would not be helpful, as we shall explain latter.

Inserting the numerical optimal values for the kinetic constants k_{ij} and k_{ji} from Table 1 into expressions for the fluxes (7) and entropies (8), we calculate the flux and total entropy production change when we optimize kinetic constants of different transitions (Tables 2 and 3). As expected, an increase or decrease in the flux relative to its measured value (Table 2, second column) led to a corresponding increase or decrease in the overall EP (Table 3, last column). An increase in the overall flux and total entropy production occurs only in the case of the last transition (Tables 2 and 3). Thus, a possible rate-limiting step in the physiological direction (from DHAP to GAP) is the fourth transition (gray rows in Tables 2 and 3), which is in accordance with [6, 19]. The rate-limiting step mentioned in these studies is proton transfer from GLU167 coupled to product release (GAP) in the forward direction, due to loop-6 opening.

We verified the consistency with Kirchhoff's laws in each optimization procedure. In the case of a cyclic reaction scheme, the first Kirchhoff law (for fluxes) states that the algebraic sum of all transition fluxes in each state of stationary system equals zero. The second Kirchhoff law (for steady-state affinities) states that the sum of affinities of all transitions within the cycle equals the total thermodynamic force. By inserting the numerical optimal values for the kinetic constants k_{ij} and k_{ji} from Table 1 into expressions for the fluxes (7), we found that all four transition fluxes are equal, irrespective of which one of four optimization scenarios has been considered, consistent with the first Kirchhoff law. Also, the total thermodynamic force $X/RT = (X_{12} + X_{23} + X_{34} + X_{41})/RT$ retains the same constant value equal to 0.6852 calculated using optimal values of the kinetic constants from Table 1, consistent with the second Kirchhoff law.

Fig. 3 The dependence of the entropy production σ_{23}/nR from expression (8) on the kinetic constant k_{23}

Table 2 Comparison of fluxes, catalytic constants k_{cat} , Michaelis constants K_M , and specificity constants k_{cat}/K_M calculated using experimental data of reference [15] for enzyme triosephosphate isomerase (*second row*), and numerically obtained optimal values of kinetic constants k_{ij} and k_{ji} for i -th transition ($i = 1, \dots, 4$; *rows 3–6*). Measured values [15] were taken as known values of kinetic constants for other than optimized transition i

Transitions between functional states	Flux [s^{-1}]	k_{cat} [s^{-1}]	K_M [M]	k_{cat}/K_M [$\text{M}^{-1}\text{s}^{-1}$]
Experimental values are used for kinetic constants	14.42	431.66	$5.50 \cdot 10^{-4}$	$78.58 \cdot 10^4$
Optimized 1. transition	7.25	431.66	$11.11 \cdot 10^{-4}$	$38.85 \cdot 10^4$
Optimized 2. transition	9.29	300.41	$6 \cdot 10^{-4}$	$50.48 \cdot 10^4$
Optimized 3. transition	7.19	210.33	$5.4 \cdot 10^{-4}$	$39.18 \cdot 10^4$
Optimized 4. transition	20.77	685.56	$6 \cdot 10^{-4}$	$112.82 \cdot 10^4$

Table 2 also shows changes in k_{cat} , K_M and the specificity constant k_{cat}/K_M (catalytic activity [20]). We have calculated this ratio, as well as k_{cat} and K_M , using expressions from reference [21]:

$$k_{\text{cat}} = \frac{k_{23}k_{34}k_{41}}{(k_{23} + k_{32})(k_{43} + k_{41}) + k_{23}k_{34} + k_{34}k_{41}} \quad (19)$$

$$K_M = \frac{k_{21}[k_{32}(k_{43} + k_{41}) + k_{34}k_{41}] + k_{23}k_{34}k_{41}}{k_{12}^*[(k_{23} + k_{32})(k_{43} + k_{41}) + k_{23}k_{34} + k_{34}k_{41}]}. \quad (20)$$

Table 3 Entropy production associated with each transition and total entropy production of the TIM enzyme (*last column*). Maximal EP values for each transition are in *bold numbers*

Entropy production in each transition (s^{-1})	$\frac{\sigma_{12}}{nR}$	$\frac{\sigma_{23}}{nR}$	$\frac{\sigma_{34}}{nR}$	$\frac{\sigma_{41}}{nR}$	Overall entropy production/ nR
For measured values of kinetic constants	0.573	2.208	0.241	6.861	9.883
For k_{12} , k_{21} optimal values	2.283	0.703	0.074	1.909	4.970
For k_{23} , k_{32} optimal values	0.236	2.947	0.115	3.067	6.365
For k_{34} , k_{43} optimal values	0.141	0.517	2.361	1.905	4.924
For k_{41} , k_{14} optimal values	1.196	4.830	0.551	7.656	14.234

Using total entropy production as the selection criterion, the optimal values for the flux (J), σ/nR , k_{cat} and k_{cat}/K_M were significantly higher than the corresponding experimental values only in the case when MaxEP was applied to the product release step (compare Tables 2 and 3). This suggests that TIM evolution still leaves room for improvement in the product dissociation step in order to improve its performance.

With regard to the trade-off between flux increase and affinity decrease in different transitions between functional states, we notice that optimizations in transitions 1, 2, and 3 lead to a much higher affinity in the corresponding transitions (not shown) and place an upper bound on the optimized flux, such that the flux is about two times smaller than its measured value (Table 2). In the last transition the combination of significant flux increase and modest affinity decrease leads to entropy production increase when that transition is optimized. Due to the steady state constraints, the only transition associated with decreased affinity is the product release transition, leading to an improved catalytic constant and catalytic activity for the TIM enzyme.

We have kept constant the total force (sum of all four affinities). This constraint can be changed by decreasing the product concentration from $0.064 \mu\text{M}$ to, for instance, $0.013 \mu\text{M}$, which leads to X/RT increase from 0.6852 to 2.2791. With the same type of EP maximization in the product release step, increase in total EP, enzyme activity (flux) and k_{cat}/K_M , due to such optimization, is no longer close to 30% (Tables 2 and 3, compare second and last row values), but is around 42% (data shown only for the EP increase in Fig. 4). As before, we have assumed that all equilibrium constants retain their experimental values.

3.2 Alternative applications of MaxEP

We now extend our investigation to address some of the uncertainties surrounding MaxEP. Namely, as was already mentioned in the introduction, it is not yet clear which entropy production should be maximized, the entropy production of individual step/steps or total

Fig. 4 Distribution of entropy productions for each TIM-enzyme functional transition before (*black columns*) and after (*extended gray area over black area in each column*) MaxEP optimization for the 4th step (product release), in the case when substrate (DHAP) and product (GAP) concentrations are $40 \mu\text{M}$ and $0.013 \mu\text{M}$, respectively

entropy production of all steps. A previous application of MaxEP to ATP-synthase [14] showed that the current kinetic design of ATP-synthase is consistent with maximization of the entropy production associated with the synthesis/hydrolysis transition. This result hints at the possibility that, more generally, the relevant entropy production to be maximized is the entropy production associated with the transitions involving substrate/product exchange. In the case of the TIM enzyme, this would correspond to the combined entropy productions of reactions 1–2 (exchange of DHAP) and 1–4 (exchange of GAP), i.e., variation of $(\sigma_{12} + \sigma_{41})/nR$ simultaneously with respect to k_{12} and k_{41} . After performing these calculations and numerical evaluations (Table 4), we found that the height of $(\sigma_{12} + \sigma_{41})/nR$ slowly increases with k_{12} increase, but has no maximal value for a finite k_{12} . For all k_{12} values higher and k_{14} values smaller than approximately 100 s^{-1} the overall entropy production divided by nR is higher than the value of 14.23 s^{-1} found after entropy production maximization solely in the GAP release transition step (see Table 3). In three dimensions there is a smoothly curved line connecting the highest $(\sigma_{12} + \sigma_{41})/nR$ values for different pairs of k_{12} and k_{14} . Only seven such pairs are shown in Table 4. As before, we used experimental values for forward kinetic constants other than the varied constants k_{12} and k_{41} .

An alternative approach is to maximize entropy production for functionally important internal enzyme transitions [4, 12] subject to appropriate constraints. The diffusion limit for ligands is the most important constraint. After considering optimal kinetic constant values k_{14} for maximal EP values in Table 4, we can conclude that the corresponding k_{14}^* values (with $[P]=0.064 \text{ }\mu\text{M}$) are well below the diffusion limit of $10^{10} \text{ M}^{-1}\text{s}^{-1}$ [22], but are around the lower estimate for the diffusion limit of $10^9 \text{ M}^{-1}\text{s}^{-1}$ [6]. All k_{12}^* corresponding to k_{12} values (with $[S]=40 \text{ }\mu\text{M}$) in Table 4 are well below the diffusion limit of $10^9 \text{ M}^{-1}\text{s}^{-1}$. The conservative choice of the k_{12} and k_{14} pair are shown in Table 4 as gray background that include experimentally determined $k_{12}=400 \text{ s}^{-1}$. In this way, we can look for the highest entropy production $(\sigma_{23} + \sigma_{34})/nR$ in internal transitions starting from the best result after optimization of external transitions, and staying as close to measured values of kinetic constants as possible. When these calculations were performed, we could not find maximal $(\sigma_{23} + \sigma_{34})/nR$, while overall EP actually decreased to 15.50 s^{-1} from the Table 4 value of 16.35 s^{-1} . Since we used simultaneous optimization by variation of k_{23} and k_{34} , the values for these kinetic constants were found too as 3240 and $10,000 \text{ s}^{-1}$, respectively, which is in slightly better agreement with measured values of these kinetic

Table 4 Sum of entropy productions due to ligand association and formation of external transition states

$k_{12}[\text{s}^{-1}]$	$k_{14} [\text{s}^{-1}]$	$(\sigma_{12} + \sigma_{41})/nR [\text{s}^{-1}]$	$(\sigma_{\text{overall}})/nR [\text{s}^{-1}]$
100	107.32	7.70	13.12
200	78.30	8.53	15.05
400	70.01	9.03	16.35
800	66.34	9.31	17.06
1000	65.66	9.37	17.22
2000	64.35	9.49	17.53
10000	63.10	9.59	17.76

constants (see Table 1). In summary, the results so far support σ_{41}/nR as the dominant contribution and the conclusion that the product release step is the most important one.

We focused here only on calculating the entropy production without considering the distribution of state probabilities. In some cases, Shannon's information entropy associated with the enzyme state probabilities $S = -\sum_{i=1}^4 p_i \ln p_i$ can also be maximized with respect to some or all forward kinetic constants [4, 14]. Optimal kinetic constants found by information entropy maximization can then be compared to their optimal values found from the MaxEP application. In the case of the TIM kinetic scheme, Shannon's entropy did not have a maximum with respect to any of four forward kinetic constants. It remained approximately the same as the value calculated from experimental data (around 0.3 as calculated from p_i distribution) or decreased after MaxEP optimizations in each of the four transitions, because state occupancy for the first transition state remained around 92% or increased even more for all optimization scenarios. Also, $(\sigma_{12} + \sigma_{41})/nR$ and Shannon's entropy could not be simultaneously optimized when one varies k_{12} and k_{41} .

4 Discussion

Entropy production is the most important function in irreversible thermodynamics. The maximum entropy production principle, or MaxEP conjecture [23], has long been neglected in physics, mainly due to lack of recognition that the principle of least dissipation [24, 25] is in reality the application of the MaxEP principle when constraints are taken into account for linear relationships between fluxes and forces [26, 27]. A tendency to elevate Prigogine's minimal entropy production theorem to the level of a general principle also contributed to confusion as noted by Ross and Vlad [28]. In fact, as Beretta and Martyushev stated [11, 29, 30], maximal EP implies minimal EP at some constrained stationary states such as the static head state for linear force–flux relationships. However, the MaxEP postulate is often considered as being of limited validity [31–33]. Some of the examples used to disprove MaxEP are outside the range of the principle's applicability or even worse violate the second law corollary that EP must always be positive-definite [11]. Dewar [34, 35] and Martyushev [10, 11, 30] pointed out the relevance of MaxEP as a physical principle in statistical physics [36], transport theory [37] and thermomechanics [38], while numerous other applications and supporting observations were found including geology [39], climatology [40], crystal growth [41], nanophysics [42], and biology [4, 12–14, 43]. It is still an open question how general MaxEP is as a selection principle in irreversible thermodynamics [11] or even as a new paradigm for biological evolution—the survival of the likeliest [44]—providing much-needed physical insight into the driving force for pre-biological and biological evolution. While recognizing MaxEP limitations [11, 30], we maintain that it is relevant for biological evolution.

There have been ambitious attempts to associate the MaxEP principle with evolution of metabolic networks [43], but our goal in this work was more focused and limited to the study of the TIM enzyme. This enzyme is often described as perfectly designed by biological evolution [6, 15]. In addition, its equilibrium constant is close to one. When equilibrium constants and all other steady-state conditions are kept constant as constraints, can anything of interest happen when kinetic constants from one or more transitions between the four

functional states are allowed to vary? Will the reaction rate, the catalytic constant k_{cat} , and the specificity constant k_{cat}/K_M remain unaltered, decrease, or increase when optimal kinetic constants in the considered transition are calculated from the requirement that entropy production is maximal in that transition? What will be the effect on total entropy production by TIM when entropy production is separately maximized in each of the four possible transitions? With the proof that an entropy production maximum can be found for each transition [12], we were sure that optimal kinetic constants can be found by using a standard optimization procedure.

From a biological viewpoint, the TIM enzyme and glycolytic pathway have been highly optimized during evolution. An increased rate of product synthesis would lead to GAP accumulation, in the absence of improved downstream enzymes utilizing it. This would cause a change of the steady state in the direction of substrate (DHAP) synthesis, because TIM is a reversible enzyme, and DHAP accumulation can lead to conversion into toxic methylglyoxal [45]. For this undesired effect, it makes no difference whether it is caused by a drastic decrease or a drastic increase in TIM-enzyme activity. One can conclude that optimal enzyme activity and rate of optimal GAP synthesis should be found as a maximization consequence of some other objective function instead of enzyme activity. One advantage of considering EP as a function to be optimized is that it can be separately explored/optimized in each of the transition steps between enzyme functional states, allowing for identification of the rate-limiting transition independently from mechanistic details of how catalysis is performed.

We found that the entropy production of the TIM enzyme, the catalytic constant k_{cat} , and the specificity constant k_{cat}/K_M are significantly higher than the corresponding experimental values when kinetic constants are optimized in the last transition (Tables 2 and 3). The optimization procedure in the product release step influences all other transitions as well. While optimization of other transitions also has this effect, only the MaxEP requirement for the product release step leads to increased entropy production in all other transitions too (Table 3). The increase with respect to EP values calculated from measured rate constants [12] is quite significant for transitions 1–2, 2–3, and 3–4, approximately doubling EP values, while for 4–1 optimization there is only a modest increase in the EP value (the last row of Table 3). Transitions 2–3 and 4–1 are responsible for about 90% of TIM enzyme overall entropy production. These are the proton transfers and loop motion/product release conformational changes [19]. Even a small EP increase in the product release step, for instance after some favorable mutation, should lead to improvement in all catalytic parameters together with overall EP increase (Tables 2 and 3).

The best EP value we found numerically (the last gray cell in Table 3) was not obtained by maximizing overall entropy production with respect to all kinetic constants simultaneously. In other words, it is possible that overall maximal EP is higher than the value we presented in Table 3. Indeed, we found higher overall EP values after simultaneous optimization of two external transitions involving the substrate or product binding/release step (Table 4). However, we showed that $(\sigma_{12} + \sigma_{41})/nR$ is not an objective function for maximization simultaneously with respect to k_{12} and k_{41} , as its height for a given k_{12} increases as k_{12} increases. We can only state that evolution toward an asymptotic state with maximum overall entropy production is possible when both external transitions are optimized and diffusion limits are taken into account, similarly to evolution of metabolic networks [43]. The entropy production $(\sigma_{23} + \sigma_{34})/nR$ for internal transitions also lacks a maximal value, and its variation with respect to kinetic constants k_{23} and k_{34} does not lead to additional increase of overall entropy production. On the contrary, it leads to a decrease

in overall EP. It also leads to better similarity with measured kinetic constants. The applications of MaxEP to internal transitions [4, 12, 13] of cyclic enzyme kinetic schemes indicated that order of magnitude or better similarity with measured kinetic constants can be reached when these transitions are optimized, but optimization scenarios were not explored for different transitions between functional states, and corresponding entropy productions were not compared. In the present work, we used overall entropy production and a less-than-perfect agreement between optimized and experimental kinetic constants as a guide to which transition between functional states is the best candidate for additional optimization. Invoking the maximal entropy principle and MaxEP for external transitions, as in Dewar et al. [14], did not help in our case to find optimal kinetic constants.

These results are the outcome of the initial assumption that none of the equilibrium constants change from their measured values for a reference steady state. Different steady states may be established after a change in steady substrate and/or product concentration, due to a change of upstream or downstream enzymatic activities (with respect to the TIM enzyme) in the glycolytic pathway. For instance, an additional EP increase in all functional transitions due to maximal EP in the product release step could be achieved by decreasing the steady-state GAP concentration (Fig. 4). Increasing enzymatic activity of the next enzyme (glyceraldehyde phosphate dehydrogenase) in the glycolytic pathway could reduce GAP concentrations. However, when product concentration drops to $0.013 \mu\text{M}$, as we assumed in calculations leading to the Fig. 4 results, the product attachment step runs uncomfortably close to the higher end of the diffusion limit restriction ($10^{10} \text{ M}^{-1}\text{s}^{-1}$) [22]. Increased temperature would help to increase the frequency of product release, if enzyme unfolding is prevented, as it is with TIM from thermophile archaeon *Pyrococcus furiosus* [46]. Its specificity constant $k_{\text{cat}}/K_{\text{M}}$ reaches values higher than $10^{10} \text{ M}^{-1}\text{s}^{-1}$ at 90°C . Concomitant entropy production by archaea's TIM enzymes should be much higher than in mesophilic organisms, but it is also due to a more complex oligomeric structure [47]. At lower temperature, it is still possible to remove the diffusion limitation by formation of enzyme aggregates [5]. For the TIM enzyme of eukaryotic cells, evolution resulted in two structural solutions for overcoming the diffusion limit without destabilizing the enzyme. The first device is the well-known dimeric structure for TIM enzymes of all mesophilic species [47] and the second is electrostatic enhancement of association rates due to electrostatic steering [48].

Compared with earlier approaches [2, 3, 22, 49, 50], our method for optimizing enzyme activity and its specificity constant is conceptually much simpler and the only one derived from a thermodynamic principle of a quite general nature. The rate of product synthesis or enzyme activity has been a favorite function for maximization, but it does not have a maximum. Without being constrained, it keeps increasing without limit. A large number of different constraints have been used in the form of inequalities for kinetic constants spanning several orders of magnitude.

The possibility to achieve a significant increase in $k_{\text{cat}}/K_{\text{M}}$ by the MaxEP requirement may be taken as an indication that the TIM enzyme is still not fully evolved, because a reaction rate increase is still possible, at least in theory, as a consequence of entropy production maximization in the product release step. It is not surprising that this suggests that the steady-state rate of catalysis is limited by the rate of loss of product from the enzyme. This has also been found after suitable mutation, in the enzyme glutathione-S-transferase [51], and it is the rate-limiting step in a number of other enzymes [5]. It is also common in biochemistry that the slowest step in the mechanism (the last one in the case of the TIM enzyme) is rate-determining.

The loop-6 opening and product release step represent the concerted fast movement of a large number of atoms over a long distance within a single protein [6, 52]. That conformational change is the physical reason for the product release step being the most important contributor to overall entropy production by the TIM enzyme. Kinetics, thermodynamics, and structural studies provide equally important insights as to what an enzyme's amino acids do when provided with a substrate in solution.

These results could be used to check the usefulness of entropy production maximization for other enzymes with cyclic reaction schemes [53]. Free-energy transduction from driving to driven thermodynamic force is possible by enzymes associated with kinetic schemes having a greater number of functional states connected with more than one cycle [7, 18]. In such cases, the secondary (driven) force can vary together with secondary flux in a driven cycle and the application of maximum entropy production requirement is numerically more complex [13]. As in this work, the goal of using the MaxEP requirement for individual transitions can be to find the rate-limiting step or steps. The goal of approaching or predicting even approximate experimental values for kinetic constants may lead to restrictions or decrease in overall EP after taking into account all known constraints.

Finding the maximal possible amount of useless energy (dissipation) may appear to be unrelated to biological evolution, but thermodynamic and biological evolution must be somehow connected, and we have proposed 10 years ago [54] that the major effect of biological evolution is an acceleration of thermodynamic evolution. A very good example is the enolization rate of DHAP to GAP, which in the presence of TIM enzyme increases by a factor of more than 10^9 over that of the uncatalyzed reaction [55] with concomitant entropy production increase by the same factor. Chemical and biological evolution of macromolecular structures facilitated their integration with thermodynamic evolution in a way which can be best described as a synergistic relationship or positive feedback. While this proposal arose after the study of far-from-equilibrium bioenergetics (photosynthesis), there is nothing in the physical foundation of the MaxEP principle that would prevent its application to enzymes characterized with a near-equilibrium equilibrium constant, such as the TIM enzyme.

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