

# Resource uptake and the evolution of moderately efficient enzymes

Florian Labourel<sup>\*,1</sup> and Etienne Rajon<sup>1</sup>

<sup>1</sup>Univ Lyon, Université Lyon 1, CNRS, Laboratoire de Biométrie et Biologie Evolutive UMR5558,  
F-69622 Villeurbanne, France

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**Abstract** — Enzymes speed up reactions that would otherwise be too slow to sustain the metabolism of self-replicators. Yet, most enzymes seem only moderately efficient, exhibiting kinetic parameters orders of magnitude lower than their expected physically achievable maxima and spanning over surprisingly large ranges of values. Here, we question how these parameters evolve using a mechanistic model where enzyme efficiency is a key component of individual competition for resources. We show that kinetic parameters are under strong directional selection only up to a point, above which enzymes appear to evolve under near-neutrality, thereby confirming the qualitative observation of other modeling approaches. A majority While the existence of kinetic parameters compiled elsewhere do spread onto this a large fitness plateau . Nonetheless could potentially explain the extensive variation in enzyme features reported, we show using a population genetics model that includes genetic drift and mutational biases, we show that this is such a very widespread distribution is an unlikely outcome of evolution on a common landscape, as mutation-selection-drift balance occupy a narrow area even when very moderate biases towards lower efficiency should prevent the occurrence of such a diversity are considered. Instead, differences between species, and within a species between metabolic pathways and in the reactions to perform, evolutionary context encountered by each enzyme should be involved, such that each evolves on an individual, unique landscape. Our results point to drift and effective population size playing an important role, along with the kinetics of nutrient transporters, the tolerance to high concentrations of intermediate metabolites, and the reversibility of reactions. Enzyme concentration also shapes selection on kinetic parameters, suggesting but we show that the joint evolution of concentration and efficiency , facilitated by the plateau, should matter. Interestingly, the position of an enzyme along the metabolic pathway is does not key for its evolution, contrasting with the prediction of models assuming that fitness depends on a precise level of flux control, rather than on competitive abilities yield extensive variance in evolutionary outcomes when documented costs to protein expression are applied.

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<sup>\*</sup>Corresponding author: [florian.labourel@univ-lyon1.fr](mailto:florian.labourel@univ-lyon1.fr)

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## 1 Introduction

Living organisms need to uptake nutrients and metabolize them and metabolize nutrients, relying on enzymes to catalyze catalyse chemical reactions along metabolic pathways. Accordingly, and despite being intrinsically reversible (Haldane, 1930; Klipp and Heinrich, 1994), *in vivo* enzyme-catalyzed reactions are commonly thought of as an irreversible two-step process (Bar-Even *et al.*, 2011, 2015; Michaelis and Menten, 1913; Johnson and Goody, 2011):



where  $k_f$  and  $k_r$  are the rates of association and dissociation between enzyme and substrate, and  $k_{cat}$  is the turnover number, that is the rate of formation of the product  $P$  from  $ES$  complexes. The first part of this chemical equation describes the encounters between the enzyme  $E$  and the substrate  $S$ ; the enzyme will be efficient if  $ES$  complexes form often and do not dissociate before the substrate has been turned into a product, which is reflected by the constant  $K_M = \frac{k_r + k_{cat}}{k_f}$ . The efficiency  $v$  of an enzyme – the rate at which it makes a product  $P$  from  $S$  – depends on these two constants through equation (2):

$$v = k_{cat} \cdot [E_{tot}] \cdot \frac{[S]}{K_M + [S]}, \quad (2)$$

under the assumption that the concentration  $[S]$  is approximately constant and that of  $[ES]$  is at equilibrium steady-state (Michaelis and Menten, 1913; Briggs and Haldane, 1925).

At first glance, Natural selection Selection is presumed to optimize enzymatic efficiency by pushing  $k_{cat}$  upwards and  $K_M$  downwards to universal physical limits. Enzyme efficiencies are for instance limited by the diffusion properties of their aqueous environment, which sets an upper bound of approximately  $10^8 - 10^{10} M^{-1} s^{-1}$  for the ratio  $k_{cat}/K_M$  (Alberty and Hammes, 1958; Zhou and Zhong, 1982).

$k_{cat}/K_M$  (Alberty and Hammes, 1958; Zhou and Zhong, 1982). Nearly optimal enzymes indeed seem to exist, as exemplified by triosephosphate isomerase (TIM) whose ratio is close to this theoretical ceiling (Knowles and Alberly, 1977). But they are uncommon: most enzymes appear to be only moderately efficient and far off these physical limits – including enzymes immediately flanking TIM in the glycolysis metabolic pathway (Davidi *et al.*, 2018). Indeed, Bar-Even *et al.* (2011) have analysed a dataset of several hundreds of enzymes and found a wide diversity among enzyme parameters, thus sketching a puzzling pattern that has far more in common with a zoo than it looks like variations around an archetypal form.

To date, three sources of explanations have been invoked to explain both this variance in parameter estimates and the fact that many look suboptimal: (1) *in vitro* estimates do not reflect the *in vivo* functioning of enzymes, (2) physical constraints vary between enzymes such that their achievable optima differ, and (3) Evolution fails to produce optimum properties.

Surely, This wide distribution of enzyme features could partly be explained by differences between enzyme behavior *in vivo* and *in vitro*. Such differences are expected, first because diffusion in a test tube is hardly comparable to diffusion in the cytoplasm (Ellis, 2001; Rivas *et al.*, 2004; Zhou *et al.*, 2008; Rivas and Minton, 2018). As the cytoplasm gets packed, the cell approaches a state where molecules are less mobile, hindering substrate-enzyme encounters (Muramatsu and Minton, 1988; Zimmerman and Minton, 1993; Blanco *et al.*, 2018). In this regard,  $K_M$  values are thus likely underestimated *in vitro*, and enzymes should perform less efficiently *in vivo*. Simultaneously, macromolecular crowding can sometimes improve catalytic activity *in vivo*, making specificity constants  $k_{cat}/K_M$  higher than their *in vitro* estimates (Ralston, 1990; Ellis, 2001; Jiang and Guo, 2007; Pozdnyakova and Wittung-Stafshede, 2010). These Crowding effects are obviously important for our understanding of enzyme evolution but do not alone provide a reasonable explanation to the reported patterns insofar as some of them should be partially correlated between enzymes (Tabaka *et al.*, 2014), and , alone, they are definitely too weak to explain the wide variability across enzymes insofar as their reported estimates are rather weak Davidi *et al.* (2016). typically lie in the range of one order of magnitude (Davidi *et al.*, 2016).

Another source of explanation to the observed distribution of enzymes (in)efficiencies is a failure of Evolution to consistently optimize them, possibly due to physical constraints. Indeed, Heckmann *et al.* (2018) have shown how a variety of  $k_{cat}$ s may evolve provided that some of them are physically constrained. Besides the diffusion limit already mentioned, constraints on enzyme evolution also might include an intrinsic trade-off (Gudelj *et al.*, 2010; Stiffler *et al.*, 2015) that originates from the dependency of both  $k_{cat}$  and  $K_M$  on intermediate energy profiles (Heinrich *et al.*, 1991). Nonetheless, this trade-off is scarcely observable among evolved enzymes – Bar-Even *et al.* (2011) report a coefficient of determination around 0.09 for the correlation between  $\log_{10}(k_{cat})$  and  $\log_{10}(K_M)$  – suggesting that it can be overcome. Other constraints may exist and be specific of a given reaction (Klipp and Heinrich, 1994) – *e.g.* reaction reversibility – potentially explaining a part of the variance in enzyme efficiencies. Estimating It remains that estimating constraints on all individual enzymes appears like a daunting task, which could be guided, in part, by the identification of deviations from evolutionary predictions.

Enzyme Following this idea, the premise of our theoretical investigation into the origins of enzyme diversity is that it results mainly from unconstrained evolution, such that the reported differences may be caused by the combined action of selection and genetic drift. It is important to notice that the information we have is

partial, as an enzyme's activity is the joint result of its kinetic constants and cellular concentration, perhaps also contributing to the reported variance in the former. In fact, Davidi *et al.* (2016)'s method to determine *in vivo* turn-over rates lends some credence to the idea that increased levels of expression make up for lower kinetic constants (Davidi *et al.*, 2018). It is therefore obvious that an enzyme's expression needs to be considered as another dimension of its activity, especially since it has been shown that the evolutionary tuning of gene expression can happen very quickly (Dekel and Alon, 2005).

Concomitantly, an enzyme's activity can be impacted by protein misfolding, which reduces the effective enzyme concentration (Tokuriki and Tawfik, 2009; Yue *et al.*, 2005; Drummond *et al.*, 2005; Echave and Wilke, 2017) while also impacting fitness by enhancing protein erroneous interactions (Yang *et al.*, 2012) and the formation of toxic protein aggregates (Bucciantini *et al.*, 2002; Sabate *et al.*, 2010; Geiler-Samerotte *et al.*, 2011). Protein stability is thus under strong purifying selection to avoid the deleterious effects of misfolding (Drummond and Wilke, 2008). Accordingly, it has been shown that proteins have evolved to stand beyond a stability threshold (Bloom *et al.*, 2005), although marginally (Taverna and Goldstein, 2002).

Because mutations are on average destabilizing, this definitely narrows down the spectrum of adaptive mutations (Shoichet *et al.*, 1995; DePristo *et al.*, 2005; Weinreich *et al.*, 2006; Tokuriki *et al.*, 2007, 2008; Lunzer *et al.*, 2010). Nevertheless, several studies have reported the existence of a genotype space where activity can be optimized without compromising stability (Schreiber *et al.*, 1994; van den Burg and Eijssink, 2002; Bloom *et al.*, 2004; Knies *et al.*, 2017; Miller, 2017). Even when improving function requires the fixation of destabilizing mutations, compensatory mutations can in principle cancel out stability losses arising from active site evolution (DePristo *et al.*, 2005; Tokuriki *et al.*, 2008; Tokuriki and Tawfik, 2009; Storz, 2018). Adaptive evolution may even be facilitated by preexisting mutational robustness against misfolding (Bloom *et al.*, 2006, 2007). Therefore, although the requirement of a stable, correctly folding protein may sometimes slow down the evolutionary process, it is rather unlikely that stability explains the distribution of enzyme kinetic parameters albeit marginally.

Enzyme kinetics evolution has often been considered theoretically through the lens of flux control (Burns *et al.*, 1985; Fell, 1992; Kacser *et al.*, 1995)(Burns *et al.*, 1985; Clark, 1991; Fell, 1992; Kacser *et al.*, 1995; Yi and Dean, 2019). Indeed, (Kacser and Burns, 1973) along with (Heinrich and Rapoport, 1974) brought to light that the control of the flux in a metabolic pathway is shared between all enzymes, in what is known as the summation theorem (Kacser and Burns, 1973; Heinrich and Rapoport, 1974). Thence, because the sum of control coefficients must equal 1 within a pathway, if all enzymes have similar kinetic parameters, none of them exerts a strong influence (Dean, 1995). But if one enzyme departs from this trend and becomes inefficient, it exerts a strong control at the expense of others (Dykhuisen and Dean, 1990). This leads to diminishing-returns epistasis in which the fitness landscape flattens because, as an enzyme becomes more efficient, subsequent mutations have smaller effects (Kacser and Burns, 1973; Dykhuisen *et al.*, 1987; Tokuriki *et al.*, 2012), a

finding that **have has** since received empirical confirmation (Fell, 1992; Lunzer *et al.*, 2005)(Fell, 1992; Dean, 1995; Lunzer *et al.*, 2005; Yi and Dean, 2019; Chou *et al.*, 2014).

(Hartl *et al.*, 1985) and (Dean *et al.*, 1986) have considered such a fitness landscape under a population genetics framework to conclude that enzymes may **be evolving quickly reach a fitness plateau and evolve** on nearly neutral landscapes (Ohta, 1992). More recently, based on similar evolutionary models and a fitness function coming from flux balance analysis, Heckmann *et al.* (2018) have furthered the idea, compellingly showing how diminishing returns epistasis could generate the wide expected variety of  $k_{cat}$ s at the level of an organism. Nonetheless, these studies fall short of explaining why inefficient enzymes having stronger control do not evolve higher activities (Yi and Dean, 2019). In these models as in most, an enzyme’s efficiency is captured by its activity, generally represented by a composite of  $k_{cat}$ ,  $K_M$   $K_M$  and enzyme concentration (Hartl *et al.*, 1985; Kaltenbach and Tokuriki, 2014)(Hartl *et al.*, 1985; Clark, 1991; Chou *et al.*, 2014; Kaltenbach and Tokuriki, 2014), such that the distinct evolutionary dynamics of these parameters, together with an enzyme’s concentration, is ignored. This reduction of an enzyme’s dimensionality goes against the empirical observation that each dimension may have a differential impact on fitness in the context of antibiotic resistance (Walkiewicz *et al.*, 2012; Stiffler *et al.*, 2015; Rodrigues *et al.*, 2016) and that each is thus necessary to predict evolutionary outcomes (Walkiewicz *et al.*, 2012).

Perhaps more importantly, (Heinrich *et al.*, 1991) and (Schuster *et al.*, 2008) have pointed out that these **modelling** frameworks assume a constant value for either or both concentrations of the first substrate and of the final product (Orth *et al.*, 2010), whereas Evolution should instead maximize the amount of final products generated. (Klipp and Heinrich, 1994) found that the aforementioned concentrations indeed have a major influence on optimal rate constants under certain assumptions. Likewise, nutrient uptake is most often not considered explicitly in existing models of enzyme evolution, while it is obviously critical in the competition for resources (Dykhuizen and Dean, 1994).

Nutrient uptake occurs when metabolites move inwards across cell membranes; it may rely on membrane permeability only (passive diffusion) or involve channels and carrier proteins, be they transporters or cotransporters (Stein, 1986a). Here we build a model that explicitly includes passive (PD hereafter) or facilitated diffusion (FD) followed by an unbranched metabolic pathway to study how resource availability coupled to transport modulates the evolution of enzymes along the pathway. In ecological scenarios where individuals compete for resources, Natural Selection should favour genotypes that maximize the net intake of molecules and their transformation, which are linked under both PD and FD.

Based on this premise, we **show confirm** that the evolution of **the first enzyme’s enzyme** kinetic parameters  $k_f$  and  $k_{cat}$  takes place on cliff-like fitness landscapes where a fitness plateau covers a wide part of the relevant parameter space. Kinetic parameters have co-dependent but distinct evolutionary dynamics – and thus distinct sensitivities to certain parameters of the model – such that the shape of the plateau can be

modulated by changing parameters of the model within realistic ranges.

We further show that most empirical estimates for kinetic parameters compiled by Bar-Even *et al.* (2011) are spread on the fitness plateau of a generic fitness landscape. But we also We show that this fitness landscape depends on features of transporters that initiate a metabolic pathway, along with parameters that vary among enzymes within a pathway, like the tolerance to high concentrations of intermediate metabolites or the reversibility of reactions.

We further demonstrate, using a simple population genetics model, that this is a highly unexpected evolutionary outcome when we introduce biases – even very mild – for the mutations of kinetic parameters the evolutionarily expected features of an enzyme should be predictable, even though enzymes evolve near-neutrally on the fitness plateau. This is because the model includes slightly biased mutations that tend to produce a majority of less efficient enzymes. We thus explore the effect of several parameters that may make the fitness landscape of each enzyme unique, thus explaining, at least in part, their exceptional diversity. postulate that the wide variety of enzyme features reported might be explained in a large part by differences in the shape of their fitness landscapes. While testing this hypothesis will require extensive information about individual enzymes, we made a small step in this direction, showing that enzymes involved in metabolic pathways with different types of transporters exhibit differences that our model qualitatively predicts.

## 2 Results

### 2.1 Passive diffusion is generally inadequate to sustain cell metabolism

In the version of our model in which intake relies on passive diffusion (PD), the net uptake of a nutrient is a direct outcome of its concentration gradient, and therefore of how efficiently the first enzyme catalyses its transformation inside the cell. Assuming that fitness is proportional to the flux of product of this reaction, we find that the fitness landscape has a cliff-like shape with fitness increasing steeply as parameters  $k_{\text{cat}}$  and  $k_f$  increase (see Supplementary materials, section Text S1). The precise shape will not be commented in detail here, for it is very similar to landscapes obtained under facilitated diffusion (FD, treated in the rest of this manuscript).

Importantly, our results indicate that PD can only sustain a small part of the metabolism of most living cells given cell permeabilities reported in the literature (Wood *et al.*, 1968; Milo *et al.*, 2010), suggesting that this process may not be a determining factor in the evolution of enzymes along metabolic pathways. Indeed, even extremely efficient enzymes, harbouring values of  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_M$  close to their physical limits, yield low inward fluxes that approach  $10^{-2} \text{ mM.s}^{-1}$  when considering a spherical cell with a diameter  $D = 1\mu\text{m}$ . To get a sense of how low these fluxes are, we calculated the maximum cell size that they can theoretically sustain. Considering that basal metabolic demands are approximately proportional to the cell volume and using estimates by Lynch and Marinov (2015) for this relationship, we predicted the

maximum size enabled by sugar passive diffusion (see 4Materials and Methods). Setting a (conservatively high) medium concentration in glucose  $[G] = 1\text{M}$  yields a theoretical volume ceiling  $V_{est} = 0.84\mu\text{m}^3$ .

Nearly all eukaryotes, and most prokaryotes are *de facto* larger than this threshold (Heim *et al.*, 2017), which might help explain the apparent ubiquity of FD. While this demonstration hinges on numbers for sugar uptake, which may arguably be the task requiring the highest flux, PD may be limiting for many other metabolites (Boer *et al.*, 2010), depending on their permeability and availability in the environment: even for very high amino-acids concentrations that may only be met in multicellular organisms (Schmidt *et al.*, 2016) and assuming the highest observed permeability for such metabolites (Chakrabarti, 1994), these levels are orders of magnitude lower than with FD (see Supplementary material - section Text S1 for PD results).

Our calculations therefore suggest that primordial cells, preceding the evolution of FD, must have been extremely small. Current-sized cells, however, must predominantly rely on FD when concentration gradients can be maintained, and on active transport otherwise.

## 2.2 General shape of the fitness landscape under facilitated diffusion

For most metabolites, FD relies on the specific binding of the substrate to transmembrane carrier proteins (transporters hereafter), followed by its translocation to the other side of the membrane (Danielli, 1954; Kotyk, 1967; Stein, 1986b). Our model builds on ter Kuile and Cook (1994)’s approach to model FD, considering the simplifying assumption of symmetric transport. Within this framework, FD operates on the concentration gradient (Bosdriesz *et al.*, 2018) and is susceptible to saturation, represented by constant  $K_T$  – similar to  $K_M$  in the Michaelis-Menten equation – and an interaction constant  $\alpha$  (see Methods for details). We assessed how this saturation phenomenon influences the selection pressure acting on forward enzyme kinetic parameters ( $k_f$  and  $k_{cat}$ ) under various scenarios.

In order to depict a fitness landscape representative of an average enzyme, we first consider a situation where transporters saturate at an intermediate external concentration of the substrate with an intermediate affinity induce a moderately low rate  $V_{Tm}$  and saturate with a relatively high affinity  $K_T$  (FIG. 1, notice that affinity increases when  $K_T$  decreases). In this situation, the inward equilibrium flux flux at steady-state (which, as argued in the introduction, can be considered representative of fitness) forms a plateau when the upstream enzyme in the metabolic pathway has high  $k_{cat}$  and  $k_f$ . This low equilibrium flux elasticity coincides with the saturation theory (Wright, 1934; Kacser and Burns, 1973)(Wright, 1934; Kacser and Burns, 1973; Hartl *et al.*, 1985; Dykhuizen *et al.*, 1987; Dean, 1995; Yi and Dean, 2019), especially with its version incorporating facilitated diffusion (ter Kuile and Cook, 1994)(ter Kuile and Cook, 1994; Dean, 1995). The flux plateau is delineated by parallel isoclines (solid and interrupted lines in FIG. 1) oriented in the bottom-right direction of the landscape for intermediate values of  $k_{cat}$  and  $k_f$ , such that decreasing  $k_f$  by one order of magnitude can be compensated



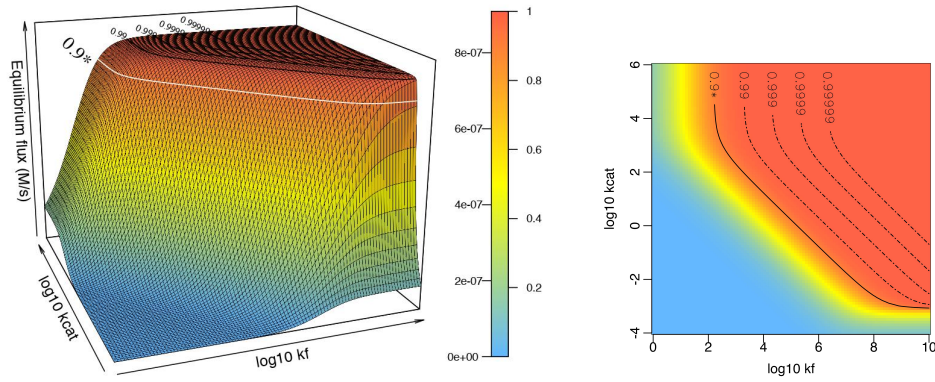


Figure 1: The flux of product following substrate uptake by transporters and conversion by a dedicated enzyme depends on the kinetic parameters  $k_f$  and  $k_{cat}$  of the enzyme. This landscape is generic in the sense that we avoid focusing based on sugars – arguably extreme cases in terms of flux intensity – for a general description, considering a moderate moderately low flux at saturation  $V_{Tm} = 1\mu M.s^{-1}$  close to those measured for amino acids and nucleosides in *E.coli* (Zampieri *et al.*, 2019). We also set the transport saturation ratio  $[S_{out}]/K_T$  to 10 such that the FD process approaches saturation, and relatively high transporter affinity  $K_T = 50\mu M$ , also in line with estimates for nucleosides (Griffith and Jarvis, 1996; Xie *et al.*, 2004) (see Material and Methods). Other parameter values include  $k_r = 10^3 s^{-1}$  and  $[E_{tot}] = 1mM$ . The color gradient shows indicates the absolute and normalized (such that the with a maximum flux equals of 1) values of equilibrium flux.

by a similar increase in  $k_{cat}$ . While this mutual dependency holds even for high  $k_f$  values as long as  $k_{cat}$  is not critically low (*i.e.* when  $k_{cat} > 10^{-3}$ ), it stops when  $k_{cat} \geq 10^3$ , where increasing  $k_{cat}$  no longer improves fitness. Besides, the influence of  $k_{cat}$  and  $k_f$  is not strictly equivalent, since the increase in flux is more gradual in response to  $k_f$ .

Furthermore, and contrary to the textbook picture whereby most biological reactions are not limited by diffusion at all (Bar-Even *et al.*, 2011; Sweetlove and Fernie, 2018), increasing an enzyme’s association rate  $k_f$  – be it through its diffusivity or its binding rate – may still enhance the equilibrium flux when diffusion is substantially faster than catalysis.

### 2.3 Properties of facilitated diffusion modulate the landscape

To explore the effect of FD kinetics on the evolution of enzymes in the metabolic pathway, we studied the influence of  $K_T$  – the affinity of the transporter for the substrate – and  $V_{Tm}$  – the maximum transport rate – still assuming that the substrate is close to saturation ( $[S_{out}]/K_T = 10$ ). We considered ranges of empirical estimates for sugars (high flux with low to moderate affinity in FIG. ??, (Stein, 1986b; Maier *et al.*, 2002)), nucleosides (Griffith and Jarvis, 1996) and amino acids (Stein, 1986b; Zampieri *et al.*, 2019) (weak to moderate flux with moderate to high affinity in FIG. ??).

Both the affinity and rate of a transporter have an impact on the (normalized) flux landscape for upstream enzymes, the black isocline (corresponding to 0.9) delineates the fitness plateau. Each plot represents the landscape obtained with a pair of values for transporters affinity  $K_T$  and saturation  $V_{Tm}$ . Moving one step to the right means that  $V_{Tm}$  increases by 1.5 orders of magnitude – from  $10^{-6}$  (low flux) to  $10^{-3} M.s^{-1}$  (high) – and one step up means that  $K_T$  decreases by 2 orders of magnitude, starting at  $10^{-1} M$  (low affinity). Increasing  $K_T$  extends the plateau only towards the left part of the landscape, allowing enzymes with lower  $k_f$  on the plateau, whereas



decreasing  $V_{Tm}$  extends the plateau in both directions. Other parameter values:  $k_r = 1000/s$ ,  $[E_{tot}] = 1mM$  and  $[S_{env}] = 10 \times K_T$ .

We find that increasing the transport flux  $V_{Tm}$  exerts a positive selection pressure on kinetic parameters for the upstream enzyme (*i.e.* for increasing  $k_{cat}$  and  $k_f$ ). The plateau is shifted accordingly (see FIG. 2-A), towards the top-right corner of the landscape, at a distance that corresponds to the magnitude of the change in  $V_{Tm}$ . Increasing the affinity of the transporter (*i.e.* decreasing  $K_T$ ), however, selects for higher  $k_f$  (the isoclines are displaced to the right and the fold change is similar to that of  $K_T$ ) but has no other visible influence on  $k_{cat}$  than increasing its codependency with  $k_f$ , a result that holds regardless of the flux at saturation  $V_{Tm}$ . (notice that we only considered high  $V_{Tm}$ s, larger than in the average case, because these cases are more likely to be under directional selection).

This specific effect on the affinity of the upstream enzyme is likely due to a competition between the transporter – which can transport the substrate in both directions – and the enzyme, which harvests the substrate at a rate that depends on the dissociation constant  $K_D = k_r/k_f$ . It should be noted that nutrients under lower demands – *e.g.* amino acids – are generally less concentrated in the environment, often coinciding with a higher affinity of their transporter. Therefore, the possible combinations of flux and affinity likely occupy a restricted space of possibilities where flux and affinity are negatively linked (Gudelj *et al.*, 2010; Bosdriesz *et al.*, 2018), which as can be seen in FIG. ??-ASM Figs. S3-A,E,I results in landscapes that mainly differ by the minimum value of  $k_{cat}$  on the plateau. In FIG. 2-A, we have considered ranges of empirical estimates for sugars (high flux with low to moderate affinity) (Stein, 1986b; Maier *et al.*, 2002), nucleosides (Griffith and Jarvis, 1996) and amino acids (Stein, 1986b; Zampieri *et al.*, 2019) (weak to moderate flux with moderate to high affinity), which indeed mainly correspond to these combinations.

So far we have considered transporters saturated by high external substrate concentrations. Relaxing this assumption has little impact on the fitness landscape, except that very low values of  $k_{cat}$  (*i.e.* lower than  $10^{-2}$  in FIG. 2-B) can only sustain high fluxes at saturation (see the low influx of transporters far from saturation, but fail to keep up with higher influxes in richer environments).

## 2.4 Enzymes differ among metabolic pathways

We then superimposed empirical estimates of kinetic parameters over our theoretical fitness landscapes, after substituting parameter  $k_f$  for its usual empirical counterpart,  $k_{cat}/K_M$ . Because  $k_{cat}/K_M = k_f k_{cat}/(k_r + k_{cat})$ , this approximation only holds when  $k_{cat} \gg k_r$ . Representing the fitness landscape in this parameter space sets an inaccessible area in the bottomright part of the landscapes where  $k_f$  would exceed the diffusion limit (grey area on FIG. 2). Diminishing returns shall thus be the rule for the first enzyme whatever the extracellular environment, 3). For purposes of inclusiveness, we used  $k_r = 10^2 s^{-1}$  by default – noting that this limit would be displaced upwards for larger  $k_r$  (and downwards otherwise).

We otherwise used sets of parameters that correspond to typical features of sugar and amino acid/nucleoside

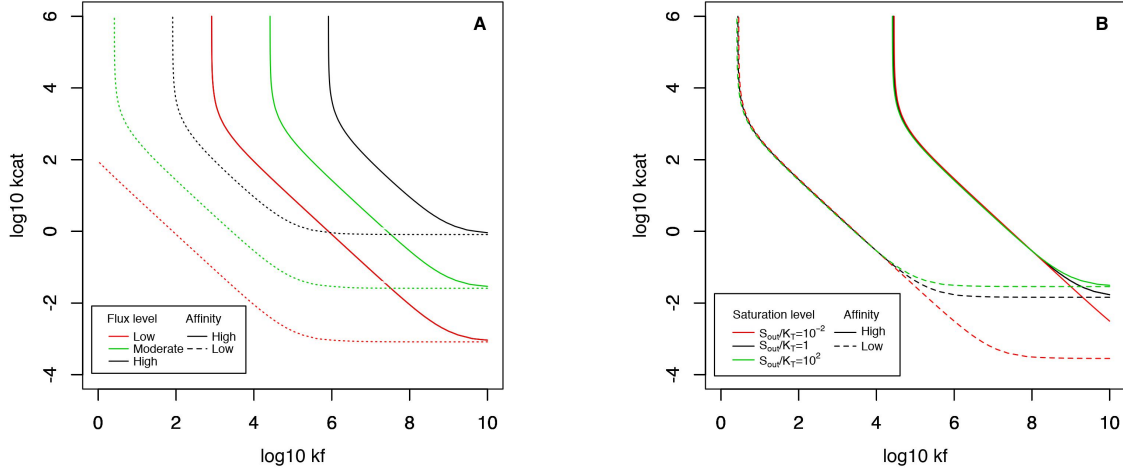


Figure 2: The shape Features of a transporter have an impact on the fitness plateau – represented flux landscape for each condition through upstream enzymes, as shown by the 0.9 isocline (see FIG. 1 for details) isoclines – above which the relative flux is little dependent on  $> 90\%$  – that delineate the saturation fitness plateau for each set of the parameter. A: low ( $K_T = 0.1M$ ) and high ( $10\mu M$ ) transporter affinities are considered, in combination with low ( $V_{Tm} = 10^{-6}M$ ), moderate ( $10^{-4.5}M$ ) or high maximum flux ( $10^{-3}M$ ). Only Increasing  $K_T$  extends the plateau only towards the left part of the landscape, allowing enzymes downstream low affinity transporters show slightly relaxed selection with lower  $k_f$  on  $k_{cat}$  when the external concentration plateau, whereas decreasing  $V_{Tm}$  extends the plateau in both directions. B: the shape of the substrate fitness plateau is lower than however little dependent on the affinity constant saturation of the transporter,  $K_T$ . The example shown is for a transporter with moderate flux ( $V_{Tm} = 10^{-4.5}M.s^{-1}$ ); the effect is identical for other higher  $V_{Tm}$  (, see Figure S2 of SM Fig. S2). Other parameter values:  $k_r = 1000/s$ ,  $[E_{tot}] = 1mM$  and  $[S_{env}] = 10 \times K_T$ .

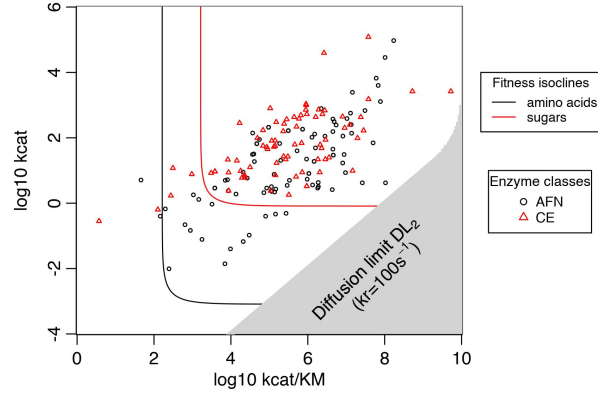


Figure 3: *in vitro* experimental estimates of kinetic parameters  $k_{cat}$  and  $k_{cat}/K_M$  exhibit different distributions for enzymes involved in different categories of pathways – as identified by Bar-Even *et al.* (2011) – namely (AFN): amino acids, fatty acids, and nucleotides and (CE): carbohydrates and energy. Corresponding fitness landscapes – differing by transporter features – are superimposed, with the parameter space narrowed down due to the diffusion limit (grey area, set for  $k_r = 10^2s^{-1}$ ). The isoclines shown correspond to parameter values typical of sugar transporters ( $K_T = 5mM, V_{Tm} = 1mM.s^{-1}$ , in red) (Maier *et al.*, 2002) or amino acids transporters (same as in FIG. 1, in black).

transporters to obtain FIG. 3. Because we have previously shown that changing the affinity or maximum flux of transporters may move the fitness plateau, our model predicts that enzymes involved in the corresponding pathways (e.g. of sugars and amino acids) should have their own specific distributions. We see that enzymes

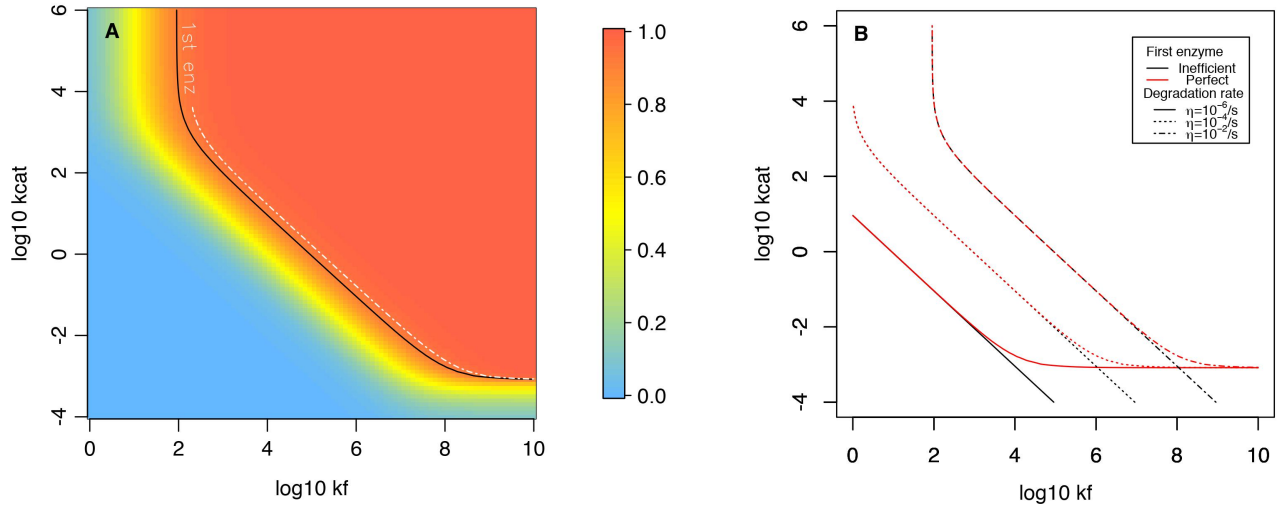


Figure 4: Downstream enzymes exhibit similar fitness landscapes as those upstream, with a dependency to degradation parameter  $\eta_d$ . These enzymes are involved in a moderate flux pathway whose pace is driven by the same uptake kinetics as in FIG. 1 where  $V_{Tm} = 1\mu M/s$ ,  $K_T = 50\mu M$  and  $[S_{out}] = 10K_T$ . Settings for the second enzyme also correspond to a model case with  $k_r = 1000s^{-1}$  and  $[E_{tot}] = 1mM$ . (A) corresponds to : a high degradation rate  $\eta_d = 10^{-1}s^{-1}$  that limits metabolite intracellular concentration to  $[S]_{in} = 10\mu M$  (for the selected  $V_{Tm}$  value  $\eta_d = 10^{-2}/s$ ), with the upstream enzyme being perfect and concentrated ( $k_f = 10^{10}M^{-1}.s^{-1}$ ,  $k_{cat} = 10^6s^{-1}$ ,  $k_r = 10^3s^{-1}$ ,  $[E_{tot}] = 1mM$ ) and results in a fitness landscape plateau for the second enzyme very similar to that of the first enzyme when it faces similar conditions (findings are relatively similar for sugar like transporters, as reported ; in SM - Figure S4), while plateau isoclines drawn the case presented the first enzyme is considered “perfect” in (B) also show results for a second degradation rate limiting this concentration order to  $[S]_{in} = 10mM$ , in draw the context fitness landscape of an upstream concentrated the second enzyme being either perfect (see above  $k_f = 10^{10}M^{-1}.s^{-1}$ ,  $k_{cat} = 10^6s^{-1}$ ,  $k_r = 10^3s^{-1}$ ,  $[E_{tot}] = 1mM$ ). B: decreasing the degradation rate allows less efficient enzymes (with lower  $k_{cat}$  or  $k_f$ ) to reach the fitness plateau. Considering the first enzyme to be inefficient ( $k_f = 10^2M^{-1}.s^{-1}$ ,  $k_{cat} = 10^{-2}s^{-1}$ ,  $k_r = 10^3s^{-1}$ ). Decreasing  $\eta_d$  makes instead of perfect marginally changes the cell fitness landscape by making organisms tolerant to higher concentrations of intermediate metabolites extremely low  $k_{cat}$ . Other parameter values are identical to FIG. 1 (the product of the first reaction) findings are relatively similar for sugar-like transporters, hence relieving the selection pressure on the second enzyme and moving the plateau towards the bottomleft corner as reported in SM - Fig. S6).

involved in the central carbohydrate metabolism as categorized by Bar-Even *et al.* (2011) have on average higher  $k_{cat}$  and this phenomenon typically occurs within the known behavioral range for both catalysts and transporters  $K_M$  than those metabolising amino-acids and nucleotides. Our superimposition with the predicted fitness plateaus in FIG. 3 suggests that there may indeed be an explainable difference between enzymes contributing to carbohydrate processing (in red) and to that of other primary metabolites (in black, e.g. amino acids). We acknowledge that this result implicitly suggests that enzymes within a pathway have evolved on a common fitness landscape, spreading neutrally onto the fitness plateau. This is by no means our interpretation, as this subset of the full dataset includes enzymes that differ in many other ways that, as we will see, make each enzyme evolve on its own fitness landscape and thereby potentially explain a large part of this observed variance.

## 2.5 Downstream enzymes also evolve on cliff-like fitness landscapes

In this section, we intend to draw fitness landscapes for enzymes catalysing reactions downstream in One of the factors that makes enzymes different along a pathway is their position, such that the fitness landscape in FIG.1 may only

hold for the most upstream enzyme in a pathway. Indeed, because the metabolic pathway. Because the flux of the first product in a pathway increases with the substrate gradient across the cell membrane, the upstream enzyme of a given metabolic pathway is selected for efficiency within the limits mentioned in the previous two sections as described above. In contrast, this selective selection pressure does not apply directly downstream; at steady-state, even inefficient enzymes can in principle process newly formed substrate molecules at an elevated rate, assuming that the concentration of the substrate is allowed to reach any equilibrium steady-state value. This is an obviously unreasonable assumption, since a part of this standing substrate should be lost by outward diffusion or degradation, or may interfere with other cellular processes due to the surging involvement in non-specific or promiscuous interactions (Khersonsky and Tawfik, 2010; Schäuble *et al.*, 2013). We included a degradation term in the model, proportional to the constant (Jones *et al.*, 2015; Bosdriesz *et al.*, 2018). The loss of fitness may therefore result from the loss of metabolites in a way that can be modelled by a constant degradation rate  $\eta_d$  (Chou *et al.*, 2014) (assuming that the external environment is infinite, the degradation term can as well represent an efflux). Modelling the cost of accumulation this way is likely also consistent with Highly concentrated metabolites may also be involved in widespread non-specific (Keller *et al.*, 2015) or promiscuous interactions (Khersonsky and Tawfik, 2010; Schäuble *et al.*, 2013; Peracchi, 2018) that may interfere with other cellular processes; this is well captured by the linear cost as non-specific interactions that should follow Michaelis-Menten kinetics albeit with much lower affinities and rates than the focal reaction, which means their rates should follow a, hence following an approximately linear relationship up to very high cellular concentrations. (see Materials and Methods for more details). However for some reactions the accumulation of metabolites may result in the production of toxic compounds (Lilja and Johnson, 2017; Niehaus and Hillmann, 2020), hence triggering toxicity best modelled as a non-linear fitness cost (Clark, 1991; Wright and Rausher, 2010).

We first consider a “perfect”, highly concentrated upstream enzyme ( $k_f = 10^{10} M^{-1} s^{-1}$ ,  $k_{cat} = 10^6 s^{-1}$ ,  $k_r = 10^3 s^{-1}$ ,  $[E_{tot}] = 10^{-3} M$ ) and study focus on the second enzyme in the pathway, showing that it evolves on a fitness landscape that has a similar shape than described above, still hitting a plateau (FIG. 4, with the same parameterization as FIG. 1). The degradation rate creates a ceiling for the concentration of the product of the first reaction, such that reducing  $\eta_d$  allows for higher concentrations (see SM - Figure S3 Fig. S4) and makes the flux tolerant to second enzymes with lower  $k_f$ s, whereas selection on  $k_{cat}$  is barely impacted by this parameter. The plateau is therefore extended to the left when high product concentrations are enabled at low  $\eta_d$  (see FIG. 4-B). The shape of the plateau is little impacted by changes in the efficiency of the first enzyme, especially when it stands on the plateau. These conclusions are independent of characteristics of results are almost independent of the transporter initiating the pathway, although degradation rates limiting metabolite concentrations to similar levels pushes the plateau to the right slightly more when compared to the landscape of the first enzyme (see SM - Figure S4 Fig. S6 for the case of low affinity- moderate affinity, high flux transporters). We can notice that the second enzyme’s kinetic parameters evolve in response to the net rate  $v$  of the upstream reaction and to the

presence of any process in competition for its product (like efflux or degradation) , such that these results are expected to hold for any enzyme downstream.

## 2.6 Most enzymes lie on the fitness plateau

*in vitro* experimental estimates for enzyme kinetic parameters spread on the plateau of a generic theoretical fitness landscape. The parameter space considered corresponds to the usual empirical estimates for enzyme kinetic parameters  $k_{cat}$  and  $k_{cat}/K_M$ . The space is narrowed down here due to the diffusion limit, outlined by the grey area when  $k_r$  is low ( $10^2 s^{-1}$ ), that impedes high values for  $k_{cat}/K_M$  under low values for the ratio between  $k_{cat}$  and  $k_r$ . In (A), the full dataset from (Bar-Even *et al.*, 2011) is plotted in the parameter space and contrasted with the fitness landscape of the most upstream enzyme involved in amino acid or nucleoside metabolism (same parameters for transport than in Fig. 1,  $[E_{tot}] = 10^{-3} M$  and  $k_r = 10^2 s^{-1}$ ). Most enzymes fall on the plateau of the fitness landscape. A few outliers overcome the diffusion limit, suggesting that  $k_r$  might be lower than  $10^2 s^{-1}$  for these enzymes. In (B), the dataset is divided into classes identified by Bar-Even *et al.* (2011), with the acronym denoting enzymes involved in the central metabolism including (AFN): amino acids, fatty acids, and nucleotides - and (CE): carbohydrates and energy. The isoclines shown correspond to parameter values typical of sugar transporters ( $K_T = 5 mM, V_{Tm} = 1 mM.s^{-1}$ ) (Maier *et al.*, 2002) or amino acids transporters (same as in (A)). With a few exceptions, enzymes seem to spread on the corresponding fitness plateau.

We then superimposed empirical estimates of kinetic parameters over our theoretical fitness landscapes, after substituting parameter  $k_r$  by  $k_{cat}/K_M$ . The shape of the negative relationship between metabolite concentration and fitness can be important (Figs S7-S9 in SM), as it can make the fitness landscape of an enzyme dependent of the overall flux of the metabolic pathway, and therefore on other enzymes in the pathway. Indeed, low general fluxes (as modelled by an inefficient first enzyme in Figs. S7-S8) make the metabolite concentration below its toxicity threshold, therefore making organisms tolerant to enzymes with lower  $k_f$  for its usual empirical counterpart,  $k_{cat}/K_M$ . Because  $k_{cat}/K_M \approx k_f k_{cat}/k_r$ , this approximation only holds when  $k_{cat} \gg k_r$ . No less significantly, representing the fitness landscape in this parameter space sets an area in the bottomright part of the landscapes where  $k_f$  exceeds the diffusion limit (grey area on FIG. 3) . For purposes of inclusiveness, we used  $k_r = 10^2 s^{-1}$  by default – noting that this limit would be displaced upwards for larger  $k_r$  – and otherwise used the same set of parameters as in FIG. 1 to obtain FIG. 3. We see that a vast majority of the enzymes in the Bar-Even *et al.* (2011) dataset belong to the fitness plateau. The implicit suggestion that this could be a result of Evolution on a common fitness landscape is surprising, as the dataset includes enzymes from many Eukaryote and Prokaryote species that contribute to various pathways. We instead anticipate that differences in evolutionary context should explain a part of the observed variance. We also note a strong linear positive relationship between  $k_{cat}$  and  $k_{cat}/K_M$  ( $R^2 = 0.4$ ). This illustrates how focusing on  $k_{cat}/K_M$  can be misleading if one's interest lies in mechanistic processes and their implications. Indeed, as obvious as it may sound,  $k_{cat}$  and  $k_{cat}/K_M$  are not independent from each other, especially when  $k_{cat} < k_r$  (which is the case for a majority of the data points in FIG. 3). Concluding on whether a positive relationship between  $k_{cat}$  and  $k_{cat}/K_M$  exists depends on the exact cost function applied, with a linear cost generating epistasis for  $k_{cat}$  only and a non-linear cost possibly impacting both  $k_f$  and  $k_{cat}$ . Estimating  $k_r$  for each enzyme.  $k_{cat}$ .

## 2.6 The reversibility of reactions also matters

Because we have previously shown that different transporters may yield plateaus with different shapes (*e.g.* for sugars and amino acids), our model predicts that enzymesinvolved in Reversibility is an intrinsic feature of chemical reactions that cannot be directly overcome by Evolution (Haldane, 1930; Cornish-Bowden, 1979). A highly reversible reaction corresponds to a large intrinsic equilibrium constant  $K_{eq} = [S]_{eq}/[P]_{eq}$  (Klipp and Heinrich, 1994), and results in higher backward than forward rates in the following chemical equation:



where  $k_{inh}$  represents the rate at which enzyme and product combine back. Such a (reversible) reaction could in principle influence the selective pressure acting on the following enzyme in the pathway, for both enzymes compete to process the same metabolite  $P_1$ . We thus quantified how reversibility affects the evolution of an enzyme downstream (SM Figs S10 and S11).

The equilibrium constant  $K_{eq}$  has a similar (non-linear) impact on the fitness landscape of the second enzyme to that of the corresponding pathways should have their own specific distributions. Representing findings by (Bar-Even *et al.*, 2011) suggests that there may indeed be an explainable difference between enzymes contributing to carbohydrate processing (in red in FIG. 3-B) and to that of amino acids (a part of the black dots in FIG. 3-B). This apparent discrepancy in fitness landscapes across pathways starting with different transporters can only explain a small part of the wide distribution of kinetic parameters reported by Bar-Even *et al.* (2011), thus requiring a careful study of other evolutionary, ecological and chemical parametersdegradation rate, with a highly reversible upstream enzyme exerting a selection pressure downstream towards an increase of kinetic parameters (SM Fig. S10-A). Indeed, increasing  $K_{eq}$  moves the fitness plateau toward the upper-right corner in the  $(k_f, k_{cat})$  parameter space, hence selecting for more efficient downstream enzymes. The effect appears linear, except for very low values of  $K_{eq}$  where metabolite accumulation exerts a dominant role in shaping the fitness landscape (through the degradation rate  $\eta_d$ , set to a low residual value). Therefore, the reversibility of the upstream reaction appears like a critical parameter for the evolution of an enzyme.

## 2.7 Evolutionary dynamics of enzyme kinetic parameters

How much variance in evolutionary outcomes these differences in fitness landscapes may explain is contingent on the interplay between selection, mutation and drift. Small differences in an isocline position should indeed be of little importance if populations perform random walks on the fitness plateau, for instance. To approach how populations evolve on the fitness landscapesdrawn through our mathematical modelour mathematically derived fitness landscapes, we built a simple population genetics model that includes, besides selection, mutation and drift. In this individual based model, in which absolute fitness is directly proportional to the flux of product at equilibrium arising from the first enzyme at steady-state – which itself equals the net inward flux of nutrients. We consider the

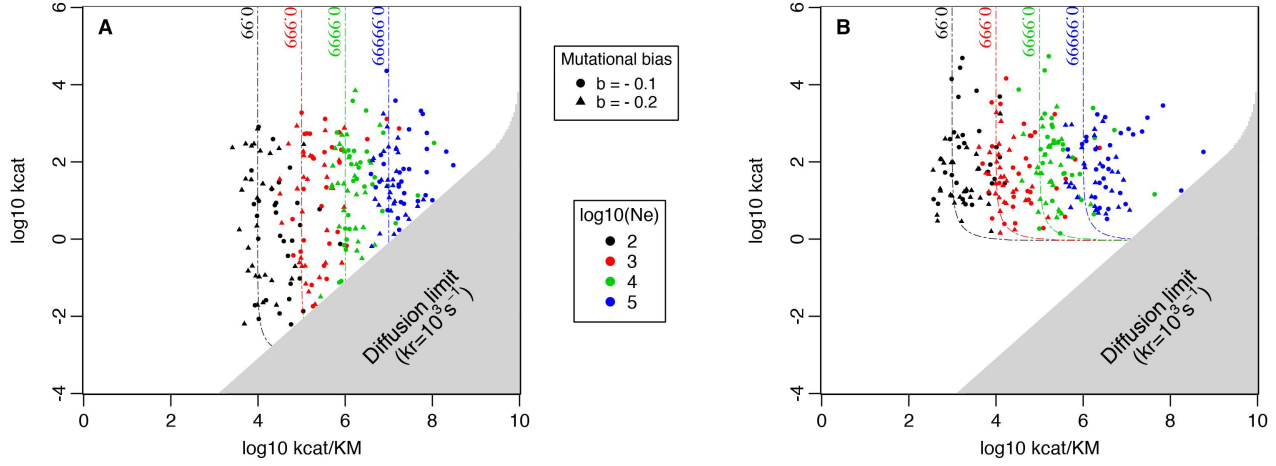


Figure 5: Simulations of a population genetics model indicate Population genetic simulations predict that enzymes should spread on reach a plateau corresponding to their effective population size  $N_e$  only predictable set of features when mutations are unbiased; mutations mutation biases towards lower efficiencies make enzymes stick to iso-clines are considered (see SM fig. S17 for the case of an absence of bias). Indeed, such the mutation selection drift equilibrium establishes close to an isocline indicative of effective selection that their evolution is predictable depends on the effective population size  $N_e$ . The case cases considered here is are that of a transporter with a low flux at saturation and high affinity (A;  $V_{Tm} = 1 \mu M s^{-1}$  and  $K_T = 10 \mu M$ ) – under various scenarios: four and one with a high flux at saturation but low affinity (B;  $V_{Tm} = 1 mM s^{-1}$  and  $K_T = 100 mM$ ) with effective population sizes ranging from  $10^2$  to  $10^5$  (different colors) and three cases two strengths of the mutational biases bias ( $b = 0$  corresponds to the absence of mutational bias was also considered, see SM). We ran Each of 30 independent simulations for each scenario, each is represented a dot in the “empirical” parameter space ( $k_{cat}$ ,  $k_{cat}/K_M$ ). Only, but only  $k_{cat}$  and  $k_f$  were susceptible to evolve, while  $k_r$  was is set to  $10^3 s^{-1}$  such that the grey part of the parameter space is inaccessible to enzymes due to that would otherwise exceed the diffusion limit. At evolutionary equilibrium, enzyme efficiencies reach a plateau delineated by an isocline indicative of effective selection. In (A), no mutational bias results in enzymes spreading onto the plateau, some reaching very high  $k_{cat}$  and/or  $k_{cat}/K_M$  values, while in (B) enzyme efficiencies stick to their predicted isoclines – under the Nearly Neutral Theory of Evolution (Ohta, 1992) – owing to the over-representation of mutations reducing enzyme efficiencies.

flux of the first enzyme (see Methods and previous Results sections for details), as presumably the evolutionary dynamics should be similar for downstream enzymes with similar landscapes. Two different levels of metabolic demands were considered, corresponding to parameter values used to draw of amino acids/nucleosides and sugar transporters (panels (A) and (I) in FIG. ?? in order to control that differences in the delineation of the plateau (in the parameter space) do not affect the evolutionary equilibrium. For the enzyme of a given individual SM Fig. S3). In this instance of the model, only  $k_{cat}$  and  $k_f$  were susceptible to evolve through mutations. Mutational effects on  $\log_{10} k_{cat}$  and  $\log_{10} k_f$  were drawn from independent normal distributions with mean  $b \leq 0$ , with and the absolute value of  $b$  setting the intensity of mutational a mutational bias towards less efficient parameter values, as has been widely documented in many contexts (Eyre-Walker and Keightley, 2007; Serohijos *et al.*, 2012; Heckmann *et al.*, 2018). The standard deviation of the distribution of mutational effects was set to equals 0.3 such that most mutations explore the neighbouring parameter space, with rare mutations allowing changes seldom changing a parameter by more than one order of magnitude (one  $\log_{10}$  unit) at a time, accordingly to values reported in (Carlin *et al.*, 2016). in compliance with empirical estimates (Carlin *et al.*, 2016). Since the relation between kinetic parameters may be constrained



– e.g. due to shared properties of the energy profile of a reaction – we tested the influence of negative and positive relationships using bivariate normal distributions, with three different values of  $\rho$  (see Materials and Methods for details).

Backwards reaction rates of an enzyme directly upstream have a strong impact on the fitness landscape. Both plots show results of the influence of the reversibility of the first reaction on the fitness landscape of the next enzyme. Parameters are identical as in FIG. 1, corresponding to the model case for amino acids, with  $k_r = 10^3 s^{-1}$ .  $K_{eq}$  equals  $[S]_{eq}/[P]_{eq} = k_r k_{inh}/k_{cat} k_f$  (Klipp and Heinrich, 1994) and quantifies the degree of reversibility, a low  $K_{eq}$  featuring low reversibility and vice versa. The first enzyme is a nearly perfect forward enzyme with  $k_f = 10^8 M^{-1}$  and  $k_{cat} = 10^4 s^{-1}$ . Reversibility was equally spread between the two backwards parameters (e.g.  $K_{eq} = 10^2$  yields  $k_r = 10^1 k_{cat}$  and  $k_{inh} = 10^1 k_f$ ), and a low degradation rate was considered ( $\eta_d = 10^{-4} s^{-1}$ ). In (A), results are plotted in the theoretical parameter space ( $k_f$ ,  $k_{cat}$ ), showing that any increase in reversibility increases the pressure on enzyme kinetics by the same magnitude – except when reactions are highly non-reversible (in red). In (B), the same results are shown in the experimenter parameter space of the second enzyme, showing that there is an increased pressure on  $k_{cat}/K_M$  under higher reversibility. While the plateau is not moved upwards, indicative of a selection on  $k_{cat}$  independent on reversibility when  $k_{cat}/K_M$  is fixed, positive selection for this parameter may still arise due to the diffusion limit that precludes the access to the lowest  $k_{cat}$  at high  $k_{cat}/K_M$ . The diffusion limit should play an important role for enzymes with a high dissociation rate  $k_r$ , as illustrated by the delineation of the diffusion limit (grey area or grey dashed lines) corresponding to several  $k_r$  values (eg. DL<sub>3</sub> stands for  $k_r = 10^3 s^{-1}$ ; the star indicates that it is the case represented in (A)).

Without any In the absence of mutational bias ( $b = 0$ ), simulated enzymes spread all over the fitness plateau, as expected (FIG. 5A Fig. S16-A for low flux, Figure S7A of SM Fig. S17A otherwise). The onset of the plateau depends on the strength of drift and hence derive from the effective population size  $N_e$ , following the classical expectation that selection becomes inefficient when  $N_e \times s < 1$  (Kimura, 1968)(Wright, 1931; Kimura, 1968). Introducing a mutational bias that makes enzyme kinetics less efficient on average has a strong effect on both  $k_{cat}$  and  $k_f$ , preventing simulated enzymes from improving far above the drift barrier (FIG. 5B -A for low flux, Figure S7B of SM FIG. 5-B otherwise). Even weak biases ( $b = 0.1$   $b = -0.1$ ) lead to enzymes evolving in the vicinity of the isocline where  $N_e \times s \approx 1$ . Increasing the strength of this bias to 0.2 only slightly decreases the population variance around this expectation. Finally, mutational correlations do not impact much the distribution of evolutionary outcomes (SM Fig. S18).

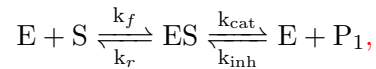
In light of this Our results suggest a strong effect of the effective population size on enzyme evolution, the seeming adequation between the plateau and the data in FIG. 3 appears puzzling, as the lowest such that species with  $N_e$  considered here do not encompass those traditionally found for unicellular organisms that mostly exceed above  $10^5$  (Bobay and Ochman, 2018). In the same vein, (Bobay and Ochman, 2018, most unicellular organisms) should express extremely efficient enzymes. This appears to not be the case, as for instance Eukaryotes and Prokaryotes datasets display similar ranges display similar enzymes despite large differences in effective population sizes (Bar-Even *et al.*, 2011). It is thus possible that the role of genetic drift is not well captured by a model that considers small parts of a large system in isolation, or more generally that

the link between fitness and flux is not as straightforward as we have assumed. As we will later discuss, this conundrum might resolve when considering the smaller size of organisms forming large populations, making them more sensitive to noise in gene expression and favouring higher concentrations. Notwithstanding this issue, the prediction of enzymes evolving a predictable set of kinetics parameters when mutational biases are considered contrasts with the broad variability reported (Davidi *et al.*, 2018), thereupon requiring further investigation.

## 2.8 Explaining the variance within a pathway

So far, our model predicts that enzymes intervening in a given pathway should evolve on a common fitness landscape, provided that the accumulation of intermediate metabolites is highly detrimental. This should produce similar enzymes along a pathway, which is directly contradicted by empirical estimates, suggesting that each enzyme instead evolves on its own fitness landscape. In this section we show that (1) physical constraints, among which in first place is the reversibility of reactions and (2) the joint evolution of kinetic parameters and other evolving parameters, can explain large differences among enzyme kinetic parameters by influencing actual *in vivo* efficiencies.

Reversibility is an intrinsic feature of chemical reactions that cannot be directly overcome by Evolution (Haldane, 1930; Cornish-Bowden, 1979). A highly reversible reaction corresponds to a large intrinsic equilibrium constant  $K_{eq} = [S]_{eq}/[P]_{eq}$  (Klipp and Heinrich, 1994), and results in higher backward than forward rates in the following chemical equation:



where  $k_{inh}$  represents the rate at which enzyme and product combine back. Such a (reversible) reaction could in principle influence the selective pressure acting on the following enzyme in the pathway, for both enzymes compete to process the same metabolite  $P_1$ . We thus quantified how reversibility affects the evolution of an enzyme downstream (FIG. ?? ; using the same model case parameters used throughout the paper (see FIG. 1 for details) and considering a nearly perfect first enzyme for consistency:  $k_{cat} = 10^4 s^{-1}, k_f = 10^8 M^{-1} s^{-1}$ ).

The equilibrium constant  $K_{eq}$  has a similar (non-linear) impact on the fitness landscape of the second enzyme than the degradation rate, with a highly reversible upstream enzyme exerting a selection pressure downstream towards an increase of kinetic parameters (FIG. ??-A). Indeed, increasing  $K_{eq}$  moves kinetic parameters strongly suggests that a large part of the broad variance in enzyme features is due to differences in the selective context experienced by each, thereupon requiring further investigation on the dependency of the position of the fitness plateau toward the upper-right corner in the  $(k_f, k_{cat})$  parameter space, hence selecting for more efficient downstream enzymes. The effect appears linear, except for very low values of  $K_{eq}$  where it fades – such that a 100-fold change in  $K_{eq}$  has little impact – because the main issue when reactions are highly non-reversible becomes, again, metabolite accumulation. Therefore, the reversibility of the upstream reaction appears like a critical parameter for the evolution of an enzyme, able to generate large changes in evolutionarily expected kinetic parameters. When using the “empirical” parameter space (FIG. ??-B), we observe that increasing reversibility only selects for higher  $k_{cat}/K_M$ . Interestingly, the unbinding rate  $k_r$  of an enzyme, which is correlated to its reversibility, may eventually favour a positive codependency between the forward rates  $k_{cat}$

and  $k_f$  as the latter is no longer sufficient to ensure a high  $k_{cat}/K_M$  (see FIG. ??-B and Figure S9 of SM for this influence in the theoretical parameter space). to parameters of our model.

## 2.8 The joint evolution of enzyme concentrations and kinetic parameters

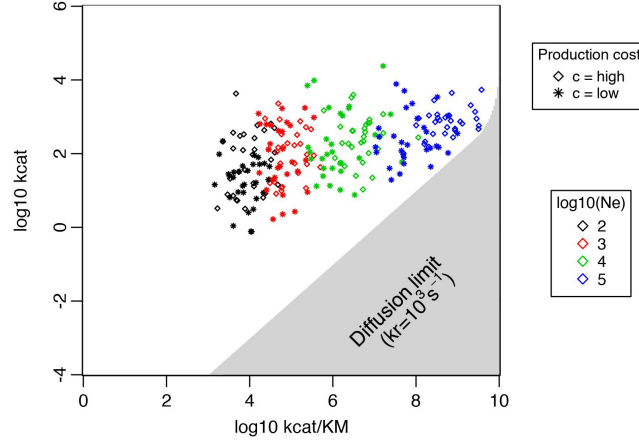


Figure 6: High Simulations of the joint evolution of enzyme concentration  $[E_{tot}]$  alleviate selection on both  $k_{cat}/K_M$  and  $k_{cat}$  kinetic parameters, with a twofold cost of enzyme overexpression (the direct metabolic cost and the indirect cost of cell packing). The parameters for transport case considered here is that of a transporter with a high flux at saturation and low affinity ( $V_{Tm} = 1mMs^{-1}$  and  $K_T = 1mM$ ) under a high mutational bias on kinetic constants ( $b = -0.2$ ). Two different costs of protein production  $c$  are considered along with four effective population sizes ranging from  $10^2$  to  $10^5$ . We ran 30 independent simulations for each scenario, each represented by a dot in the same than “empirical” parameter space as described in FIGfig. 1, with  $k_r = 10^3 s^{-1}5$ .

Hitherto, we have considered enzymes to be highly concentrated, an assumption that we relax here now since it is an important component of the presumed kinetic activity (Koshland, 2002). Predictably, increasing the concentration of the first or second enzyme in a pathway releases the selection on their kinetic parameters (Noor *et al.*, 2016), producing larger fitness plateaus as an enzyme concentration increases (see FIG. ?? for the first enzyme and Figure S10 of SM for the second). Because the concentration is subject to heritable changes (Schaeffe *et al.*, 2013) SM - Figs S12-B and S13-B for this influence in different contexts). Due to the compensatory effects between concentration and activity, we anticipate that the joint evolutionary dynamics of the concentration and kinetic parameters should yield a negative correlation between them, as reported by Davidi *et al.* (2016, 2018)(who focused on  $k_{cat}[E]_{tot}$ ), due to the compensatory effect of concentration.

Despite their common role on reaction efficiency, enzyme concentration expectedly responds to very different selection pressures than kinetic parameters, as increased gene expression levels come with costs (Wagner, 2005; Lang *et al.*, 2009; Scott *et al.*, 2010; Noor *et al.*, 2016; Kafri *et al.*, 2016). Indeed, producing extra proteins requires both energy and matter (Novick and Weiner, 1957; Stoebel *et al.*, 2008; Wagner, 2005; Lynch and Marinov, 2015) and may impede the efficiency of physical processes that rely on an optimal intermediate content (Dong *et al.*, 1995; Dill *et al.*, 2011; Andrews, 2020). We designed a new instance of our population genetics

model to study the tangled evolution of kinetic constants and enzyme concentration, introducing two of these costs: (1) the cost of producing proteins  $c$ , considered to be proportional to concentration (Wagner, 2005; Chou *et al.*, 2014; Lynch and Marinov, 2015), and (2) the exponential cost of an increase in macromolecular crowding, which hinders diffusion and thus slows down reactions (Dill *et al.*, 2011; Schavemaker *et al.*, 2018; Andrews, 2020) (see SM Fig. S15 for the resulting fitness landscapes of enzyme concentration).

The two types of costs result in a different shape of the fitness landscape, with the noticeable difference that evolutionarily expected concentration depends on  $N_e$  when the cost of production is considered (SM - Fig. S19) but not with crowding effects (SM - Fig. S20). With a combination of the two costs, enzyme concentrations decrease with  $N_e$  and production costs, resulting in the evolution of higher kinetic constants (FIG. 6). This is because at higher effective sizes, direct costs of protein production are large enough to incur effective selection for lower protein expression. This is no longer the case when  $N_e$  decreases, such that the major force driving the optimization of enzyme concentration becomes that opposing macromolecular crowding, which is less sensitive to  $N_e$  (as shown in Fig. S19 in SM). The balance between these two selective forces, and the dependency to  $N_e$ , obviously depend on the relative importance of these costs (SM - Fig. S20), itself depending on many parameters (protein length, molecular weight, etc.) that should only make enzymes marginally different within a given species (when their activity evolves on similar fitness landscapes).

### 3 Discussion

Most enzymes have been considered to be only moderately efficient (Bar-Even *et al.*, 2011), if not sloppy (Bar-Even *et al.*, 2011, 2015)(Bar-Even *et al.*, 2015). This claim was put into perspective by Newton *et al.* (2018) who argued that the link between fitness and enzyme efficiencies is complex and may be partly enzyme-dependentenzyme dependent, such that all enzymes may not evolve on a common fitness landscape. Through this work, we have developed a model where enzyme efficiencies are mechanistically linked to fitness through the impact of nutrient gradients on the production of metabolites. Our results emphasize that enzymes might in fact be quite efficient and , possibly, as efficient as Evolution allows.

We have modelled two processes of nutrient uptake that rely heavily on concentration gradients: passive (PD) and facilitated diffusion (FD). Our results apply to the numerous metabolic pathways that start with them, and also possibly to the widely used secondary active transport (SAT) in which FD of ion-substrate complexes follows the active extraction of a cotransported ion. Indeed, SAT can be described by similar equations as FD, with the difference that energy is required for extraction (Stein, 1986c).

Our results indicate that an enzyme’s forward kinetic parameters an enzyme’s fitness landscape – and the resulting mutation-selection-drift balance –  $k_{cat}$  and  $k_f$  – evolve on cliff-like fitness landscapes with a plateau – where the flux of energy generated increases on a log scale – may indeed be largely context dependent, possibly explaining a large part of the extreme observed variance in enzyme features.

At first sight, all enzymes evolve on fitness landscapes that have the same general shape, with a fitness plateau surrounded by a steep slope. This conclusion seems to agree qualitatively with the assumptions of previous models (Hartl *et al.*, 1985; Kaltenbach and Tokuriki, 2014), which we confirm here from a nutrient driven perspective. Importantly, our framework allows to consider the joint evolution of two kinetic parameters,  $k_f$  and  $k_{cat}$ . While this shape is usual in models of enzyme evolution (Hartl *et al.*, 1985; Kaltenbach and Tokuriki, 2014; Yi and Dean, 2019), in our model the landscape is drawn in the parameter space formed by the two forward kinetic parameters  $k_{cat}$  and  $k_f$ , instead of a composite “efficiency” whose relevance is questionable (Koshland, 2002; Eisinger *et al.*, 2007), and to pinpoint model parameters that have a differential impact on each. This, along with our broad comparison of model predictions with a massive dataset, initiates an attempt to predict evolutionary trends at the level of individual enzymes. We show that this should be possible in principle, under the assumption that mutations of efficient enzymes are slightly biased towards making them less efficient, which limits evolutionary predictions to a narrow range in the parameter space.

Overall, (Eisinger *et al.*, 2007; Koshland, 2002). Our model allows to predict the precise position of the fitness plateau in various contexts, showing that model parameters may have a selective impact on  $k_f$ ,  $k_{cat}$ , or both, thereby confirming the relevance of considering their distinct evolutionary dynamics.

We have shown that the exact position of the plateau is important through a population genetics model including mutational biases that produce less efficient enzymes at a slightly higher frequency. Despite their small effect, these biases are sufficient to have a significant impact on the evolutionary dynamics occurring on the fitness plateau, preventing enzymes to explore the parameter space far away from an isocline whose precise value can be predicted. Because the mutation-selection-drift balance occupies a narrow part of the landscape, this makes the evolution of an enzyme should result, in principle, highly predictable. Likewise, we anticipate that differences between enzymes should largely be explained by differences in the shapes of their individual fitness landscapes.

Overall, the selective pressure acting on an enzyme results from an interplay between (1) ecological and biochemical factors— metabolic demands, richness of the environment, reversibility of reactions – that draw the landscape, and (2) evolutionary factors – effective population size, distribution of mutational effects – governing how organisms move through evolutionary times on these landscapes several biochemical factors. We have effectively found that the shape of the fitness landscape depends on is first governed by features of the transporter, namely initiating a pathway, especially the maximum flux they can sustain and their affinity for the substrate, each having different effects on  $k_{cat}$  and  $k_f$ . These features are themselves evolving quantities, and the joint evolution of transporters and intracellular enzymes remains to be studied. Yet, using . Using parameters that correspond to empirical estimates for sugars and amino acids/nucleosides, we have found that enzymes intervening in contributing to subsequent metabolic pathways should be different, with those in the “sugars” pathway being selected for faster kinetics. The focus in this study was not on interactions within a pathway and high-order epistasis, but our framework suggests that all enzymes but the first in a pathway should evolve on fitness landscapes that do not depend on their position, as long as all upstream enzymes belong to the fitness plateau. If one enzyme

is noticeably inefficient, however, selection will push downstream enzymes towards a wider plateau, as a larger region of the parameter space can accommodate such a lower flux.

We also notice that different constraints may be met along a pathway and make each enzyme While sharing a common transporter, enzymes along a pathway are also subject to a variety of local chemical contexts, making each evolve on its own fitness landscape, possibly explaining a part of the large variance reported at this scale unique fitness landscape. This could explain, at least in part, the large within-pathway variance of enzyme kinetic parameters. Physical constraints may for instance act differentially on different enzymes, as exemplified by the intrinsic reversibility of a reaction that fuels the selective pressure towards higher efficiency in downstream enzymes. This may contribute to explain the high efficiency of a few specific enzymes, and thereby why there exists some “equilibrium enzymes” pushing reactions close to equilibrium (Williamson *et al.*, 1967; Davidi *et al.*, 2018). Aside from physical constraints, actual enzyme kinetic parameters are entangled with many more biochemical parameters, making the prediction of their evolution complex. enzymes like TIM (Williamson *et al.*, 1967; Davidi *et al.*, 2018).

More specifically, we have examined the effect of one such parameter (but not its evolution), the concentration of the enzyme One way to compensate for low kinetic constants is to enhance the level of expression of an enzyme, as confirmed by our model – concentration indeed has a strong influence on the fitness landscape of  $k_f$  and  $k_{cat}$ . Nonetheless, concentration and kinetic parameters face very distinct selection regimes: while the latter are both under directional selection, vanishing at high efficiencies, concentration is under stabilizing selection – owing to a combination between its positive impact on the flux and the adverse costs to high expression – as already pinpointed by Chou *et al.* (2014). Their joint evolution is complex because the position of the concentration optimum depends on an enzyme’s kinetic constants, whose fitness landscape itself depends on concentration. This results in a slightly increased variance in enzyme efficiencies compared to simulations with fixed concentrations, along with a complex relationship with genetic drift, because small populations tend to tolerate higher enzyme concentrations and, therefore, evolve less efficient enzymes.

It should be noted that our model does not consider another selection pressure on enzyme concentrations that arises from noise in gene expression, showing that it indeed exerts an important role and is likely involved in the variance in  $k_f$  and  $k_{cat}$ , for low efficiency can be buffered by high levels of expression and vice versa. How enzyme levels of expression and efficiencies jointly evolve, including the role of the cellular content on these efficiencies, should be the next step forward in the understanding of enzyme evolution. Because a cell is packed with many organites and biomolecules, making diffusion less efficient and  $k_f$ s lower *in vivo* than their *in vitro* counterparts, opposing forces govern the evolution of enzyme concentrations. Increasing concentrations may thus come at a great cost by slightly but steadily decreasing the actual efficiency of many catalysts at once, in turn favouring the evolution of highly efficient enzymes unless physical constraints prevent such an improvement. as argued by Wang and Zhang (2011). Indeed, low expression results in detrimental noise that should be avoided by pushing enzyme concentrations towards higher values in small organisms like Prokaryotes (see SM section Text S6 for an estimate of this effect). This could result in a different relationship between  $N_e$  and enzyme efficiencies than considered here,

possibly explaining the confusing observation that species with larger populations (and smaller sizes) do not express markedly more efficient catalysts. Furthermore, an enzyme’s effective concentration can also increase through compartmentalization (Ovádi and Saks, 2004; Diekmann and Pereira-Leal, 2013; Cornejo *et al.*, 2014) and substrate channeling (Welch and Easterby, 1994; Huang *et al.*, 2001; Sweetlove and Fernie, 2018), within the limits imposed by noise, and modify the selective pressure acting on kinetic parameters.

Our work provides a mechanistic model for enzyme evolution, based on the assumption that This illustrates that rather than making precise predictions, our study aims at making the strong claim that selection acting on enzyme features is important for their diversity, possibly largely overcoming the diversity arising from neutral processes. If this is indeed the case, trends in enzyme evolution can be predicted – as it was shown empirically in the context of antibiotic resistance (Walkiewicz *et al.*, 2012) – and further improvements of this model should allow to predict the expected features of individual enzymes. Such improvements are made easier by the use of a mechanistic framework, where fitness arises as enzymatic efficiency helps ingesting nutrients and win the competition for resources. This framework could even be enriched by other dimensions relevant to the genotype-phenotype-fitness map (Bershtein *et al.*, 2017; Echave, 2019; Kinsler *et al.*, 2020).

Unfortunately, mechanistic does not mean free of a definition of fitness, as we have here assumed that the latter is proportional to metabolic flux, hence considering each flux in isolation. Fitness instead results from a wide range of metabolic pathways that combine together and should all be competitive to certain degrees. How high-order global epistasis builds up (Weinreich *et al.*, 2013; Otwinowski *et al.*, 2018; Reddy and Desai, 2020), and genetic drift acts in this context, is far from obvious ; but (Iwasa *et al.*, 2004; Weinreich and Chao, 2005; Weissman *et al.*, 2009). But this should not impact much how enzymes evolve in old, overall efficient pathways, as any impediment in efficiency should have an a relatively independent effect on fitness in this context, as captured by our model. Yet, this could make selection weaker in some or all pathways , as exemplified by Heckmann *et al.* (2018), and therefore help explain why they appear to be evolutionary suboptimal. But more importantly, such a model would Understanding these complex interactions between pathways would nevertheless be crucial to understand how metabolic pathways arose and improved, likely from a highly inefficient state during early steps in the evolution of life on Earth (Kacser and Beeby, 1984; Schmidt *et al.*, 2003; Heckmann *et al.*, 2018).

## 4 Materials and Methods

### 4.1 Quantifying the maximum size for cells using passive diffusion

If a cell is to be viable, it has, at least, to uptake enough glucose to compensate for basal metabolism – metabolism that allows to maintain the same cell size for non-actively growing cells (Lynch and Marinov, 2015) – leading to the following equation:  $\phi_{PD} = C_M$ , with  $\phi_{PD}$  the uptake through passive diffusion and  $C_M$  the basal metabolism demand. To calculate the maximum size a cell can reach using only passive



diffusion, we relied on the formula  $C_M = 0.39V^{0.88}(10^9 \text{ ATP/hr})$  estimated in (Lynch and Marinov, 2015). We also assumed the cell to be of spherical shape, both concentrations – inside and outside the cell – to be constant with the cellular concentration staying so low that it can be overlooked, meaning that the uptake resulting from passive diffusion can merely be written as  $\phi_{PD} = P \cdot [S_{\text{out}}] \cdot \frac{SA_{\text{sphere}}}{V_{\text{sphere}}}$ , where  $SA_{\text{sphere}}$  and  $V_{\text{sphere}}$  are the surface area and the volume of a sphere, and  $P$  represents the cell permeability and was measured to  $10^{-6} \mu\text{m}^{-1}$  (Wood *et al.*, 1968) for glucose. Finally, we considered that each glucose yields 30 ATP molecules (Rich, 2003).

## 4.2 Flux sustained by the first enzyme

When assessing the flux of product made by the first enzyme in a pathway, both (PD) and (FD) result in similar sets of equations; we focus on FD here (see Text S5 - Mathematical appendix in Supplementary material for a comparison with PD). FD typically relies on the specific binding of substrate molecules – located outside the cell – by transmembrane carrier proteins, followed by their translocation into the cytoplasm (Danielli, 1954; Wilbrandt and Rosenberg, 1961; Kotyk, 1967)(Danielli, 1954; Wilbrandt and Rosenberg, 1961; Kotyk, 1967; Bosdriesz *et al.*, 2018). This specific process obeys Michaelis Menten-like kinetics when transport is assumed to be symmetric (Koty, 1967), which can be modelled through Briggs-Haldane equations (Briggs and Haldane, 1925; Haldane, 1930; Stein, 1986b):

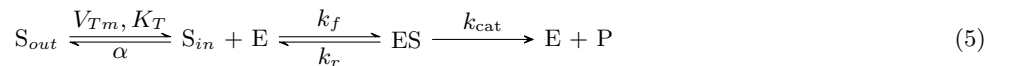
$$\frac{d[S_{\text{in}}]}{dt} = V_{Tm} \cdot \frac{[S_{\text{out}}] - [S_{\text{in}}]}{K_T + ([S_{\text{out}}] + [S_{\text{in}}]) + \alpha \cdot \frac{[S_{\text{out}}][S_{\text{in}}]}{K_T}} \quad (4)$$

with:

$$\left\{ \begin{array}{l} V_{Tm}: \text{the maximum rate of a given carrier protein;} \\ K_T: \text{a constant inversely proportional to the transport efficiency;} \\ \alpha : \text{the Kotyk interactive constant capturing the disequilibrium between bound and free transporters.} \end{array} \right.$$

By construction,  $\alpha$  cannot exceed 1 Kotyk (1967) and is close to this upper limit for sugars (*e.g.*  $\alpha = 0.91$  for glucose (Teusink *et al.*, 1998), so we set  $\alpha = 1$  by default in this study, maximizing the effect of interaction).

A model including both FD and irreversible substrate conversion by an enzyme therefore corresponds to the following chemical equation:



Using analytical tools (see ter Kuile and Cook (1994) and Bosdriesz *et al.* (2018), rederived in Supple-

mentary material - Text S5 Mathematical appendix), the flux can be determined through the following set of equations:

$$[ES]^* = \frac{k_f[S_{in}]^*}{k_r + k_{cat} + k_f[S_{in}]^*} \cdot [E_{tot}] \quad (6)$$

$$v = \frac{d[P]}{dt} = k_{cat}[ES]^* \quad (7)$$

where:

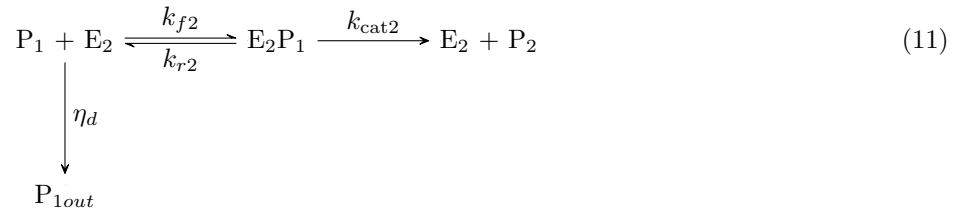
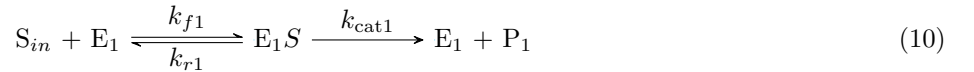
$$[S_{in}]^* = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \quad (8)$$

with:

$$\begin{cases} a = k_f k_{cat} [E_{tot}] (1 + \frac{[S_{out}]}{K_T}) + k_f V_{Tm} \\ b = k_f k_{cat} [E_{tot}] ([S_{out}] + K_T) + (k_{cat} + k_r - k_f [S_{out}]) V_{Tm} \\ c = -V_{Tm} [S_{out}] (k_r + k_{cat}) \end{cases} \quad (9)$$

### 4.3 Multiple enzymes model

In order to study the evolution of downstream enzymes, we considered an unbranched metabolic pathway in which the product formed by the first reaction serves as the substrate for a second reaction **whose flux determines fitness**. Theoretically, as there is nothing prohibiting increase in product concentrations – for it is not considered reversible at this point – any second enzyme should be able to sustain any metabolic demand. We penalized large increases in cellular concentrations through a degradation process of the product of the first reaction, occurring at rate  $\eta_d$  ( $\times$  this concentration). **Under these new assumptions, the The** chemical reactions occurring after uptake (Michaelis Menten part of Eq.5) are described by the following equations:



Such a system may reach a steady-state at which the cellular concentrations of the substrate  $S_{in}$  and

of the first product  $P_1$  are constant. At this point, the net instantaneous uptake of substrate (through the membrane) equals the instantaneous production of  $P_1$  which, in turn, equals the addition of the instantaneous sum of the instantaneous amount of degraded  $P_1$  and the instantaneous production of  $P_2$ , according to the principle of mass conservation. It yields the following system of equations:

$$V_{Tm} \cdot \frac{([S_{out}] - [S_{in}])}{K_T + ([S_{out}] + [S_{in}]) + \alpha \cdot \frac{[S_{out}][S_{in}]}{K_T}} = V_{m1} \cdot \frac{[S_{in}]}{K_{M1} + [S_{in}]} \quad (12)$$

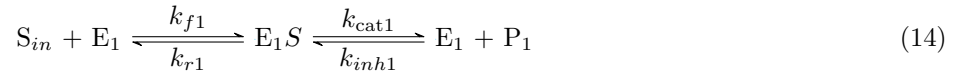
$$V_{m1} \cdot \frac{[S_{in}]}{K_{M1} + [S_{in}]} = V_{m2} \cdot \frac{[P_1]}{K_{M2} + [P_1]} + \eta_d \cdot [P_1] \quad (13)$$

where appear the traditional Michaelis-Menten kinetic parameters for the ( $i^{eth}$ ) reaction:

$$\begin{cases} V_{m_i} = k_{cat_i} [E_{tot_i}] \\ K_{M_i} = \frac{k_{r_i} + k_{cat_i}}{k_{f_i}} \end{cases}$$

To test the potential influence of toxicity, we defined the absolute fitness as a function of both the flux and a toxicity factor whose influence is represented through a sigmoid function and reflects the non-linearity nature of toxic effects (Clark, 1991; Wright and Rausher, 2010):  $f = \phi(1 - \frac{[P]}{[P] + T})$

In an independent section, we also introduced reversibility to further the analysis, through the modification of equation 10(10), which becomes:



Such a phenomenon is described by the more general form of Haldane equation (Haldane, 1930; Cornish-Bowden, 1979), which changes the contribution of the first reaction ( $V_{m1} \cdot \frac{[S_{in}]}{K_{M1} + [S_{in}]}$ ) in both equations (12) and (13) to:

$$V_{m1+} \cdot \frac{[S_{in}]}{K_{M1+} + [S_{in}] + K_I [P_1]} - V_{m1-} \cdot \frac{[P_1]}{K_{M1-} + [P_1] + [S_{in}]/K_I}$$

with  $V_{m1+}$  and  $K_{M1+}$  respectively corresponding to the previous  $V_{m1}$  and  $K_{M1}$ , while:

$$\begin{cases} V_{m1-} = k_{r1} [E_{tot1}] \\ K_I = k_{inh1} / k_{f1} \\ K_{M1-} = K_{M1+} / K_I \end{cases}$$

To solve these systems, which are not easily tractable analytically – for they rely on cubic equations – we implemented the Newton method (Atkinson, 1989) aiming to find  $[S_{in}]^*$  and  $[P_1]^*$ . We ran the algorithm starting from very low values of concentration (both set to  $10^{-20}M$ ) to determine numerically the equilibrium without facing convergence problems. The final flux can then be determined through the “production” part of

equation (13), *i.e.*  $V_{m2} \cdot \frac{[P_1]}{K_{M2} + [P_1]}$ .

#### 4.4 Validation of the method and computing of the fitness landscapes

To validate the approach, we compared equilibrium results obtained with Raphson-Newton algorithm to those obtained when simulating the process with Euler explicit schemes for a set of (3x3) kinetic values –  $k_{cat}$  and  $k_f$  – encompassing three orders of magnitude (see SM - Section Text 5 for further details).

In each section, we then determined the flux. We then drew fitness landscapes after determining the flux – assumed to be to be linearly related to fitness – achieved for enzyme kinetic parameters  $k_{cat}$  and  $k_f$  varying by 10 orders of magnitude, setting  $k_r$  to  $10^3 s^{-1}$  – within the range found for several enzymes (Klipp and Heinrich, 1994; Knowles and Alberly, 1977) – unless stated otherwise. Except in the section dedicated to the influence of enzyme concentration, we set the enzyme concentration such that  $[E_{tot}] = 1mM$ , lying nearby the highest observed values (Albe *et al.*, 1990; Noor *et al.*, 2016). Other parameters are detailed on a case-by-case basis as they may change depending on the goal of each section. To compare with the data and visualize results in the experimenter’s parameter space, we also determined the flux and plotted simulation results using  $k_{cat}$  and  $\frac{k_{cat}}{K_M}$ , also making them vary by 10 orders of magnitude. Fitness was considered to be linearly related to the flux in a pathway. To compute fitness landscapes, we thus We divided the parameter space in 100 equidistant values on a log-scale log-equidistant values (250 for representations with  $k_{cat}/K_M$  to draw obtain a cleaner demarcation for the diffusion limit).

#### 4.5 Population genetics model

We built an evolutionary model based Evolutionary simulations rely on a Wright-Fisher process including selection, to determine how enzymes should evolve on the selective effects of mutations displacing enzymes on mathematically-derived fitness landscapes. We considered the two fitness landscapes corresponding to opposite cases – Two fitness landscapes were considered: weak flux, high affinity (A on Fig.?? Fig. S3 A of SM) and high flux, low affinity (I on Fig.??)– for Fig. S3 I of SM), both with saturated facilitated diffusion ( $S_{out} = 10K_T$ ), with  $[S_{out}] = 10K_T$  and the following constant parameters:  $k_r = 10^3 s^{-1}$  and  $[E_{tot}] = 10mM$ . Mutations followed reproduction and were drawn at random from Gaussian distributions, at rates  $[E_{tot}] = 1mM$ . Mutations occur at a rate  $\mu = 10^{-1}/N_e$  making the quantity of mutations per unit time similar between simulations. Along with a similar following reproduction, with an effect sampled in Gaussian distributions with dispersion ( $\sigma = 0.3$ ), three cases of average mutational biases were considered for the log-values of forward parameters ( $k_{cat}$  and  $k_f$ ), which affected them independently: no mutational bias. The mean effect of a mutation is given by parameter  $b$ , hence representing the mutation bias – absent with  $b = 0$ , low ( $m = 0$ ), a low mutational bias ( $m = -0.1$  so that approximately 1 mutation out of 3 is positive) and a high mutational bias ( $m = -0.2$  so that approximately 1 mutation out of 4 is positive and 1% of them exceed 0.5). To determine the effect of  $N_e$ , we tested 4 different values of the parameters, ranging from

$N_e = 10^2$  to  $10^5$ .  $b = -0.1$ ) or high ( $b = -0.2$ ). Kinetic parameters were initiated to the inefficient values of  $k_{cat} = 10^{-3}s^{-1}$  and  $k_f = 10^2M^{-1}s^{-1}$  and  $k_f$  was limited to values under that allowed by the diffusion limit –  $10^{10}M^{-1}s^{-1}$  ( $k_{cat}$  was also limited to  $10^6s^{-1}$  when  $b = 0$  to avoid physical outliers). To analyse simulation outcomes, we picked out the kinetic and fitness values of the most represented genotype when multiple variants were segregating. 30 simulations were ran for each set of parameters. Finally, we verified that evolutionary steady-states were reached and considered it was the case when at least the average fitnesses (over all simulations) of the last three time-steps were not significantly different one from another (see SM Figures S5 and S6 of SM).

We also simulated the case where mutations between parameters are correlated. We tested both positive and negative mutational relationships through a bivariate Gaussian distribution whose correlation coefficient were set to  $\rho = -0.8$ ,  $\rho = -0.5$ ,  $\rho = +0.5$  (see SM Figure 18 for the results with a moderate flux).

#### 4.6 Evolution of enzyme concentrations

Finally, we simulated the joint evolution between kinetic parameters and enzyme concentration, repeating the previous procedure with concentration as an evolvable quantity and the fitness function including deleterious effects of extra gene expression (see SM section Text S5 for the effect of each cost considered independently one from another). Mutations affected either levels of expression or kinetic constants, with those affecting levels of expression (in log values) being drawn from Gaussian distributions with mean 0 and  $\sigma = 0.2$  to comply with existing estimates (Landry *et al.*, 2007; Metzger *et al.*, 2016; Hodgins-Davis *et al.*, 2019). Because sugars are directly involved in energy metabolism, we computed these simulations for the case of a high flux which can more readily be linked to the cost of expression.

As explained above, producing extra proteins is costly, both energetically and because it may increase a cell's crowding. The cost of protein production is considered to be proportional to the steady-state enzyme concentration, with a slope  $c$ . Empirical estimates suggest that  $c$  should be in the range  $[10^{-4}, 10^{-3}]$  (Wagner, 2005; Lynch and Marinov, 2015), such that producing an extra mM of a protein would impede the whole energy budget by one 10000<sup>th</sup> to one 1000<sup>th</sup> (we also consider  $c = 10^{-5}$  and  $10^{-2}$  in the SM). Accordingly, we calculate the absolute fitness  $f = \Phi - [E_{tot}]c$ , where  $\Phi$  is the flux generated by the enzyme.

The influence of crowding was computed by penalizing  $k_f$  through an effective  $k_{f,act} = k_f \cdot 10^{-([E_{tot}] + [M_b])/[2M_b]}$ , where  $[M_b] = 2.5 \cdot 10^{-3}M$  represents the basal protein concentration of a viable cell and also constitutes a scaling factor that complies with Andrews (2020) log-linear influence of crowding or glass transition effects described by (Dill *et al.*, 2011).

## 4.7 Data availability

All the enzyme data used in this work to compare fitness landscapes and measured values were recovered from (Bar-Even *et al.*, 2011), and so was the classification of reactions with regards to metabolic groups. Thanks to their authors and publisher, datasets are publicly available at <https://pubs.acs.org/doi/10.1021/bi2002289>. Apart from that, no new data were generated in support of this research.

## 5 Supplementary Material

Supplementary material is available online.

## References

- Albe, K. R., Butler, M. H., and Wright, B. E. 1990. Cellular concentrations of enzymes and their substrates. *J Theor Biol*, 143(2): 163–95.
- Alberty, R. A. and Hammes, G. G. 1958. Application of the theory of diffusion-controlled reactions to enzyme kinetics. *The Journal of Physical Chemistry*, 62(2): 154–159.
- Andrews, S. S. 2020. Effects of surfaces and macromolecular crowding on bimolecular reaction rates. *Phys Biol*, 17(4): 045001.
- Atkinson, K. 1989. *An Introduction to Numerical Analysis*. John Wiley and Sons, New York, 2nd edition.
- Bar-Even, A., Noor, E., Savir, Y., Liebermeister, W., Davidi, D., Tawfik, D. S., and Milo, R. 2011. The moderately efficient enzyme: Evolutionary and physicochemical trends shaping enzyme parameters. *Biochemistry*, 50(21): 4402–4410.
- Bar-Even, A., Milo, R., Noor, E., and Tawfik, D. S. 2015. The moderately efficient enzyme: Futile encounters and enzyme floppiness. *Biochemistry*, 54(32): 4969–4977.
- Bershtein, S., Serohijos, A. W., and Shakhnovich, E. I. 2017. Bridging the physical scales in evolutionary biology: from protein sequence space to fitness of organisms and populations. *Current Opinion in Structural Biology*, 42: 31–40.
- Blanco, P. M., Garcés, J. L., Madurga, S., and Mas, F. 2018. Macromolecular diffusion in crowded media beyond the hard-sphere model. *Soft Matter*, 14: 3105–3114.
- Bloom, J., Wilke, C., Arnold, F., and Adami, C. 2004. Stability and the evolvability of function in a model protein. *Biophysical Journal*, 86: 2758–2764.

- Bloom, J. D., Silberg, J. J., Wilke, C. O., Drummond, D. A., Adami, C., and Arnold, F. H. 2005. Thermodynamic prediction of protein neutrality. *Proceedings of the National Academy of Sciences of the United States of America*, 102(3): 606.
- Bloom, J. D., Labthavikul, S. T., Otey, C. R., and Arnold, F. H. 2006. Protein stability promotes evolvability. *Proceedings of the National Academy of Sciences*, 103(15): 5869.
- Bloom, J. D., Lu, Z., Chen, D., Raval, A., Venturelli, O. S., and Arnold, F. H. 2007. Evolution favors protein mutational robustness in sufficiently large populations. *BMC Biology*, 5(1): 29.
- Bobay, L.-M. and Ochman, H. 2018. Factors driving effective population size and pan-genome evolution in bacteria. *BMC Evolutionary Biology*, 18(1): 153.
- Boer, V. M., Crutchfield, C. A., Bradley, P. H., Botstein, D., and Rabinowitz, J. D. 2010. Growth-limiting intracellular metabolites in yeast growing under diverse nutrient limitations. *Molecular biology of the cell*, 21(1): 198–211.
- Bosdriesz, E., Wortel, M. T., Haanstra, J. R., Wagner, M. J., de la Torre Cortés, P., and Teusink, B. 2018. Low affinity uniporter carrier proteins can increase net substrate uptake rate by reducing efflux. *Scientific Reports*, 8(1): 5576.
- Briggs, G. E. and Haldane, J. B. 1925. A note on the kinetics of enzyme action. *The Biochemical journal*, 19(2): 338–339.
- Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C. M., and Stefani, M. 2002. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature*, 416(6880): 507–511.
- Burns, J., Cornish-Bowden, A., Groen, A., Heinrich, R., Kacser, H., Porteous, J., Rapoport, S., Rapoport, T., Stucki, J., Tager, J., Wanders, R., and Westerhoff, H. 1985. Control analysis of metabolic systems. *Trends in Biochemical Sciences*, 10(1): 16.
- Carlin, D. A., Caster, R. W., Wang, X., Betzenderfer, S. A., Chen, C. X., Duong, V. M., Ryklansky, C. V., Alpekin, A., Beaumont, N., Kapoor, H., Kim, N., Mohabbot, H., Pang, B., Teel, R., Whithaus, L., Tagkopoulos, I., and Siegel, J. B. 2016. Kinetic characterization of 100 glycoside hydrolase mutants enables the discovery of structural features correlated with kinetic constants. *PLOS ONE*, 11(1): e0147596–.
- Chakrabarti, A. C. 1994. Permeability of membranes to amino acids and modified amino acids: Mechanisms involved in translocation. *Amino Acids*, 6(3): 213–229.



- Chou, H.-H., Delaney, N. F., Draghi, J. A., and Marx, C. J. 2014. Mapping the fitness landscape of gene expression uncovers the cause of antagonism and sign epistasis between adaptive mutations. *PLOS Genetics*, 10(2): e1004149–.
- Clark, A. G. 1991. Mutation-selection balance and metabolic control theory. *Genetics*, 129(3): 909–923.
- Cornejo, E., Abreu, N., and Komeili, A. 2014. Compartmentalization and organelle formation in bacteria. *Curr Opin Cell Biol*, 26: 132–138.
- Cornish-Bowden, A. 1979. *Chapter 2 - Introduction to enzyme kinetics*, pages 16–38. Butterworth-Heinemann.
- Danielli, J. 1954. Morphological and molecular aspects of active transport. In *Symposia of the Society for Experimental Biology*, volume 8, pages 502–516, DOWNING ST, CAMBRIDGE. COMPANY BIOLOGISTS LTD UNIV CAMBRIDGE DEPT ZOOLOGY.
- Davidi, D., Noor, E., Liebermeister, W., Bar-Even, A., Flamholz, A., Tummli, K., Barenholz, U., Goldenfeld, M., Shlomi, T., and Milo, R. 2016. Global characterization of in vivo enzyme catalytic rates and their correspondence to in vitro  $k_{cat}$  measurements. *Proceedings of the National Academy of Sciences*, 113(12): 3401.
- Davidi, D., Longo, L. M., Jabłońska, J., Milo, R., and Tawfik, D. S. 2018. A bird’s-eye view of enzyme evolution: Chemical, physicochemical, and physiological considerations. *Chemical Reviews*, 118(18): 8786–8797.
- Dean, A. M. 1995. A molecular investigation of genotype by environment interactions. *Genetics*, 139(1): 19–33.
- Dean, A. M., Dykhuizen, D. E., and Hartl, D. L. 1986. Fitness as a function of  $\beta$ -galactosidase activity in *Escherichia coli*. 48(1): 1–8.
- Dekel, E. and Alon, U. 2005. Optimality and evolutionary tuning of the expression level of a protein. *Nature*, 436(7050): 588–592.
- DePristo, M. A., Weinreich, D. M., and Hartl, D. L. 2005. Missense meanderings in sequence space: a biophysical view of protein evolution. *Nature Reviews Genetics*, 6(9): 678–687.
- Diekmann, Y. and Pereira-Leal, J. 2013. Evolution of intracellular compartmentalization. *Biochem J*, 449(2): 319–331.

- Dill, K. A., Ghosh, K., and Schmit, J. D. 2011. Physical limits of cells and proteomes. *Proceedings of the National Academy of Sciences*, 108(44): 17876.
- Dong, H., Nilsson, L., and Kurland, C. G. 1995. Gratuitous overexpression of genes in escherichia coli leads to growth inhibition and ribosome destruction. *Journal of Bacteriology*, 177(6): 1497.
- Drummond, D. A. and Wilke, C. O. 2008. Mistranslation-induced protein misfolding as a dominant constraint on coding-sequence evolution. *Cell*, 134(2): 341–352.
- Drummond, D. A., Bloom, J. D., Adami, C., Wilke, C. O., and Arnold, F. H. 2005. Why highly expressed proteins evolve slowly. *Proceedings of the National Academy of Sciences of the United States of America*, 102(40): 14338.
- Dykhuizen, D. E. and Dean, A. M. 1990. Enzyme activity and fitness: Evolution in solution. *Trends Ecol Evol*, 5(8): 257–262.
- Dykhuizen, D. E. and Dean, A. M. 1994. Predicted fitness changes along an environmental gradient. *Evolutionary Ecology*, 8(5): 524–541.
- Dykhuizen, D. E., Dean, A. M., and Hartl, D. L. 1987. Metabolic flux and fitness. *Genetics*, 115(1): 25–31.
- Echave, J. 2019. Beyond stability constraints: A biophysical model of enzyme evolution with selection on stability and activity. *Molecular Biology and Evolution*, 36(3): 613–620.
- Echave, J. and Wilke, C. O. 2017. Biophysical models of protein evolution: Understanding the patterns of evolutionary sequence divergence. *Annu Rev Biophys*, 46: 85–103.
- Eisenthal, R., Danson, M. J., and Hough, D. W. 2007. Catalytic efficiency and  $k_{cat}/k_M$ : a useful comparator? *Trends in Biotechnology*, 25(6): 247–249.
- Ellis, R. J. 2001. Macromolecular crowding: obvious but underappreciated. *Trends in Biochemical Sciences*, 26(10): 597–604.
- Eyre-Walker, A. and Keightley, P. D. 2007. The distribution of fitness effects of new mutations. *Nature Reviews Genetics*, 8(8): 610–618.
- Fell, D. A. 1992. Metabolic control analysis: a survey of its theoretical and experimental development. *The Biochemical journal*, 286 ( Pt 2)(Pt 2): 313–330.
- Geiler-Samerotte, K. A., Dion, M. F., Budnik, B. A., Wang, S. M., Hartl, D. L., and Drummond, D. A. 2011. Misfolded proteins impose a dosage-dependent fitness cost and trigger a cytosolic unfolded protein response in yeast. *Proceedings of the National Academy of Sciences*, 108(2): 680.

- Griffith, D. A. and Jarvis, S. M. 1996. Nucleoside and nucleobase transport systems of mammalian cells. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes*, 1286(3): 153–181.
- Gudelj, I., Weitz, J. S., Ferenci, T., Claire Horner-Devine, M., Marx, C. J., Meyer, J. R., and Forde, S. E. 2010. An integrative approach to understanding microbial diversity: from intracellular mechanisms to community structure. *Ecology Letters*, 13(9): 1073–1084.
- Haldane, J. B. S. 1930. *Enzymes*. Longmans, Green, London, New York.
- Hartl, D. L., Dykhuizen, D. E., and Dean, A. M. 1985. Limits of adaptation: the evolution of selective neutrality. *Genetics*, 111(3): 655–674.
- Heckmann, D., Zielinski, D. C., and Palsson, B. O. 2018. Modeling genome-wide enzyme evolution predicts strong epistasis underlying catalytic turnover rates. *Nature Communications*, 9(1): 5270.
- Heim, N. A., Payne, J. L., Finnegan, S., Knope, M. L., Kowalewski, M., Lyons, S. K., McShea, D. W., Novack-Gottshall, P. M., Smith, F. A., and Wang, S. C. 2017. Hierarchical complexity and the size limits of life. *Proceedings of the Royal Society B: Biological Sciences*, 284(1857): 20171039.
- Heinrich, R. and Rapoport, T. A. 1974. A linear steady-state treatment of enzymatic chains. general properties, control and effector strength. *Eur J Biochem*, 42(1): 89–95.
- Heinrich, R., Schuster, S., and Holzhütter, H. G. 1991. Mathematical analysis of enzymic reaction systems using optimization principles. *Eur J Biochem*, 201(1): 1–21.
- Hodgins-Davis, A., Duveau, F., Walker, E. A., and Wittkopp, P. J. 2019. Empirical measures of mutational effects define neutral models of regulatory evolution in *saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences*, 116(42): 21085.
- Huang, X., Holden, H. M., and Raushel, F. M. 2001. Channeling of substrates and intermediates in enzyme-catalyzed reactions. *Annual Review of Biochemistry*, 70(1): 149–180.
- Iwasa, Y., Michor, F., and Nowak, M. A. 2004. Evolutionary dynamics of invasion and escape. *J Theor Biol*, 226(2): 205–214.
- Jiang, M. and Guo, Z. 2007. Effects of macromolecular crowding on the intrinsic catalytic efficiency and structure of enterobactin-specific isochorismate synthase. *Journal of the American Chemical Society*, 129(4): 730–731.
- Johnson, K. A. and Goody, R. S. 2011. The original michaelis constant: Translation of the 1913 michaelis–menten paper. *Biochemistry*, 50(39): 8264–8269.

- Jones, C. M., Hernández Lozada, N. J., and Pfleger, B. F. 2015. Efflux systems in bacteria and their metabolic engineering applications. *Applied microbiology and biotechnology*, 99(22): 9381–9393.
- Kacser, H. and Beeby, R. 1984. Evolution of catalytic proteins. *Journal of Molecular Evolution*, 20(1): 38–51.
- Kacser, H. and Burns, J. A. 1973. The control of flux. *Symp Soc Exp Biol*, 27: 65–104.
- Kacser, H., Burns, J. A., Kacser, H., and Fell, D. A. 1995. The control of flux. *Biochemical Society Transactions*, 23(2): 341–366.
- Kafri, M., Metzl-Raz, E., Jona, G., and Barkai, N. 2016. The cost of protein production. *Cell Reports*, 14(1): 22–31.
- Kaltenbach, M. and Tokuriki, N. 2014. Dynamics and constraints of enzyme evolution. *J Exp Zool B Mol Dev Evol*, 322(7): 468–487.
- Keller, M. A., Piedrafitra, G., and Ralser, M. 2015. The widespread role of non-enzymatic reactions in cellular metabolism. *Current opinion in biotechnology*, 34: 153–161.
- Khersonsky, O. and Tawfik, D. S. 2010. Enzyme promiscuity: a mechanistic and evolutionary perspective. *Annu Rev Biochem*, 79: 471–505.
- Kimura, M. 1968. Evolutionary rate at the molecular level. *Nature*, 217(5129): 624–626.
- Kinsler, G., Geiler-Samerotte, K., Petrov, D. A., Cooper, V. S., Barkai, N., and Gresham, D. 2020. Fitness variation across subtle environmental perturbations reveals local modularity and global pleiotropy of adaptation. *eLife*, 9: e61271.
- Klipp, E. and Heinrich, R. 1994. Evolutionary optimization of enzyme kinetic parameters; effect of constraints. *Journal of Theoretical Biology*, 171(3): 309–323.
- Knies, J., Cai, F., and Weinreich, D. M. 2017. Enzyme efficiency but not thermostability drives cefotaxime resistance evolution in tem-1  $\beta$ -lactamase. *Molecular Biology and Evolution*, page msx053.
- Knowles, J. R. and Albery, W. J. 1977. Perfection in enzyme catalysis: the energetics of triosephosphate isomerase. *Accounts of Chemical Research*, 10(4): 105–111.
- Koshland, D. E. 2002. The application and usefulness of the ratio kcat/km. *Bioorganic Chemistry*, 30(3): 211–213.

- Kotyk, A. 1967. Mobility of the free and of the loaded monosaccharide carrier in *saccharomyces cerevisiae*. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 135(1): 112–119.
- Landry, C. R., Lemos, B., Rifkin, S. A., Dickinson, W. J., and Hartl, D. L. 2007. Genetic properties influencing the evolvability of gene expression. *Science*, 317(5834): 118.
- Lang, G. I., Murray, A. W., and Botstein, D. 2009. The cost of gene expression underlies a fitness trade-off in yeast. *Proceedings of the National Academy of Sciences*, 106(14): 5755.
- Lilja, E. E. and Johnson, D. R. 2017. Metabolite toxicity determines the pace of molecular evolution within microbial populations. *BMC Evolutionary Biology*, 17(1): 52.
- Lunzer, M., Miller, S. P., Felsheim, R., and Dean, A. M. 2005. The biochemical architecture of an ancient adaptive landscape. *Science*, 310(5747): 499–501.
- Lunzer, M., Golding, G. B., and Dean, A. M. 2010. Pervasive cryptic epistasis in molecular evolution. *PLOS Genetics*, 6(10): e1001162–.
- Lynch, M. and Marinov, G. K. 2015. The bioenergetic costs of a gene. *Proceedings of the National Academy of Sciences of the United States of America*, 112(51): 15690–15695.
- Maier, A., Völker, B., Boles, E., and Fuhrmann, G. F. 2002. Characterisation of glucose transport in *Saccharomyces cerevisiae* with plasma membrane vesicles (countertransport) and intact cells (initial uptake) with single Hxt1, Hxt2, Hxt3, Hxt4, Hxt6, Hxt7 or Gal2 transporters. *FEMS Yeast Research*, 2(4): 539–550.
- Metzger, B. P. H., Dubeau, F., Yuan, D. C., Tryban, S., Yang, B., and Wittkopp, P. J. 2016. Contrasting frequencies and effects of cis- and trans-regulatory mutations affecting gene expression. *Molecular Biology and Evolution*, 33(5): 1131–1146.
- Michaelis, L. and Menten, M. 1913. Die kinetik der invertinwirkung. *biochemische zeitschrift. Biochemische Zeitschrift*, 49: 333–369.
- Miller, S. R. 2017. An appraisal of the enzyme stability-activity trade-off. *Evolution*, 71(7): 1876–1887.
- Milo, R., Jorgensen, P., Moran, U., Weber, G., and Springer, M. 2010. Bionumbers—the database of key numbers in molecular and cell biology. *Nucleic acids research*, 38(Database issue): D750–D753.
- Muramatsu, N. and Minton, A. P. 1988. Tracer diffusion of globular proteins in concentrated protein solutions. *Proceedings of the National Academy of Sciences*, 85(9): 2984.

- Newton, M. S., Arcus, V. L., Gerth, M. L., and Patrick, W. M. 2018. Enzyme evolution: innovation is easy, optimization is complicated. *Current Opinion in Structural Biology*, 48: 110–116.
- Niehaus, T. D. and Hillmann, K. B. 2020. Enzyme promiscuity, metabolite damage, and metabolite damage control systems of the tricarboxylic acid cycle. *The FEBS Journal*, 287(7): 1343–1358.
- Noor, E., Flamholz, A., Bar-Even, A., Davidi, D., Milo, R., and Liebermeister, W. 2016. The protein cost of metabolic fluxes: Prediction from enzymatic rate laws and cost minimization. *PLOS Computational Biology*, 12(11): e1005167.
- Novick, A. and Weiner, M. 1957. Enzyme induction as an all-or-none phenomenon. *Proceedings of the National Academy of Sciences*, 43(7): 553.
- Ohta, T. 1992. The nearly neutral theory of molecular evolution. *Annual Review of Ecology and Systematics*, 23(1): 263–286.
- Orth, J. D., Thiele, I., and Palsson, B. Ø. 2010. What is flux balance analysis? *Nature biotechnology*, 28(3): 245–248.
- Otwinowski, J., McCandlish, D. M., and Plotkin, J. B. 2018. Inferring the shape of global epistasis. *Proceedings of the National Academy of Sciences*, 115(32): E7550.
- Ovádi, J. and Saks, V. 2004. On the origin of intracellular compartmentation and organized metabolic systems. *Molecular and Cellular Biochemistry*, 256(1): 5–12.
- Peracchi, A. 2018. The limits of enzyme specificity and the evolution of metabolism. *Trends in Biochemical Sciences*, 43(12): 984–996.
- Pozdnyakova, I. and Wittung-Stafshede, P. 2010. Non-linear effects of macromolecular crowding on enzymatic activity of multi-copper oxidase. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1804(4): 740–744.
- Ralston, G. B. 1990. Effects of "crowding" in protein solutions. *Journal of Chemical Education*, 67(10): 857.
- Reddy, G. and Desai, M. M. 2020. Global epistasis emerges from a generic model of a complex trait. *bioRxiv*, page 2020.06.14.150946.
- Rich, P. R. 2003. The molecular machinery of keilin’s respiratory chain. *Biochemical Society Transactions*, 31(6): 1095–1105.

- Rivas, G. and Minton, A. P. 2018. Toward an understanding of biochemical equilibria within living cells. *Biophysical reviews*, 10(2): 241–253.
- Rivas, G., Ferrone, F., and Herzfeld, J. 2004. Life in a crowded world. *EMBO reports*, 5(1): 23–27.
- Rodrigues, J. V., Bershtein, S., Li, A., Lozovsky, E. R., Hartl, D. L., and Shakhnovich, E. I. 2016. Biophysical principles predict fitness landscapes of drug resistance. *Proc Natl Acad Sci U S A*, 113(11): E1470–8.
- Sabate, R., de Groot, N. S., and Ventura, S. 2010. Protein folding and aggregation in bacteria. *Cellular and Molecular Life Sciences*, 67(16): 2695–2715.
- Schaefer, B., Emerson, J. J., Wang, T.-Y., Lu, M.-Y. J., Hsieh, L.-C., and Li, W.-H. 2013. Inheritance of gene expression level and selective constraints on trans- and cis-regulatory changes in yeast. *Molecular Biology and Evolution*, 30(9): 2121–2133.
- Schäuble, S., Stavrum, A. K., Puntervoll, P., Schuster, S., and Heiland, I. 2013. Effect of substrate competition in kinetic models of metabolic networks. *FEBS Letters*, 587(17): 2818–2824.
- Schavemaker, P. E., Boersma, A. J., and Poolman, B. 2018. How important is protein diffusion in prokaryotes? *Frontiers in Molecular Biosciences*, 5: 93.
- Schmidt, J. A., Rinaldi, S., Scalbert, A., Ferrari, P., Achaintre, D., Gunter, M. J., Appleby, P. N., Key, T. J., and Travis, R. C. 2016. Plasma concentrations and intakes of amino acids in male meat-eaters, fish-eaters, vegetarians and vegans: a cross-sectional analysis in the epic-oxford cohort. *European Journal of Clinical Nutrition*, 70(3): 306–312.
- Schmidt, S., Sunyaev, S., Bork, P., and Dandekar, T. 2003. Metabolites: a helping hand for pathway evolution? *Trends in Biochemical Sciences*, 28(6): 336–341.
- Schreiber, G., Buckle, A. M., and Fersht, A. R. 1994. Stability and function: two constraints in the evolution of barstar and other proteins. *Structure*, 2(10): 945–51.
- Schuster, S., Pfeiffer, T., and Fell, D. A. 2008. Is maximization of molar yield in metabolic networks favoured by evolution? *J Theor Biol*, 252(3): 497–504.
- Scott, M., Gunderson, C. W., Mateescu, E. M., Zhang, Z., and Hwa, T. 2010. Interdependence of cell growth and gene expression: Origins and consequences. *Science*, 330(6007): 1099–1102.
- Serohijos, A. W. R., Rimas, Z., and Shakhnovich, E. I. 2012. Protein biophysics explains why highly abundant proteins evolve slowly. *Cell Rep*, 2(2): 249–256.



- Shoichet, B. K., Baase, W. A., Kuroki, R., and Matthews, B. W. 1995. A relationship between protein stability and protein function. *Proceedings of the National Academy of Sciences of the United States of America*, 92(2): 452–456.
- Stein, W. D. 1986a. *CHAPTER 1 - Physical Basis of Movement across Cell Membranes*, chapter 1, pages 1–68. Academic Press.
- Stein, W. D. 1986b. *CHAPTER 4 - Facilitated Diffusion: The Simple Carrier*, chapter 4, pages 231–361. Academic Press.
- Stein, W. D. 1986c. *CHAPTER 5 - The Cotransport Systems: Two Substrates That Are Carried on a Single Transporter*, chapter 5, pages 363–474. Academic Press.
- Stiffler, M. A., Hekstra, D. R., and Ranganathan, R. 2015. Evolvability as a function of purifying selection in tem-1  $\beta$ -lactamase. *Cell*, 160(5): 882–892.
- Stoebel, D. M., Dean, A. M., and Dykhuizen, D. E. 2008. The cost of expression of *Escherichia coli lac* operon proteins is in the process, not in the products. *Genetics*, 178(3): 1653.
- Storz, J. F. 2018. Compensatory mutations and epistasis for protein function. *Current opinion in structural biology*, 50: 18–25.
- Sweetlove, L. J. and Fernie, A. R. 2018. The role of dynamic enzyme assemblies and substrate channelling in metabolic regulation. *Nature Communications*, 9(1): 2136.
- Tabaka, M., Kalwarczyk, T., Szymanski, J., Hou, S., and Holyst, R. 2014. The effect of macromolecular crowding on mobility of biomolecules, association kinetics, and gene expression in living cells. *Frontiers in Physics*, 2: 54.
- Taverna, D. M. and Goldstein, R. A. 2002. Why are proteins marginally stable? *Proteins: Structure, Function, and Bioinformatics*, 46(1): 105–109.
- ter Kuile, B. H. and Cook, M. 1994. The kinetics of facilitated diffusion followed by enzymatic conversion of the substrate. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1193(2): 235–239.
- Teusink, B., Diderich, J. A., Westerhoff, H. V., van Dam, K., and Walsh, M. C. 1998. Intracellular glucose concentration in derepressed yeast cells consuming glucose is high enough to reduce the glucose transport rate by 50%. *Journal of bacteriology*, 180(3): 556–562.
- Tokuriki, N. and Tawfik, D. S. 2009. Stability effects of mutations and protein evolvability. *Current Opinion in Structural Biology*, 19(5): 596–604.

- Tokuriki, N., Stricher, F., Schymkowitz, J., Serrano, L., and Tawfik, D. S. 2007. The stability effects of protein mutations appear to be universally distributed. *Journal of Molecular Biology*, 369(5): 1318–1332.
- Tokuriki, N., Stricher, F., Serrano, L., and Tawfik, D. S. 2008. How protein stability and new functions trade off. *PLoS Computational Biology*, 4(2): e1000002.
- Tokuriki, N., Jackson, C. J., Afriat-Jurnou, L., Wyganowski, K. T., Tang, R., and Tawfik, D. S. 2012. Diminishing returns and tradeoffs constrain the laboratory optimization of an enzyme. *Nature Communications*, 3(1): 1257.
- van den Burg, B. and Eijssink, V. G. H. 2002. Selection of mutations for increased protein stability. *Curr Opin Biotechnol*, 13(4): 333–337.
- Wagner, A. 2005. Energy Constraints on the Evolution of Gene Expression. *Molecular Biology and Evolution*, 22(6): 1365–1374.
- Walkiewicz, K., Benitez Cardenas, A. S., Sun, C., Bacorn, C., Saxer, G., and Shamoo, Y. 2012. Small changes in enzyme function can lead to surprisingly large fitness effects during adaptive evolution of antibiotic resistance. *Proceedings of the National Academy of Sciences*, 109(52): 21408.
- Wang, Z. and Zhang, J. 2011. Impact of gene expression noise on organismal fitness and the efficacy of natural selection. *Proceedings of the National Academy of Sciences of the United States of America*, 108(16): E67–E76.
- Weinreich, D. M. and Chao, L. 2005. Rapid evolutionary escape by large populations from local fitness peaks is likely in nature. *Evolution*, 59(6): 1175–1182.
- Weinreich, D. M., Delaney, N. F., DePristo, M. A., and Hartl, D. L. 2006. Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science*, 312(5770): 111.
- Weinreich, D. M., Lan, Y., Wylie, C. S., and Heckendorn, R. B. 2013. Should evolutionary geneticists worry about higher-order epistasis? *Curr Opin Genet Dev*, 23(6): 700–707.
- Weissman, D. B., Desai, M. M., Fisher, D. S., and Feldman, M. W. 2009. The rate at which asexual populations cross fitness valleys. *Theor Popul Biol*, 75(4): 286–300.
- Welch, G. R. and Easterby, J. S. 1994. Metabolic channeling versus free diffusion: transition-time analysis. *Trends Biochem Sci*, 19(5): 193–197.
- Wilbrandt, W. and Rosenberg, T. 1961. The concept of carrier transport and its corollaries in pharmacology. *Pharmacological Reviews*, 13(2): 109.

- Williamson, D. H., Lund, P., and Krebs, H. A. 1967. The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem J*, 103(2): 514–527.
- Wood, R. E., Wirth, F. P. J., and Morgan, H. E. 1968. Glucose permeability of lipid bilayer membranes. *Biochim Biophys Acta*, 163(2): 171–178.
- Wright, K. M. and Rausher, M. D. 2010. The evolution of control and distribution of adaptive mutations in a metabolic pathway. *Genetics*, 184(2): 483.
- Wright, S. 1931. Evolution in mendelian populations. *Genetics*, 16(2): 97.
- Wright, S. 1934. Physiological and evolutionary theories of dominance. 68(714): 24–53.
- Xie, H., Patching, S. G., Gallagher, M. P., Litherland, G. J., Brough, A. R., Venter, H., Yao, S. Y. M., Ng, A. M. L., Young, J. D., Herbert, R. B., Henderson, P. J. F., and Baldwin, S. A. 2004. Purification and properties of the escherichia coli nucleoside transporter nupg, a paradigm for a major facilitator transporter sub-family. *Mol Membr Biol*, 21(5): 323–336.
- Yang, J.-R., Liao, B.-Y., Zhuang, S.-M., and Zhang, J. 2012. Protein misinteraction avoidance causes highly expressed proteins to evolve slowly. *Proceedings of the National Academy of Sciences*, 109(14): E831.
- Yi, X. and Dean, A. M. 2019. Adaptive landscapes in the age of synthetic biology. *Molecular biology and evolution*, 36(5): 890–907.
- Yue, P., Li, Z., and Moult, J. 2005. Loss of protein structure stability as a major causative factor in monogenic disease. *J Mol Biol*, 353(2): 459–473.
- Zampieri, M., Hörl, M., Hotz, F., Müller, N. F., and Sauer, U. 2019. Regulatory mechanisms underlying coordination of amino acid and glucose catabolism in escherichia coli. *Nature Communications*, 10(1): 3354.
- Zhou, G.-Q. and Zhong, W.-Z. 1982. Diffusion-controlled reactions of enzymes. *European Journal of Biochemistry*, 128(2-3): 383–387.
- Zhou, H.-X., Rivas, G., and Minton, A. P. 2008. Macromolecular crowding and confinement: Biochemical, biophysical, and potential physiological consequences. *Annual Review of Biophysics*, 37(1): 375–397.
- Zimmerman, S. B. and Minton, A. P. 1993. Macromolecular crowding: Biochemical, biophysical, and physiological consequences. *Annual Review of Biophysics and Biomolecular Structure*, 22(1): 27–65.