Authors' Response to Reviews of

Resource uptake and the evolution of moderately efficient enzymes

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Molecular Biology and Evolution

EC: ***Editor's Comment***, **RC:** ***Reviewers' Comment***, AR: Authors' Response, ☐ Manuscript Text

1. Editor

EC: ***We have received two reviewers' comments. Although they found the work interesting, they both raised major concern about the approach. Particularly, the model needs to include more biophysical properties of the protein energy landscape (aka folding stability) and also it is lacking comparison with the other modules built for protein evolution.***

Dear editor,

Thank you for giving us the opportunity to submit a revised draft of our manuscript entitled "Resource uptake and the evolution of moderately efficient enzymes". We deeply appreciate the time you and the reviewers dedicated to evaluate our manuscript and are very grateful for the insightful comments provided, which definitely helped us improve our work by putting its findings in a wider perspective.

We have identified three main comments from the reviewers:

- First, that we have not considered other important properties of proteins in our model, especially their stability and their resulting propensity to misfold. We agree that a discussion on this topic was lacking in the previous version of the manuscript. We corrected this by stating explicitly that the premise of our work is the existence of a genotype space where stability is sufficiently high – and misfolding sufficiently rare – such that enzyme kinetics can evolve through mutations on this space. This makes it clear that one underlying assumption is that such a space exists, and in particular that there are mutations that change enzyme activity while remaining above a stability threshold as exemplified in (Schreiber et al., 1994; Bloom et al., 2004; Knies et al., 2017; Miller, 2017) for instance. We discuss that this is mainly a requirement for evolution to be fast, as compensatory mutations on stability can in principle ensure the stability requirements (Tokuriki et al., 2008; Tokuriki and Tawfik, 2009; Storz, 2018) but may be more or less common depending on the evolutionary context (Weinreich et al., 2006; Lunzer et al., 2010; Tomala et al., 2019). We also discuss the role of mutational robustness on stability in making more mutations accessible, possibly improving evolvability in large populations (Bloom et al., 2006, 2007). Please note that we chose to treat this issue discursively and did not perform simulations; otherwise, it would have pushed us to make fuzzy phenomenological choices in contradiction with the mechanistic approach used to model the functioning of enzymes, as discussed in our response to reviewer #2.
- Second, that we have not considered all dimensions of an enzyme's phenotype. We also agree, and we have addressed this issue by running two new sets of simulations: one where a protein's concentration also evolves, which required extensive work to document and model the direct cost of protein expression

(Lynch and Marinov, 2015; Novick and Weiner, 1957; Stoebel et al., 2008; Wagner, 2005) and the indirect cost caused by molecular crowding (Andrews, 2020; Dill et al., 2011; Dong et al., 1995), and one where we consider the influence of pleiotropic mutations (Stiffler et al., 2015; Heinrich et al., 1991) affecting simultaneously k_{cat} and k_f. Our results appear to be robust to these changes, though the joint evolution between kinetic parameters and concentration can explain a small part of the observed variance.

- Last, that the relation to past theoretical work on enzyme evolution remained unclear. We now clarify that the shape of the fitness landscape with a plateau at high catalytic efficiencies is a result that seems extremely robust to modelling choices, be they to consider fitness as a capability to cope with antibiotics (Knies et al., 2017; Bershtein et al., 2017; Stiffler et al., 2015), as a flux of energy responding to a given flux of nutrients (Dean, 1995; Dykhuizen et al., 1987; Hartl et al., 1985; Yi and Dean, 2019) or, as we introduce, as an emerging feature of the competition for external resources. We also make it clear that our population genetics model contradicts the expectation that enzymes will spread onto the fitness plateau (Clark, 1991), because the steady-state resulting from the mutation-selection-drift balance will generally occupy a narrow part of the landscape. Accordingly, understanding the extensive variance in enzyme features reported in the literature requires a careful theoretical examination of how the plateau moves depending on various parameters. As far as we know, the present work is the first attempt to provide such an explanation in the case of metabolic pathways (first steps have been made for antibiotic resistance (Walkiewicz et al., 2012; Rodrigues et al., 2016)).

You will find below a point-by-point response to the reviewers' comments and concerns.

2. Reviewer #1

2.1. Comments to the Author

RC: ***The authors provide a theoretical evaluation of how the well known inefficiencies of enzyme function (or perhaps their suboptimal physical properties) may be an inherent attribute of the evolutionary fitness landscape. The authors study both a passive and facilitated diffusion model wherein a molecule such as a sugar first moves across the membrane and is then used as the first substrate of a non-branched multi-enzyme metabolic pathway. A second enzyme then uses the product of first enzyme. Using largely classical Michaelis-Menten approaches the authors plot kcat vs kf (kforward) and the consequent productive flux of the product made from the transported sugar. They note that the fitness landscape (product flux is used as a proxy for fitness in this case) shows a rather flat plateau that spans a considerable range of values of kcat and kf. This rather large plateau suggests that enzyme evolution within these systems are largely unresponsive to selective forces that would be more suggested by a more peak-like fitness landscape. The authors posit that this is unexpected and a central theme of the manuscript. The plateau also features a cliff-like fall off that essentially circumscribes kcat/kf defined plateau in both single enzyme and two enzyme projections (Fig 1 and Fig 4). Thus the major conjecture is that the plateau captures the wide range of enzyme values reported in a data set from Bar-Even et al 2011 (Fig 5). They go on to construct a population genetics based model that includes selection, mutation and drift.***
The question of why enzymes are not selected for optimality seems more linked to the idea of "sufficiency" than making the best enzyme. Protein engineers have shown for decades that catalytic efficiency in enzymes can be improved, but if it makes no difference in fitness these changes are not fixed within the population. That there are many ways of achieving sufficiency via changes in kcat, protein turnover, expression, etc is in itself not surprising and would seem to account for the breadth of enzyme character-

istics seen in Bar-Even 2011. There are important questions that limit my enthusiasm for the work that should be addressed including significant previous research from the Dean group that has already experimentally mapped out biochemical fitness landscapes that include comparable features. These questions are detailed below.

We thank the reviewer for his/her insightful comments. In our model, fitness arises as a result of the ability of cells to uptake nutrients from the external environment, which itself relies on the efficiency of a metabolic pathway. Therefore, there always exists a fitness optimum for enzymes close to their physical limits. We think that this is a slightly different situation than selection for sufficiency, in which – as we understand it – the fitness landscape becomes completely flat above some flux threshold.

Nonetheless the two situations may in some situations yield similar results, as the landscape becomes effectively flat above some threshold in our model – the threshold itself essentially depends on effective population size. The results of our population genetics model should thus apply to selection for sufficiency as well. In particular, we show that Evolution should reach a very precise mutation-selection-drift balance, close to the fitness cliff, assuming that enzymes are not physically constrained below the plateau and that mutations are slightly biased towards lower efficiency. The existence of a plateau is thus by no means an explanation to the reported diversity of enzyme kinetic parameters, therefore confirming the intuition that enzyme evolution can in principle be predicted (Walkiewicz et al., 2012). This requires that we understand how the mutation-selection-drift balance changes with the fitness landscape, which itself is shaped by the biochemical context encountered by an enzyme. Making this point, and investigating the dependency of the fitness landscape to our model's parameters, is the main object of our study as we hope to have clarified, first in the introduction:

> Based on this premise, we confirm that the evolution of 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 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 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 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.

... and in the conclusion, with these two statements:

> Because the mutation-selection-drift balance occupies a narrow part of the landscape, this makes the evolution of an enzyme, 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.

> 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, and further improvements of this model should allow to predict the expected features of individual enzymes.

Please find below a point-by-point response to your concerns.

2.2. Major concerns

RC: ***The shape and large plateau feature is strongly a product of the reversibility and total amount of enzyme. In the model, the authors set these parameters as constants using a high enzyme concentration (1 mM) and low reversibility. The reversibility and total protein are certainly under selection as the authors correctly suggest on p15 and therefore it would seem to me that the plateau itself is just as readily defined by those parameters. Why are variables that are so tightly linked to the shape and fitness landscape considered less important than the ones selected? Kcat and protein concentration are both selectable attributes and many studies have shown that expression and protein stability are just as important.***

We agree that other dimensions of enzyme kinetics that we have considered as parameters in the model may evolve, in particular an enzyme's concentration. Our initial focus on k_{cat} and k_f was on par with the Bar-Even dataset that only contains proxies for these kinetic parameters. We have addressed the reviewer's concern regarding enzyme concentrations by running evolutionary simulations where enzyme expression was subjected to mutations, along with k_f and k_{cat}. Enzyme expression, however, is peculiar because it is costly, contrary to k_f and k_{cat}, such that it does not evolve on a cliff-like plateau (see (Yi and Dean, 2019), Figure 6 for instance). We have considered two documented costs to build our model: the energy budget required from mutants with increased expression to produce the extra enzymes (a linear cost on enzyme concentration), and the crowding effect on cellular traffic that impacts diffusion exponentially. We have also considered a combination of the two costs. The details of the evolutionary model for enzyme expression are given in the last result section, and the results are shown on Figure 6. The intermediate results with the effect of each cost in isolation can be found in the section Text S5.2. Our results are generally not impacted, as selection for enzyme expression is stabilizing. Still, under these two assumptions, the selection against the protein burden pushes enzymes towards higher efficiencies within population having higher N_es, an effect which should nonetheless be counterbalanced by the adverse selective pressure of noise in gene expression, at least in Prokaryotes whose cell size is smaller. Discussion of these results can be found in the discussion section and is quoted here:

> 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. 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, 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 described in our results, possibly explaining the confusing observation that species with larger populations (and smaller sizes) do not express markedly more efficient catalysts.

We did not study further the evolution of k_r and K_{eq} for two reasons: first because the effect of changing this parameter is already presented in the manuscript and the precise results are shown in our investigation of the shape of fitness landscapes (see Figures S10,S11 and S12-A of SM for these effects), which we shown to be the main driver of evolution, and second because reversibility is an intrinsic feature of reactions that makes k_f, k_{cat} and k_r interdependent due to their common involvement in K_{eq}, which is a fixed quantity. This codependency may play a role in the optimal kinetics of an enzyme, as discussed by (Klipp and Heinrich, 1994), but can only be studied quantitatively on a case-by-case basis. Nonetheless, this approach is in agreement with the clarified goals of our study, which is first to show that Neutral Evolution cannot account for enzyme variability and second to show that there exists several specific constraints (toxicity, reversibility,metabolic demand) acting on enzymes and virtually making their fitness landscape unique.

As the reviewer points out, stability is another dimension that should be considered. We agree and now discuss the importance of protein stability. Our model makes the assumption that stability and kinetic parameters can evolve somewhat independently – which is compatible with mutations being pleiotropic on average – either because some mutations can change function with little impact on stability, or that reduced stability can be compensated. While our model includes the former type of mutations, the latter type would only slow Evolution down and give a special role to the evolution of robustness. Our assumption that Evolution proceeds on a space of stable enzymes where activity can change by mutation is now introduced in the introduction:

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 Eijsink, 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.

RC: ***It was unclear why the authors built the model such that the intermediate of enzyme 1 (P1) is "highly detrimental" (p 13) and required a branching path (ηd) to remove it. The authors state that the pathway is non-branched but here it seems it is branched in a specific way that is not necessarily consistent with either their statement or a correct biological context. Are most intermediates highly detrimental? Why was this necessary? As shown in Fig 4B the size of the plateau is dependent on this variable when the second enzyme is present.***

We now clarify that "non-branched" means that the metabolic pathway from the first substrate to the final product is linear and that no alternative pathway useful to the cell exists. Yet an outward flux of nutrients from this unique pathway is considered. As stated in the manuscript, this outward flux of metabolites prevents the unrealistic accumulation of metabolites processed by inefficient enzymes:

> In contrast, this 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 steady-state value.

This assumption is biologically relevant first because intermediate metabolites can diffuse (passively) towards the external environment, where it is supposedly in low concentration (exactly like sugars do in the case studied in the first section of results and SM). In this case the constant η_d is a diffusion constant; but it may also correspond to non specific interactions trapping the metabolite through undesired promiscuous activities (Khersonsky and Tawfik, 2010; Schäuble et al., 2013; Peracchi, 2018). We clarify that non specific interactions is an inherent property of biomolecules whose behavior can be approached by the linear cost above (as has already been done in one of the references suggested by rev. #1 (Chou et al., 2014)), because it follows Michaelis Menten kinetics with a (very) low affinity, as stated in the manuscript:

> This is an obviously unreasonable assumption, since a part of this standing substrate should be lost by outward diffusion or degradation (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 η_d (Chou et al., 2014) (assuming that the external environment is infinite, the degradation term can as well represent an efflux). 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 should follow Michaelis-Menten kinetics albeit with much lower affinities, hence following an approximately linear relationship up to very high cellular concentrations

We show that differences in this linear degradation rate could contribute to explain part of the variance in reported enzyme features, as described in the manuscript:

> 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 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 η_d allows for higher concentrations (see SM Figure S4) and makes the flux tolerant to second enzymes with lower k_fs, 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 η_d (see FIG. 4-B).

Second, the metabolite could be toxic, either due to these non-specific interactions or to more specific ones, producing damaged metabolites for example (Niehaus and Hillmann, 2020). In this revised version of the

manuscript, we have also considered cytotoxicity through a non-linear cost, as already modelled in (Clark, 1991; Wright and Rausher, 2010). Our results suggest that the shape of the toxicity / loss of metabolites function can influence the shape of the fitness landscape and in particular the epistatic relationships within a pathway, because coping with toxicity effects is dependent on the level of flux, as discussed in the end of the same section (and extensively in the dedicated section of the SM):

> The shape of the negative relationship between metabolite concentration and fitness can be important (Figures 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 and k_{cat}. Taken together, these results show that the precise epistatic relationship between enzymes in a pathway will depend 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}.

Finally, these effects are certainly ubiquitous but should vary in importance between metabolites, as the reviewer #1 highlighted. This should thus contribute to the observed variability in enzyme features as we intended to show.

RC: ***A feature that is striking in the fitness landscapes (Fig 1 and Fig 4) is the steep cliff like loss of fitness. Essentially fitness goes from essentially completely fit (using the 0.9 isocline in Fig 1) to near 0 fitness over what appears to be about a two-fold change of either kf or kcat. The authors may wish to look at Walkiewicz et al PNAS 2012 which provides an experimentally measured relationship of fitness to an enzyme performance in which a drug diffuses across a membrane and is inactivated prior to reducing fitness essentially an inverse relationship but one that measures protein levels and kinetics to show a fairly steep loss of fitness as well. Though not a sugar it might be useful as an experimental example. While experimentally validated fitness landscapes are not common there is a significant body of knowledge that is not included/evaluated in the manuscript.***

AR: Thanks for pointing this reference to us. We have added a short discussion on drug resistance empirical fitness landscapes that includes this paper:

> 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 it is necessary to predict evolutionary outcomes (Walkiewicz et al., 2012).

As described in a previous answer, it is interesting to see that in all these systems initiated by uptake the dimensionality of the landscape matters. Besides, in (Walkiewicz et al., 2012), they have also shown in the context of antibiotic resistance that fitting the right fitness landscape allows to predict evolutionary outcomes much alike what we have started to address here in the wider context of metabolism. It was thus logical to mention it in the discussion when we speak about the predictability of Evolution:

> 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.

RC: ***Also Yi and Dean (Mol Biol Evol 2019) provide a comprehensive review of their work and that of others including a prominent example from Chou et al 2014 PLOS Genet (Fig 6 in the review) of methanol catabolism (FlhA and FghA) that displays excellent examples diminishing returns and sign epistasis that shows a multi-enzyme plateau with steep fitness. Also examine Box 3 of the review for the same flat plateau featuring only in this example (from Dean et al 1995 Genetics) it is a metabolic network with a facilitated diffusion permease.***

AR: Thanks again for pointing us to another very relevant reference. We now discuss these works more extensively in the introduction:

> Enzyme kinetics evolution has often been considered theoretically through the lens of flux control (Burns et al., 1985; Clark, 1991; Fell, 1992; Kacser et al., 1995; Yi and Dean, 2019). Indeed, 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 (Dykhuizen 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; Dykhuizen et al., 1987; Tokuriki et al., 2012), a finding that has since received empirical confirmation (Fell, 1992; Dean, 1995; Lunzer et al., 2005; Yi and Dean, 2019; Chou et al., 2014).

Throughout their work, they find that transporters have a high control on flux, which our work confirms, such that transporters should therefore be under strong directional selection. Their evolution should thus not be addressed outside the context of global epistasis (Weinreich et al., 2013; Otwinowski et al., 2018; Reddy and Desai, 2020), as we mention in the discussion. More importantly, they do not predict expected kinetic constants under the mutation-selection-drift balance, which is an important part of our argumentation that enzyme kinetics evolution can be predicted, such that determinants of the fitness landscape matter.

Chou et al. (2014) show that the combination between diminishing returns and costs of proteins and metabolites should lead to stabilizing selection discriminating against high activities. This is because activity is studied through the only dimension of enzyme expression, which is under balancing selection precisely owing to its costs. We designed a new instance of our model to test the outcome of the joint evolution between enzyme kinetics and levels of expression, showing that high levels of expression are indeed counterselected and enzyme kinetics remain dependent on N_e.

There is an an obvious factor that we discuss but did not include, which is noise in gene expression. This latter could help explain why enzyme kinetic constants do not comply with expectations from the Nearly Neutral Theory in high effective population sizes but small-celled Prokaryotes. One can see SM - section Text S6 for the explanation, that we reproduce here:

> One explanation that can account for the discrepancy between these clear-cut results and empirical data is noise in gene expression, whose influence is also manifold as shown by ((Wang and Zhang, 2011). In the competition for resources, a cell needs to possess exactly the right amount of enzymes for a given set of kinetic parameters. To ensure that this happens, a cell has to possess continously at least one transcript of a specific enzyme. Since typical prokaryotic cells range around Vcell = $1\mu m^3$, for a prokaryotic

cell to have at least one transcript yields a concentration [RNAm] = $1/(6.02.10^{23}.10^{-15}) \approx 1nM$ Making the conservative hypothesis of a 1:100 ratio between transcripts and proteins, this yield a noisy concentration threshold around $[Etot] \approx 0.1M$ for this kind of cells. This is a highly conservative estimate of this threshold, for 1 transcript on average is far from sufficient to alleviate noisiness and even less to tune the kinetic activity with the needed subtelty. As a consequence, the threshold should be one or two order of magnitude higher and typically stands around 1 - $10\mu M$ for average prokaryotic cells, therefore precluding the access to low concentrations in prokaryotes in a volume dependent manner, with smaller prokaryotes being even more constrained than the above estimate. Small cells are thus subject to a complex balancing selection acting on their protein content that needs to be adressed carefully to better understand how enzyme concentrations are tuned, but in Prokaryotes at the least, concentrations are restricted within a narrow range which cannot explain most of the observed variability.

2.3. Minor concerns

RC: ***These 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 their reported estimates are rather weak (Davidi et al. , 2016)". I assume "These effects" refers to the previous references of protein crowding and so forth but it is not clear to the reviewer that the conclusion that "the reported patterns" (which patterns?) cannot be accounted for by combinations of these physical phenomena. Also enzyme complexes are often locally restrained to mitigate issues of substrate/product diffusion such as in membranes where proteins form larges assemblies to produce a more two dimensional environment.***

We agree that the formulation was awkward and hope to have made it clearer in this new version. In addition, we removed the idea that these effects might be correlated because the fact that they are weak is sufficient to make our point. You will find below the new formulation:

> Crowding effects are obviously important for our understanding of enzyme evolution but, alone, they are definitely too weak to explain the wide variability across enzymes insofar as their reported estimates typically lie in the range of one order of magnitude (Davidi et al., 2016).

It is true that enzyme and enzyme complexes might be locally restrained, a process that we mention in the discussion when talking about the process of metabolic channeling and compartmentalization. However, it still remains to be determine how ubiquitous and widespread this phenomenon is. If compartmentalization and channeling turn out to be important, they can readily be accounted for in our model as they would mainly influence an enzyme's effective concentration.

RC: ***"Our calculations therefore suggest that primordial cells, preceding the evolution of FD, must have been extremely small." This is a speculative statement. While interesting, it is not properly tested or validated in this paper and should appear in the discussion (if at all). Also that cells "must" have been small seems an overly strong assertion based on the provided information.***

We agree that this statement was too definitive and that our framework was not built to deal with this question. We thus removed it from the manuscript.

RC: ***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." I am not clear what "intervening" means in this sentence. Perhaps the authors could clarify this sentence.***

This sentence has been removed since we no longer state that enzymes involved in a given pathway should evolve on a common fitness landscape, which we think was misleading.

RC: ***"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." Should be evolutionarily suboptimal. Again though selection is for sufficiency not optimal enzyme performance.***

We agree that "appear to be evolutionary suboptimal" was inappropriate. We thus also removed this statement since the idea that Natural Selection could act differently in the context of global epistasis and lead to a different mutation-selection-drift balance was already sufficient to make our point. We also pointed to references discussing how the evolutionary process might be impacted by such phenomena:

> Fitness instead results from a wide range of metabolic pathways that combine together and should all be competitive to certain degrees. How 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 (Iwasa et al., 2004; Weinreich and Chao, 2005; Weissman et al., 2009).

3. Reviewer #2

3.1. Comments to the Author

RC: ***It has a puzzle in protein evolution why enzymes, despite the strong functional selection for their efficiency and the high fitness advantage fitness this confers, experimentally measured efficiencies of enzymes are still several orders of magnitude lower than the physical limit (Bar-Even, Biochemistry 2011). This work aims to explain this puzzle by developing a population genetics model of protein evolution based on Michaelis-Menten kinetics and flux-balance analysis. Similar models that used ab initio fitness landscapes based on flux, activity, stability, or selection against misfolding have been proposed in the past, which all exhibit a mesa-like fitness landscape. Under mutation-selection balance, such landscapes result in the near-neutral theory (Ns 1), which is also a major finding of this study. Nonetheless, in contrast to previous works that described enzyme efficiency using one parameter e=kcat/KM, here they consider the evolution of kcat and KM separately. In addition, they also investigate the role of nutrient transporters, the concentration of metabolites, and the reversibility of enzymatic reactions on protein evolution. The premise is sound, but I have major conceptual reservations with the current model and, consequently, with the conclusions.***

We thank the reviewer for his/her insightful comments and the time dedicated to evaluate and comment our draft. We do agree that the shape of the fitness landscape emerging from our framework is similar to other frameworks, which we now discuss in detail (as shown below). Nonetheless, and notwithstanding the novelty of separating the different dimensions of enzyme kinetics, our understanding was that the plateau in the fitness landscape was considered as a convenient explanation to the observed variance in enzyme features ((Clark, 1991), for instance), which were considered to explore the plateau in a nearly neutral fashion. The results of our population genetics model contradict this intuition, as we now make more explicit, thus requiring a precise understanding of the forces that shape an enzyme's fitness landscape. This makes our study relevant to uncover the principles behind the variation in enzyme features.

3.2. Major concerns

RC: ***Similar models of protein evolution on mesa-like fitness landscapes have been proposed in the past (not an exhaustive list): *fitness flux (Dykhuizen Genetics 1987; Kacser, Genetics 1981; Rodrigues, PNAS 2016; Bershtein Mol Cell 2013; Berhstein PLoS genetics 2015; Soskine Nat Rev Gen 2010); Yang Zhang Mol Systems Biology 2010). *fitness stability (Taverna Proteins 2002; Bloom Genetics 2007; Bloom PNAS 2006; Bloom PNAS 2005; Zeldovich PNAS 2007; Serohijos MBE 2014; Wylie PNAS 2011); *fitness 1-(misfolding propensity) or selection against protein misfolding (Drummond Cell 2008; Serohijos Cell Rep 2013; Yang Zhang Mol Systems Biology 2010)***
Under mutation-selection balance (Ns 1), these models have explained many observations in protein evolution, such as the distribution of folding stability (Zeldovich PNAS 2007), rate of protein evolution (Drummond Cell 2008; Serohijos Cell Rep 2013; Yang Zhang Mol Systems Biology 2010), log-normal distribution of evolutionary rates (Lobkovsky PNAS 2010), fitness effects of polymorphisms, etc. However, none of these other selective pressures on protein evolution are discussed or included in the model. After all, the selective pressures outlined above are also experienced by the enzymes tabulated by Bar-Even et al., which this current work aims to explain.

We thank the reviewer for this extensive list of references. We agree that our discussion about other models was limited in the previous version. We now explicitly mention that models of protein evolution have enabled to explain many reported patterns in molecular evolution and that we intend to address a particular and independent question treating the distribution of enzyme activities when the mutation-selection-drift balance is reached (as detailed in the answers to the next two comments). In parallel, we now describe prior theoretical work to show that those interested in central metabolism have generally considered 1-dimensional landscapes, contrasting with studies on drug resistance showing that many dimensions influence the actual efficiency of an enzyme. This discussion can be found in the introduction:

> 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 quickly reach a fitness plateau and evolve on nearly neutral landscapes (Ohta, 1992). 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} and enzyme concentration (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 it is necessary to predict evolutionary outcomes (Walkiewicz et al., 2012).

Moreover, it is often assumed that fitness plateaus should come with neutral evolution, which is not the case here and thus questions the fact that the plateau could explain enzyme kinetics variability.

We also clarified that our goal at this stage was neither to explain the full dataset of Bar-Even (2011) – which includes many enzymes involved in secondary metabolism for which the relation with fitness is less direct than in our model – nor to fully resolve the matter for the other ones, for which information is still lacking. As a consequence, we removed the plot displaying the full dataset, which was misleading, and focused on enzymes contributing to primary metabolism (namely carbohydrates, amino acids, fatty acids and nucleotides).

RC: ***Why is stability not accounted for in the model or even mentioned in the paper at all? The expectation is that fitness (protein dose or folded copies in the cell)*(activity), where abundance (probability of folding) (see Soskine Nat Rev Gen 2010; Yang Zhang Mol Systems Biology 2010; Manhart PNAS 2011, etc). The***

evolution of enzyme activity cannot be taken outside the context of folding stability (see extensive works of Bloom (Bloom Genetics 2007; Bloom PNAS 2006; Bloom PNAS 2005 for example)). More directly, even the ability to design enzymes with novel catalytic function is premised on such designed sequences exhibiting substantial energy gap between folded and unfolded states (see works of David Baker and Brian Kuhlman). Thus, I find it rather odd to include the effect of transporters that may not be relevant for all enzymes, but not stability.

Of course, stability is a prerequisite for a functional protein and we should have mentioned it. Thanks to the reviewers' comments on this question, we now explicitly state that one underlying assumption of our model is that evolution proceeds on a space of genotypes with high enough stabilities to ensure a correct folding while differing by their enzyme kinetic features. We also discuss that this may be a simplification of reality, especially as mutations may be pleiotropic for enzyme function and stability. In consequence, evolution may rely on the presence of compensatory mutations for stability, which we argue could slow down but not prevent the evolution of enzyme kinetic parameters, or give a special importance to robustness against destabilizing mutations:

> 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 Eijsink, 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.

We also clarified that in our framework, the selective pressure is first governed by the flux of metabolite an enzyme has to process such that our results should hold even if no transporters directly drive the pathway in which a specific enzyme is embedded (see Figure S9 of the SM).

RC: ***To first order, almost all random mutations affect folding stability (Tokuriki JMB 2007), but 10% of them affect will hit the active site and has strong effects on activity. Moreover, there is a known trade-off that mutations that improve activity are more likely destabilizing, which is a major source of mutational epistasis in protein evolution. These are additional arguments to include stability in the evolution of KM, kcat, or their ratio. Integration of stability in pop-gen models of protein evolution is well established and should be easily incorporated into their fitness landscape (see references above). Doing so, it will be easier to relate the conclusions of this work to prior publications.***

We agree that including stability in our model would be an important addition, which we have seriously considered. One way of doing so would have been to include a probability of folding in our model where protein concentration is also considered, such that the cost of producing misfolded (inactive) proteins would be immediate due to the cost of protein production. This is now discussed in the introduction:

> 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).

However, we think that modelling the interaction between stability and function requires a different approach

considering the underlying mechanisms at the protein sequence level, and thus perhaps based on one or a few well-known enzymes (as was studied for instance by (Knies et al., 2017)). First, because while the cost of misfolding seems immediate, the negative effect of overstability requires more developments, and second because the pleiotropic effect of mutations arising from this interaction should be key and needs to be modelled carefully. Moreover and as hinted in our previous answer, robustness to destabilizing mutations should play an important role, adding much complexity to the evolutionary dynamics.

We therefore chose to treat this issue discursively, with an explicit mention of our assumption that a space of genotypes with high stabilities exists and can be navigated through, and with an extensive discussion on how our results could be affected by selection for stability. The fact that more mutations affect folding stability than activity does not undermine this idea and may even bring some evidence for it. Indeed, our (quite speculative) opinion is that there is at least some degree of independence between stability and function as found in (Schreiber et al., 1994; van den Burg and Eijsink, 2002; Bloom et al., 2004; Knies et al., 2017; Miller, 2017) that allows compensatory mutations for stability (possibly on sites affecting only stability as reviewer #2 points out), such that the speed of evolution could be impacted but not its endpoint, or marginally. We agree that this assumption may not always hold as argued by (Tomala et al., 2019), and, in fact, this suggests an interesting perspective to the field as it may already be testable whether or not specific enzymes are less efficient in species needing higher protein thermostability.

RC: ***If indeed cellular factors, such as transporters, have a stronger effect on enzyme evolution than selection against misfolding (Drummond Cell 2008) for example, then I would expect that having or not having a transporter will have a stronger effect than codon adaptation index (CAI) for 10K enzymes cataloged by Bar-Even.***

We have never stated that transporters have a stronger effect than misfolding. We clarify in the current version – where misfolding and stability are indeed discussed – that selection against misfolding is strong and that high enough stability is a prerequisite for a functional protein. As discussed in the answer to the previous comment, the interaction between stability and function is far from straightforward, making trends of the CAI or other genomic metrics of selection quite difficult to anticipate at this stage.

RC: ***The rationale for including the role of transporters is that the data by Bar-Even from various pathways predict different equilibrium distribution under mutation-selection balance. However, the authors provide no statistical test for the comparison between model and experimental data in Fig. 5B. A simple K-S test between the predicted distribution and data will be informative.***

We should have made clearer that our models cannot predict the distribution shown in fig. 5B (now fig.3) with the amount of information that we currently have. The data points in fig.3 show different enzymes that indeed share similar transporters, but that may otherwise evolve in quite different contexts. For instance, some may be preceded in the metabolic pathway by a highly reversible enzyme, which would move their own fitness plateau towards high k_f and k_{cat}. Likewise, different population sizes, enzyme concentrations, cytotoxicity of metabolites, etc. should account for differences in the mutation-selection-drift balance between enzymes and contribute to the distribution. We have made the choice to not introduce random variation in these features to try and reproduce the distributions observed, which would have made the suggested test possible but based on multiple arbitrary choices. Instead we have clarified this matter in the text, especially that the comparison with the data is a small first step to indicate that predicting enzyme evolution may be possible in the future, as it has been attempted in the specific case of drug resistance (Walkiewicz et al., 2012; Rodrigues et al., 2016). This precision is present at the end of the introduction :

> We thus 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.

In the results section dedicated to the data, we now also insist on differences existing within a pathway that prevent a generic prediction at this stage:

> 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 involved in the central carbohydrate metabolism as categorized by Bar-Even et al. (2011) have on average higher k_{cat} and 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.

RC: ***An important readout from these ab initio fitness protein fitness landscape and pop-gen models is the rate of protein evolution (Ka, Ks, and Ka/Ks) as a function of the model parameters (stability, activity, abundance, etc.) (see reference above for the analytic work and simulation). Thus, what would be the expected rate of evolution as a function of Kcat, Km, or abundance from their model? Are these predictions validated by the enzymes in Bar-Even.***

On the one hand, our model was not built to understand protein evolution at the level of sites and as such, insights about the rate of protein site evolution are beyond its scope. Our model instead focuses on the mutation-selection-drift balance of the kinetic parameters, that we acknowledge is due to the sequence and structure of an enzyme. Despite recent advances to better characterize the link between protein residues and their structural and kinetic features, it still remains largely unattainable to make a general prediction of k_{cat} and k_f based on their sequences because, even if we were to know how mutations globally affect the energy profile - which is not the case to our knowledge - their influence on parameters would still be very dependent on a substrate's energy profile. However, this question has started to be addressed in different frameworks (Echave, 2019), which may open the door for future improvements if a way can be found to merge these frameworks.

On the other hand, we have examined the joint evolution between activity features and abundance levels in this new draft, which can help explain why there exists some highly expressed enzymes and, in turn, why these enzymes evolve more slowly at the molecular level as shown in (Drummond et al., 2005; Serohijos et al., 2012). Because we did not account for all cellular constraints acting on protein expression, our model cannot yet make precise predictions, but it is definitely promising: for example, enzymes involved in carbohydrate metabolism should on average evolve more slowly than enzymes involved in other pathways.

RC: ***The authors assume that the effects of random mutations on Kcat and KM are independent. However, from my recollection, this is a correlation between Kcat and Km for random mutations. See for example***

the well-studied enzyme beta-lactamase (Stiffler Cell 2015, particularly SI and references therein). How might relaxing this assumption affect the predictions of the model and the extent to which it can explain the data by Bar-Even?

There is indeed a known trade-off between k_{cat} and K_M, largely arising as a by-product of the mathematical formula for $K_M = \frac{k_{cat}+k_r}{k_f}$ in which increasing k_{cat} comes at the cost of the affinity $1/K_M$. This is already present in our framework because a mutation that affects k_{cat} also affects K_M and therefore has inevitably a pleiotropic effect. Yet, because there might also exists some physical pleiotropic effects due to codependencies in energy profiles (Heinrich et al., 1991), we built an instance in the model where mutations on k_{cat} and k_f were correlated. We tested both negative and positive mutational correlations and shown that evolutionary outcomes were largely unresponsive to these changes, except that reaching the mutation-selection-drift balance was longer (see SM - Figures S18, S24 and S25). This approach is detailed at the end of the results section dealing with the evolution of k_{cat} and k_f:

> 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 ρ (see Materials and Methods for details).

3.3. Minor concerns

RC: ***Abstract: "[u]sing a population genetics model that includes genetic drift and mutational biases, we show that this is a very unlikely outcome of evolution on a common landscape… [instead] our results point to drift playing an important role…" This seems contradictory and confusing.***

We changed the abstract accordingly:

> While the existence of a large fitness plateau could potentially explain the extensive variation in enzyme features reported, we show using a population genetics model that such a 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 are considered. Instead, differences in the 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.

RC: ***Again, similar models have been proposed, including those that account for the diffusion of substrates across the cell membrane (Stiffler Cell 2015).***

We agree that the case examined in (Stiffler et al., 2015) - and in (Walkiewicz et al., 2012) - is relevant to our study, since both these papers deal with substrates diffusing across the membrane before being processed. Furthermore, they both show that fitness landscapes must account for the multidimensionality of enzyme efficiency. We now mention these studies in the introduction:

> 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 it is necessary to predict evolutionary outcomes (Walkiewicz et al., 2012).

Nevertheless, these models (and experiments) are specifically designed to study antibiotic resistance, contrary

to our work that focuses on metabolism. Likewise, our findings should differ on the biochemical properties influencing the landscape (*e.g.* reversibility).

RC: ***Effect of population size and mutation rate on protein evolution have been articulated before (Wylie PNAS 2011).***

Although the reference is interesting, we have now made it clearer that we are interested in enzymes involved in metabolism. It is therefore very different than the case of viruses – that rely on the host metabolism – explored in the suggested reference that arguably face distinct selective pressures. It thus appeared a little far-fetched to us to discuss it in the context of our paper.

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