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Rubisco isn't really so bad

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Abstract

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the most widespread carboxylating enzyme in autotrophic organisms. Its kinetic and structural properties have been intensively studied for more than half a century. Yet important aspects of the catalytic mechanism remain poorly understood, especially the oxygenase reaction. Because of its relatively modest turnover rate (a few catalytic events per second) and the competitive inhibition by oxygen, Rubisco is often viewed as an inefficient catalyst for CO_2 fixation. Considerable efforts have been devoted to improving its catalytic efficiency, so far without success. In this review, we re-examine Rubisco's catalytic performance by comparison with other chemically-related enzymes. We find that Rubisco is not especially slow. Furthermore, considering both the nature and the complexity of the chemical reaction, its kinetic properties are unremarkable. Although not unique to Rubisco, oxygenation is not systematically observed in enolate and enamine forming enzymes, and cannot be considered as an inevitable consequence of the mechanism. It is more likely the result of a compromise between chemical and metabolic imperatives. We argue that a better description of Rubisco mechanism is still required to better understand the link between CO_2 and O_2 reactivity and the rationale of Rubisco diversification and evolution.

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39), catalyses both the carboxylation and oxygenation of ribulose-1,5-bisphosphate (RuBP) to yield either two 3-phosphoglycerate (PGA) molecules, or one PGA and one 2-phosphoglycolate (PG). It is the most widespread carbon-fixing enzyme, being present in more than 99.5% of autotrophic organisms (Raven, 2009). As such, since its discovery in the mid 1950's, its structural, mechanistic and kinetic properties have been extensively studied. The main features of the catalytic mechanism have been explored both experimentally and computationally and structural data obtained with X ray crystallography on different Rubisco types and mutants, providing information on structure/function relationships (for a review, see (Andersson, 2008, Cleland, Andrews, Gutteridge, Hartman & Lorimer, 1998). Taken as a whole, Rubisco's mechanism is rather complicated and involves at least three intermediates and four transition states, with tight stereochemical constraints (Cleland et al. 1998). However, as pointed out elsewhere (Tcherkez, 2013), several important details of the catalytic process remain unclear. First, the sequence of proton transfers and exchanges involved in the reaction is still incompletely understood. Second, the origin of the reactivity with O_2 (oxygenase reaction) is unknown (Tcherkez, 2016). Third, the reasons for the limited turnover of Rubisco and its promiscuity (i.e. its propensity to catalyse other reactions) are not well identified, so that both "sluggishness" and "confused specificity" (Morell, Paul, Kane & Andrews, 1992) are now tacitly accepted in the literature, with limited consideration of practical constraints on the mechanism. In addition,

despite 50 years of intense research efforts, attempts to improve the catalytic performance of Rubisco have been so far unsuccessful. This simply emphasizes the need to better understand basic questions associated with Rubisco catalysis: the knowledge of precise mechanistic constraints during Rubisco kinetics will aid current research for a better enzyme.

More generally, the catalytic performance of enzymes has intrigued biochemists for over a century. Recent progress in metabolic engineering has revived interest in metabolic systems, in which enzymes are central actors (Bar-Even & Tawfik, 2013, Erb, Jones & Bar-Even, 2017). In turn, designing catalysts that can optimally sustain a functional metabolic system requires an understanding of how both biophysical and regulatory properties have constrained enzyme evolution. This task is now facilitated by the use of comprehensive databases that include many enzyme properties (kinetics, structure, thermodynamics) and can be interrogated in order to identify key chemical imperatives for catalysis.

In the present case, considerable advances have been made in the past few years in understanding the mechanisms of several enzymes closely related to Rubisco. This provides a good opportunity to reassess Rubisco's chemical mechanism and, perhaps, provide a fresh perspective on long-standing questions. Since its discovery in the 50s, Rubisco has nurtured various theories about the evolution of its mechanism and, as mentioned above, it is often believed to have a particularly slow turnover rate and a confused specificity for its gaseous substrate, CO_2. In practice, the justification of comparative adjectives, such as "slow" and "confused", remains limited. In reality how slow and nonspecific is Rubisco? Using comparisons

with other enzymes, we will show that Rubisco *(i)* is not particularly slow, *(ii)* has a good affinity for CO_2, and *(iii)* is not exceptional in being promiscuous. Furthermore, considering recent observations on enzymes adapted to similar chemical imperatives, we show that Rubisco has evolved in response to both chemical and metabolic constraints. Aspects of Rubisco's mechanism remain poorly understood, and some catalytic improvement may still be possible. However, our analysis supports the view that Rubisco should be regarded as a very good catalyst already.

How slow is Rubisco?

What does "slow" mean for an enzyme?

Answering this question necessarily requires a comparative reference point. Physico-chemical limits to catalysis have been investigated many times. Although elementary chemical reactions can theoretically be as fast as 10^{12} s^{-1}, k_{cat} values for enzymatic reactions are unlikely to exceed 10^6-10^7 s^{-1} in practice, because catalysis involves cooperative conformational changes in the enzyme structure to optimize binding of the transition state, and conformational changes need some time to occur (Hammes 2002; see also Box 1). In addition, k_{cat}/K_m, which can be viewed as the rate of productive (i.e., leading to complete catalysis) capture of the substrate (Northrop, 1998), is limited by the frequency of collisions between the enzyme and its substrate, and therefore by the diffusion rate of small molecules in solution, which cannot exceed 10^9-10^{10} M^{-1}

s^{-1} (Fersht, 1999). Some enzymes that are believed to be diffusion-limited do in fact exhibit k_{cat}/K_m values of 10^9 M^{-1} s^{-1} or more, as well as k_{cat} values exceeding 10^6 s^{-1}. It is typically the case of carbonic anhydrase (CA) and superoxide dismutase (SOD), while triose phosphate isomerase (TPI) comes not far behind. Compared to these extreme cases (so-called "perfect" catalysts), the kinetic performances of Rubisco appear rather poor, with a k_{cat} between 1 and 10 s^{-1} (on an active site basis; hexadecameric form I Rubisco harbours eight active sites, while dimeric form II has two), and a k_{cat}/K_m of about $0.5.10^5$ to 5.10^5 M^{-1} s^{-1} ($2.5.10^5$ to 5.10^5 M^{-1} s^{-1}in higher plants).

However, how does Rubisco compare to average enzymes? A recent global analysis of the database BRENDA of kinetic parameters (including nearly 2,000 enzymes with K_m values with their natural substrates) revealed that the median turnover number is 10 s^{-1}, with 60% of values within 1-100 s^{-1} (Bar-Even, Noor, Savir, Liebermeister, Davidi, Tawfik & Milo, 2011). Therefore, Rubisco's k_{cat} value looks rather unremarkable, that is, not particularly low, as Bar-Even et al. pointed out. In addition, the median K_m value for all substrates is 130 µM (Fig. 1), and increases sharply with both decreasing molecular weight and increasing hydrophobicity for substrates below 350 Da. In other words, a K_m value for CO_2 of less than 10 µM (achieved by most specific Rubisco types) is exceptionally low, demonstrating a very good affinity of the enzyme for its gaseous substrate. In comparison, although the structure of RuBP offers many more potential binding sites than the linear triatomic CO_2, the K_ms for RuBP of various Rubiscos are almost ten times higher (Pearce 2006). Yet, the affinity of Rubisco for RuBP is never

questioned. The low K_m value for CO_2 is such that Rubisco's k_{cat}/K_m falls between $0.5.10^5$ and 5.10^5 M^{-1} s^{-1}, i.e. in the mid to upper range of most frequently observed values (60% of values ranging from 10^3 to 10^6 $M^{-1}s^{-1}$), and quite similar to values observed for enzymes operating in central carbon metabolism. As first pointed out by Bar-Even and co-workers Rubisco kinetics thus look very decent amongst the vast enzymatic diversity. Taken as a whole, their kinetic survey shows that very fast (i.e. diffusion-limited) enzymes are more the exception than the rule, with only few of them achieving catalytic "perfection" (Bar-Even et al. 2011). The reasons for enzymatic efficiency of enzymes being generally modest are not entirely clear but physical, chemical, evolutionary and metabolic constraints are serious candidates, as explained below.

What is Rubisco's rate enhancement?

It should be recognized that the rate of "uncatalysed" (i.e. non-enzymatic, in solution) biochemical reactions varies enormously, ranging over more than 16 orders of magnitude whereas enzymatic k_{cat} values generally range from 1 to 1000 s^{-1}. Consequently, rate enhancements (i.e. k_{cat}/k_{noncat}; see box 1 for definition) also vary enormously amongst enzymes (from 5.10^5-fold to 10^{25}-fold; Horvat and Wolfenden 2005; Warshel 2006; Herschlag and Natarajan 2013) although it should be kept in mind that reaction mechanisms may differ between the solution and enzyme active site. In other words, some reactions appear to be much more difficult to accelerate than others. Typically, SOD, CAN and TIM, despite being among the fastest known enzymes, catalyse reactions that occur spontaneously at a reasonable velocity

(half-time of about 1 s, 5 s and 2 d, respectively) and thus their rate enhancement is modest (10^6 to 10^{10}; Wolfenden and Snider 2001; Hall and Knowles 1975).

The rate of spontaneous carboxylation of RuBP in solution is not known to the best of our knowledge. Yet, it is unlikely to be very fast given the chemical inertness of CO_2. Assessing it experimentally would be difficult due to the instability of RuBP in solution (that is, RuBP would be degraded before the first spontaneous event of carboxylation occurs). However, theoretical calculations of the energy barriers associated with the different steps of the uncatalysed carboxylation of RuBP have been performed using density functional theory (Zhan, Niu & Ornstein, 2001). k_{noncat} values derived from that study (see Box 1) for three possible reaction pathways suggest that the rate of enhancement of higher plant Rubisco is between 6.10^{15} and 4.10^{17}, which is clearly in the upper range of enzymatic accelerations. Thus, Rubisco is actually efficient in terms of rate enhancement.

Why is Rubisco not kinetically "perfect"?

The average second-order rate constant ("efficiency") of enzymes (k_{cat}/K_m) is around 10^5 M^{-1} s^{-1} (Fig. 1), that is about 10^4-10^5 times lower than the diffusion limit. One possible explanation for this is that only a very small proportion of enzyme-substrate collisions (i.e. one out of 10^4-10^5) effectively leads to a catalytic event (Bar-Even, Milo, Noor & Tawfik, 2015). Such a small proportion could be explained by either substrates or enzymes existing in multiple forms (conformational sub-states), many of which being inappropriate for catalysis. This

explanation can also be extended to conformational changes required to accommodate the geometry of intermediates and transition states. If correct, this conformational effect would be probably more severe for enzymes presenting several substrates, complex mechanisms or multiple intermediates and transition states, since it is unlikely that a single conformation can optimize the binding of several substrates and transition states (Fried & Boxer, 2017, Hammes, Benkovic & Hammes-Schiffer, 2011). This is typically the case for Rubisco, which has to accommodate at least three different transition states, the first of which is stereochemically orthogonal to the last (Cleland et al. 1998). Thus, as discussed elsewhere (Tcherkez 2013, 2016), the catalytic optimization of Rubisco throughout evolution has probably proceeded via the fine-tuning of binding properties (internal equilibrium constants). In other words, because the intrinsic steps of the mechanism are not independent, several, if not all of them, have been under selective pressure. For such an adaptive process to reach catalytic efficiency, the enzyme must remain stable under physiological conditions and fold properly. As a result, mutations that enhance catalytic parameters but disfavour stability will be counter-selected, unless destabilization is sufficiently modest to allow compensation by subsequent stabilizing mutations. The stability-activity trade-off in protein evolution induces epistatic effects leading to irregular evolutionary landscapes and evolutionary dead-ends sometimes encountered in directed evolution experiments (Kaltenbach & Tokuriki, 2014). A recent *in silico* reconstruction of the evolutionary trajectory followed by Rubisco among a large group of phylogenetically related C_3 and C_4 species has underlined the importance of such a trade-off during the C_3-to-C_4 transition.

That is, the transition is preceded by a period where stabilizing mutations are accumulated, allowing destabilizing effects arising from modifications affecting catalysis (Studer, Christin, Williams & Orengo, 2014). Interestingly, that study points out the fact that all active site residues have been conserved during the transition, despite the important stabilizing potential of mutating them, thereby demonstrating that the chemistry of the reaction catalysed by Rubisco exerts a strong constraint on its evolution. This is probably true for many other enzymes, although based on sequence comparisons, over a third of enzyme superfamilies exhibit variation in active site catalytic residues (Todd, Orengo & Thornton, 2001).

Does the metabolic environment limit Rubisco efficiency?

While all of the effects mentioned above participate in limiting enzyme efficiency, their contribution is likely to vary substantially between enzymes because of their metabolic context. In fact, very fast enzymes can be sub-divided into two types. The first one comprises detoxification enzymes (like SOD and catalase, which catalyses the dismutation of oxygen radicals to oxygen peroxide and eventually, water and oxygen). Here, the rate is critical since it should compensate for the velocity of formation of toxic molecular species. In the case of oxygen radicals, cells exposed to natural light can generate as many as 10^4 equivalents s^{-1}, and even very low concentrations of the superoxide radical anion $O_2^{\bullet -}$ can be deleterious (Traut, 2008).

The second type corresponds to enzymes operating close to equilibrium (i.e. with an *in vivo* ΔG of reaction very close to 0), such as fumarase and TIP. In this case, the thermodynamic driving force will be the main factor controlling the flux in a given direction, so that the apparent rate of the reaction *in vivo* will be substantially lower than the maximum turnover measured *in vitro* (Rohwer & Hofmeyr, 2010). Similarly, very slow enzymes are determined by their metabolic role. For example, some kinases involved in the cellular circadian clock have very low turnover rates (10^{-2} to 10^{-6} s^{-1}) to match their signalling roles (progressive phosphorylation during daytime or night-time). These examples are far from being comprehensive, but show that enzyme catalytic properties are influenced by both their metabolic role and thermodynamics, so that kinetic comparisons can be irrelevant without sufficient functional knowledge.

That said, what is the metabolic context of Rubisco? Rubisco belongs to a specific category of enzymes catalysing virtually irreversible reactions (i.e. with a strongly negative ΔG). It is the entry point of carbon into the Benson-Calvin cycle, so that it is often considered as the rate-limiting step (RLS) for the carbon assimilation flux. In that sense, it could be viewed as a committed step. Other examples in different metabolic pathways are phosphofructokinase (PFK) in glycolysis, or pyruvate dehydrogenase (PDH) in catabolism. In principle, the activity of RLS enzymes is tightly regulated *in vivo*, so that they keep operating far from thermodynamic equilibrium. Unlike enzymes that are close to equilibrium (discussed above), RLS enzymes are expected to have a maximal catalytic activity ([Enz]*k*cat or [Enz]*k*cat/*K*m) that matches the maximal *in vivo* flux of the pathway of interest. Whether this translates into a correlation

between ΔG_{actual} (effective free energy difference) and catalytic efficiency remains to be explored. Parenthetically, ascertaining this would be highly demanding because it requires measuring concentrations of metabolites within cellular compartments, as well as kinetics of all enzyme isoforms.

The impact of the metabolic context on enzyme kinetics is also apparent in the survey of Bar-Even et al. (2011) cited above. That is, in primary metabolism (carbohydrate and energy metabolism), enzymes exhibit significantly higher k_{cat} and k_{cat}/K_m values than in secondary metabolism. Nevertheless, because of the small size of the dataset, no further conclusion can be drawn on the precise mathematical form of the distribution that could give clues as to the importance of thermodynamic constraints. However, it is striking that the median k_{cat}/K_m of enzymes involved in primary metabolism is 4.1×10^5 M^{-1} s^{-1}, a value very similar to that observed for the Rubisco forms present in higher plants and multicellular red algae. Under the assumption that in high-flux pathways, enzymes have K_m values similar to the physiological concentration of their substrate (this is the case for glycolytic enzymes (Traut 2008) and typically for plant Rubisco: $K_m(CO_2) \approx 10$ µM versus solution $[CO_2]$ in equilibrium with air at 25°C ≈ 10 µM in preindustrial atmosphere– note that the concentration of Rubisco sites in a chloroplast ≈ 4 mM, is about 300 times the concentration of CO_2 in equilibrium with air) carboxylation efficiency of Rubisco cannot be considered as being poor. It rather seems to have a catalytic efficiency *(i)* that matches metabolic imperatives, and *(ii)* similar to that occurring in other reactions of primary metabolism across most living forms.

Is Rubisco reactivity towards O_2 exceptional and inevitable?

Is Rubisco "confused specificity" a unique case?

Recent data have suggested that a large number of enzymes can catalyse more than one chemical transformation. This phenomenon, referred to as enzyme "promiscuity", is now considered as being the rule rather than the exception (Atkins 2015; Khersonsky and Tawfik 2010). It is believed to have played a central role in the evolution of enzymatic functions across several protein superfamilies, because promiscuous activities can rapidly be enhanced with a limited number of mutations (Glasner, Gerlt & Babbitt, 2006). Also, enzyme promiscuity is mechanistically diverse. The most obvious and ubiquitous case is that of enzymes catalysing similar chemistry on more than one substrate at the same active site (substrate promiscuity). Other enzymes can catalyse more than one reaction type (catalytic promiscuity). Substrate and catalytic promiscuity are not mutually exclusive so that all sorts of combinations can be found amongst enzyme families (Hult & Berglund, 2007). The alkaline phosphatase superfamily is a good example of that complexity. Members of this family can catalyse hydrolytic cleavage of either phosphate, phosphonate or sulphate groups on a range of phospho- and/or sulfo-carbohydrates substrates, exhibiting widespread crosswise promiscuity, often with impressive rate enhancements for both native and promiscuous activities (Mohamed & Hollfelder, 2013, Pabis & Kamerlin, 2016). In the enolase superfamily, which shares many structural and

mechanistic features with Rubisco-catalyzed enolization of RuBP (Gerlt, Babbitt & Rayment, 2005), the functional study of the *o*-succinylbenzoate synthase (OSBS) subgroup has shown that these enzymes often have a promiscuous racemase function, and the catalytic efficiency of enolase and racemase activities match physiological (metabolic) imperatives (Brizendine et al. 2014 and references therein).

How does the concept of promiscuity apply to Rubisco? Rubisco can catalyze both carboxylation and oxygenation of an enolized RuBP form, but also exhibits almost the whole range of enol chemistry (Shloss and Hixon 1998). In fact, it can catalyse isomerisation, epimerisation or elimination reactions, although at very low rates (Pearce, 2006). Yet, side reactions at such levels of activity could be considered as being of minor importance and, perhaps, could be detected in many other enzymes under sufficient scrutiny.

Rubisco is not known to catalyse any significant reaction on a substrate other than RuBP and is thus not promiscuous for the sugar phosphate substrate (for example, Rubisco carboxylation rate with xylulose-1,5-bisphosphate is more than a 1,000 times slower than that with RuBP, Pearce 2006). The Rubisco family nevertheless includes non-carboxylating forms (Rubisco like proteins (RLP)) catalysing the enolisation of intermediates of the methionine salvage pathway (Ashida, Saito, Kojima, Kobayashi, Ogasawara & Yokota, 2003, Ashida, Saito, Nakano, Tandeau de Marsac, Sekowska, Danchin & Yokota, 2008). Complementation experiments in *Rhodospirullum rubrum* (Dey, North, Sriram, Evans & Tabita, 2015) suggest that the carboxylating forms (I, II and III) might also catalyse a reaction allowing the rescue of this

pathway when RLP expression is disrupted. However, neither the substrate nor the kinetics of this putative promiscuous reaction have been determined so far. By contrast, the oxygenase function of Rubisco is a clear case of promiscuity, with a catalytic efficiency of only 100 times lower than that of carboxylase. This might be viewed as an unusual case of promiscuity because promiscuous functions are expected to provide a physiological advantage that can be selected for under appropriate conditions (Khersonsky & Tawfik, 2010) while here, potential benefits of oxygenation (and thus of photorespiration) are uncertain (Heber and Krause 1980; Eisenhut et al 2017 and references therein; see also the discussion below). The generally accepted view is that Rubisco first evolved in an oxygen-free atmosphere, so that oxygenation remained minimal until O_2 reached higher levels (Whitney, Houtz & Alonso, 2011). The reason why it has not been subsequently eliminated during evolution then remains unclear. From a mechanistic point of view, oxygenation is believed to be a consequence of the reactivity of the carbanionic (i.e. resonance form of the enolate; see Box 1) intermediate (Andrews & Lorimer, 1978, Lorimer & Andrews, 1973). As stated by (Lorimer, 1981), a complete understanding of the mechanism (which is still lacking) would solve that question, but O_2 reactivity in carbanion-involving enzymes is far from being unique, as explained below.

O_2 reactivity in enzyme-catalysed reactions

The O_2-reactivity inevitability postulate has been challenged with the simple assumption that, if true, enzymes producing similar types of carbanionic intermediates during catalysis

should also exhibit O_2 reactivity. Extending the work of Christen and Healy (1972, 1973), which demonstrated that a number of carbanion-forming enzymes could reduce a variety of electrophiles, Abell and Schloss (1991) investigated the reactivity towards O_2 of several enzymes. Interestingly, they showed that while some did show an oxygenase or oxidase activity (acetolactate synthase isozyme II (ALS II), pyruvate decarboxylase (PDC), glutamate decarboxylase (GDC), *S. aureus* fructose-1,6-bisphosphate aldolase (FruA), and l-rhamnulose-1-phosphate aldolase (RhuA)), several others did not (rabbit muscle fructose-1,6-bisphosphate aldolase, phosphoryl(amino)imidazole carboxylase, 6-phosphogluconate dehydrogenase, TIM, and fuculose-1-phosphate aldolase (FucA)) (Table 1). Thus, the formation of a carbanion *per se* does not seem to be sufficient to cause reactivity with O_2. This is not surprising since the reaction of a carbanion in its electronic singlet state with ground state triplet O_2 should involve a considerable energy barrier because it is spin-forbidden (Fetzner & Steiner, 2010). Accordingly, the enediolate form of ascorbate reacts with O_2 in solution at very low rates (if at all) and when it reacts, it requires free metal traces to favour the reaction (Wilson et al. 1995).

What is then required for oxygen addition? The answer to this question appears to be subtle, and can be exemplified using two class II aldolases, RhuA and FucA. These enzymes catalyse the reversible cleavage of two epimers, rhamnulose-1-phosphate and fuculose-1-phosphate, respectively, yielding dihydroxyacetone phosphate (DHAP) and L-lactaldehyde (LA). They have very similar active sites, accommodating a Zn^{2+} ion, which coordinates DHAP and facilitates the abstraction of a proton from the α-carbon to form an enol during the condensation

reaction (Jiménez, Clapés & Crehuet, 2009). In the Zn^{2+}-bound form, neither of them has a detectable oxygenase activity. However, when Zn^{2+} is substituted with Co^{2+} (but also Ni^{2+}, Cu^{2+} or Mn^{2+}), RhuA exhibits a strong oxidase activity while FucA remains insensitive to O_2 (Hixon, Sinerius, Schneider, Walter, Fessner & Schloss, 1996). Crystallographic studies with a transition state analogue have revealed that, despite the similarity of their active site residues, the Zn^{2+} ion is pentacoordinated in FucA, while it is hexacoordinated in RhuA, with a H_2O molecule acting as the sixth ligand (Joerger, Mueller-Dieckmann & Schulz, 2000, Kroemer, Merkel & Schulz, 2003). Presumably, this leads to slight differences in the geometry of coordinating residues around the metal (Jiménez *et al.*, 2009). Protein environments are known to be able to modify the electronic properties of bound metals or cofactors through a network of interaction with the "second sphere" and via indirect H-bonds (Lee & Lim, 2011, Maret, 2012, Williams, 1995). In other words, subtle interactions between the rest of the protein (even involving residues at a distance) and the active site bound metal probably influence the reactivity of the carbanionic intermediate in metalloenzymes. In such carbanion-forming enzymes, a crucial role of the metal ion is to stabilize the developing negative charge on the reactive intermediate, and the metal ion species is critical to determining the presence or absence of an oxygenase (or oxidase) activity. Accordingly, in Rubisco catalysis, as in RhuA, oxygenation is modulated by the nature of the metal ion, but the ranking of oxygenation efficiency of the different metals differs between the two enzymes, and even between Rubisco forms (Christeller, 1981). Oxygenation is thus certainly controlled by the H-bonds network in its entirety, even involving residues remote from the active

site. The fact that the majority of positive mutations involved in the C_3-to-C_4 transition are relatively far (10-20 Å) from the active site (Studer et al. 2014) thus comes as no surprise.

Presumably, Rubisco-catalysed carboxylation is accompanied by a competitive reaction with O_2 for stereochemical reasons related to the primary sequence (residues) and the influence of metal coordination. This conclusion is probably broadly applicable to all carbanion-forming enzymes, irrespective of whether they involve a metal ion. Stabilization of a reactive intermediate is a common problem to all of these enzymes, and has been solved in several ways throughout evolution, not necessarily using a metal ion. Other important mechanisms involve delocalization of the charge through the formation of a Schiff base, generally with an active site lysine (e.g. in class I aldolases), or through interaction with aromatic cofactors such as pyridoxal 5'-phosphate (PLP) or thiamine diphosphate (ThDP). Again, among enzymes showing these mechanisms, a side reaction with O_2 may or may not be observed (Bunik, Schloss, Pinto, Dudareva & Cooper, 2011). It is worth mentioning here that while significant O_2-consuming activities have been well documented for PLP- and ThDP-containing enzymes (e.g. ALS II and 2-oxoglutarate dehydrogenase), the case of class I aldolases is less clear (Table 1). The only reported example is that of *S. aureus* FruA, with a rather low activity (Abell and Schloss 1991). Noticeably, no activity at all has been detected with rabbit muscle FruA (Abell and Schloss 1991). This is surprising considering that this enzyme has been shown to be able to reduce various oxidants with redox potentials ($E^{0'}$) ranging from 0.08 to 0.725 V (Healy and Christen 1973), that is, well below that of the O_2/H_2O couple ($E^{0'}$=0.82 V).

This survey allows us to conclude that: *(i)* the reactivity of the carbanionic intermediate towards O_2 is highly variable and is inherently linked to subtle variations in the way the active site stabilizes the intermediate with the metal; and *(ii)* O_2 reactivity cannot simply be summarized as an oxido-reduction problem, meaning that *(iii)* the mechanism of O_2 attack on the enolate has to account for the spin-forbidden character of the reaction (Fig. 2).

Is radical/triplet chemistry the missing link?

A group of enzymes able to catalyse oxidase or oxygenase reactions not involving transition metal ions or organic cofactors has been discovered recently. These proteins perform a variety of reactions, which have in common an initial proton abstraction to form a carbanionic intermediate (in an analogous manner to Rubisco). The carbanion then reacts with O_2, forming a peroxy intermediate, that can undergo various fates (Fetzner and Steiner 2010). Despite their catalytic diversity, these enzymes mostly use highly conjugated polyaromatic compounds as substrates, suggesting an analogy with flavin cofactors. This type of molecule is known to form relatively stable radical forms (Bauld, 1997). Hence, it has been proposed that so called "cofactor-independent" oxygenases proceed via a "substrate-assisted" mechanism, whereby the depronated substrate (carbanion) is able to directly transfer an electron to O_2 (as flavin cofactors do; (Massey, 1994)), leading to the formation of a substrate radical pair that recombines into a peroxide (Bui & Steiner, 2016, Machovina, Usselman & DuBois, 2016, Silva, 2016). This assumption still awaits experimental evidence, and other mechanisms have been proposed, like a

direct attack of the activated substrate on O_2 to form a triplet peroxide intermediate (Hernández-Ortega, Quesne, Bui, Heyes, Steiner, Scrutton & de Visser, 2015). Regardless of mechanistic details, the flavin analogy is very interesting, because the reactivity of protein-bound flavins towards O_2 has been shown to vary widely across flavoproteins, depending on their class and substrate specificity, with no obvious relation to their one electron redox potential $E^{0'}$ (Mattevi, 2006).

Again, this suggests that the active site structure dictates the formation of radical/triplet intermediates and that O_2 reactivity is not a simple redox phenomenon. Unfortunately, this also suggests that reactivity towards O_2 cannot be easily predicted from our current knowledge of enzyme mechanisms. Presumably, the common denominator to all aforementioned oxidase- and oxygenase-catalysed reactions is the decrease in the triplet-singlet energy barrier (Schloss and Hixon 1998; Schloss 2002) so that once in its radical or triplet form (i.e. with unpaired electrons; see Box 1 for definitions), the carbanion can react with O_2 (Cadenas, 1989). However, precisely because of their reactivity, radical intermediates are very short-lived and thus difficult to detect (and thus their involvement is hard to demonstrate). Such intermediates are believed to be involved in several O_2-consuming side reactions of carbanion-forming enzymes such as ALS II, glutamate decarboxylase, ornithine decarboxylase, and 2-oxoglutarate dehydrogenase (Bunik et al 2011). Similarly, it has been proposed that in Rubisco catalysis, the enolate adopts a twisted conformation, substantially lowering the energy gap between its singlet and triplet forms (Oliva, Safont, Andrés & Tapia, 2001, Tapia & Andres, 1992). In other words, CO_2 and O_2 would be

competing for two different spin states of the enolate intermediate (i.e. singlet and triplet state, respectively; Schloss and Hixon 1998; Fig. 2). By extension, it means that in O_2-consuming enzymes, the protein environment dictates O_2 reactivity via the radical or triplet character of the reacting organic molecule (either the substrate or the cofactor) (see Box 2 for further definitions). In the case of Rubisco, its CO_2/O_2 specificity would then be determined by the geometry and the probability of formation of the triplet form of the enolate. Yet, because the work of Tapia and co-workers cited above has been done *in vacuo*, or with only a very limited set of active site residues, it cannot capture the complete picture. It is likely that the electrostatic environment of enzyme active sites, structured by H-bond interactions that extend well beyond the first sphere, can accommodate all intermediates along the catalytic pathway (Fried & Boxer, 2017).

In summary, when O_2 is not directly activated via an electron transfer from a transition metal or a cofactor, either the substrate is able to provide the activating electron (Silva 2016) or O_2 addition must involve a radical or a triplet-singlet transition – likely not O_2 itself, but rather of the organic substrate – and not a redox phenomenon.

Could O_2 reactivity be a by-product of carboxylation?

There seems to be a link between the biochemical role of enzymes and O_2 reactivity. In fact, all carbanion-forming enzymes reacting with O_2 break or make a C–C bond. And in all cases (except for aldolases which can bind a range of C_3 to C_5 aldehydes onto DHAP; Schoevaart

et al 2000), CO_2 is either a product or a substrate of the reaction. For example, in the wide family of PLP-dependent enzymes, O_2-consuming side reactions are restricted to members of the decarboxylases class (Bunik et al 2011; Table 1). One may thus wonder whether there is a link between (de)carboxylation and enzyme sensitivity to O_2. Rubisco overcomes both the high thermodynamic and kinetic barriers of the reaction associated with CO_2 addition (Appel, Bercaw, Bocarsly, Dobbek, DuBois, Dupuis, Ferry, Fujita, Hille & Kenis, 2013). All carboxylases actually do so. As a first step, they all catalyse the formation of a reactive intermediate, in the form of an enol(ate) or enamine (von Borzyskowski, Rosenthal & Erb, 2013). Some of them also activate CO_2 (HCO_3^-) by covalent binding, forming a carboxyphosphate (as in phospho*enol*pyruvate carboxylation catalysis) or a carboxybiotin intermediate (biotin dependent carboxylases). This step is crucial, because CO_2 activation protects these enzymes from O_2 side reactions (Table 1). Therefore, although reaction barriers and intrinsic mechanisms for CO_2 and O_2 additions look very different, the reactivity of the catalytic intermediate towards these two gaseous substrates may be linked. That is, the specific chemical strategy used by the enzyme for CO_2 addition might contribute to O_2 reactivity.

A rationale for O_2 reactivity?

Historically, Rubisco reactivity towards O_2 has been referred to as 'inevitable' (Lorimer and Andrews 1973). From consideration of the above, it may not be so. First, it hard to believe that the enolate intermediate has a strong inherent reactivity for O_2 that could not be suppressed

by the protein environment during evolution. Second, amongst carbanion-forming enzymes, even from the same superfamily, oxygenase activity can be either completely absent or the main activity. It is possible that the chemical constraint imposed by CO_2 inertness or scarcity (especially in a low CO_2 context) is such that the observed specificity represents the best compromise allowing carboxylation at a physiologically acceptable rate. In fact, a recent catalytic survey of Rubisco from diatoms (Young, Heureux, Sharwood, Rickaby, Morel & Whitney, 2016), which possess carbon concentrating mechanisms, strongly suggests that when the pressure on K_c (apparent Michaelis constant for CO_2) is relieved (i.e. when CO_2 is not limiting), there is an alternative evolutionary path to a better specificity by suppressing oxygenase activity, without impairing carboxylase activity. Therefore, it is very likely that the oxygenase activity is the result of a trade-off: the active site structure adapts to allow maximal enolate twisting and positioning for CO_2 reactivity (at the prevailing CO_2 mole fraction) even though O_2 can also react; alternatively, the enzyme active site can tune its structure (including Mg^{2+} coordination) to decrease dramatically the probability of the enolate forming a triplet and then reacting with O_2, but CO_2 reactivity also decreases. In kinetics terms, manipulating oxygenase activity via the geometry of the enolate affects the transition states of oxygenation and carboxylation themselves, and consequently can be anticipated to change the energy barrier of CO_2 and O_2 addition (and thus specificity) as well as the $^{12}C/^{13}C$ isotope effect associated with CO_2 addition, as observed experimentally (Tcherkez, Farquhar & Andrews, 2006). Does that mean that an oxygenase-free Rubisco can be engineered? Perhaps, provided a complete

"rewiring" of the network of interactions across the protein could be achieved to suppress the propensity of the enolate to adopt a triplet form. That said, completely suppressing oxygenation and thus photorespiration has been shown to be deleterious to photosynthetic oxygenic organisms including those possessing carbon concentrating mechanisms like C_4 plants or cyanobacteria (Zelitch et al. 2009, Eisenhut et al. 2008). Although the inability to deal with 2-PG (even at low photorespiratory rates) is an obvious reason, further evidence suggests that photorespiration could be under positive selection since its metabolism represents a sink for excess reducing equivalents (André 2011 and references therein). In C_3 plants, where the photorespiratory pathway has evolved to carry large fluxes, its suppression could also destabilize plant cell metabolism due to perturbations in flux control properties of photosynthates production and consequences for N and S assimilation (Cornish-Bowden 2006, Abadie et al. 2016, Eisenhut et al. 2017, Busch et al. 2018, see also below) . In other words, the cost/benefit ratio for photorespiration is probably not as large as often assumed, and depends on a complex combination of environmental drivers (temperature, CO_2 concentration, and precipitation), as recent insights in the ecology of C_4 plants suggest (Urban et al. 2015; Christin and Osborne 2014). Benefits for crops from an oxygenase-free Rubisco are thus difficult to anticipate considering potential consequences for the metabolic network associated with the oxygenase activity and photorespiratory recovery.

Conclusion and perspectives

Taken as a whole, the present survey of enzymes and comparisons with Rubisco's chemistry show that Rubisco is neither slow nor unspecific. It behaves as do many other enzymes with a similar mechanism. In addition, it appears to have achieved acceptable kinetic properties as compared to those commonly observed in high-flux metabolic pathways. Of course, there may still be potential for improvement, but the protein has evolved to respond to a multiple chemical compromise: catalysing the complex multistep reaction of fixing CO_2 onto RuBP in an oxygenic atmosphere, at physiologically compatible rates. Such a catalytic efficacy probably explains the overwhelming success of Rubisco as the prevalent carboxylase across all eukaryotic and prokaryotic taxons. As mentioned above, the oxygenase function is probably not inevitable *per se* and results from a chemical compromise. Despite its known fundamental properties (spin-forbidden; present in several C–C bond forming or breaking enzymes; very likely involving triplet enolate), further work is required to understand its precise chemical cause. This would in turn provide clues for successful engineering of a kinetically "better" (faster and more specific) enzyme. However, is a better Rubisco desirable and would it improve plant photosynthesis? This question is quite controversial but worth mentioning here. Targeting a supposed RLS enzyme (overexpression and/or desensitization) has been attempted several times, for many metabolites in plants (starch, lipids, amino acids, vitamins…) but unfortunately, it has never met significant

success, that is, with little increase in metabolic fluxes of interest and deleterious perturbations in metabolite concentration (see Morandini 2009 and 2013 for comprehensive reviews). The reason is that strict RLS are very rare in metabolic pathways (likely for evolutionary reasons). As metabolic control theory and experimental evidence have shown, flux control coefficients of enzymes are usually rather small, so that the flux through a metabolic pathway is distributed amongst different reactions, including those working close to equilibrium. In fact, the regulation of off-equilibrium enzyme kinetics is crucial for homeostatic regulation, but fluxes and concentrations are interdependent because of reaction reversibility; as such, flux control is often based on the demand (i.e. sink strength) (Hofmeyr & Cornish-Bowden, 2000). The Benson-Calvin cycle involves large fluxes, so that it has to be tightly regulated to avoid rapid and huge metabolite accumulation and divergence from steady-state. It is in fact likely that the metabolic control is shared between enzymes in the Benson-Calvin cycle (Raines, 2003), and this is consistent with several Benson-Calvin cycle enzymes being abundant thereby allowing flux flexibility at several metabolic points (Harris & Königer, 1997, Warren & Adams, 2004). Accordingly, the flux control coefficient of Rubisco has been shown to vary between about 0.1 and 0.9, depending on environmental conditions (particularly irradiance and N availability)(Stitt & Schulze, 1994). Also, photosynthetic fluxes are partly controlled by the metabolic demand (Paul & Foyer, 2001). Therefore, targeting Rubisco alone might not be as efficient as often anticipated in increasing photosynthetic fluxes and growth in field conditions. The engineering

of Rubisco's kinetics would nevertheless certainly bring important and valuable insights into enzyme catalysis and the metabolic regulation of photosynthesis.

Future studies are warranted to provide advances in our understanding of Rubisco's mechanism and photosynthetic biochemical regulation. This research field has driven passionate debates for many years, but persisting mysteries in Rubisco chemistry exemplify the need for further effort involving several fields of expertise, from physical chemistry to evolutionary biology.

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References

Abadie C., Boex-Fontvieille E.R., Carroll A.J. & Tcherkez G. (2016) In vivo stoichiometry of photorespiratory metabolism. *Nature plants*, **2**, nplants2015220.

Abell L.M. & Schloss J.V. (1991) Oxygenase side reactions of acetolactate synthase and other carbanion-forming enzymes. *Biochemistry*, **30**, 7883-7887.

Andersson I. (2008) Catalysis and regulation in Rubisco. *J Exp Bot*, **59**, 1555-1568.

André M. (2011) Modelling $^{18}O_2$ and $^{16}O_2$ unidirectional fluxes in plants: II. analysis of rubisco evolution. *Bio Systems*, **103**, 252-264.

Andrews T. & Lorimer G. (1978) Photorespiration—still unavoidable? *FEBS Letters*, **90**, 1-9.

Appel A.M., Bercaw J.E., Bocarsly A.B., Dobbek H., DuBois D.L., Dupuis M., Ferry J.G., Fujita E., Hille R. & Kenis P.J. (2013) Frontiers, opportunities, and challenges in biochemical and chemical catalysis of CO2 fixation. *Chemical reviews*, **113**, 6621-6658.

Ashida H., Saito Y., Kojima C., Kobayashi K., Ogasawara N. & Yokota A. (2003) A functional link between RuBisCO-like protein of Bacillus and photosynthetic RuBisCO. *Science*, **302**, 286-290.

Ashida H., Saito Y., Nakano T., Tandeau de Marsac N., Sekowska A., Danchin A. & Yokota A. (2008) RuBisCO-like proteins as the enolase enzyme in the methionine salvage pathway: functional and evolutionary relationships between RuBisCO-like proteins and photosynthetic RuBisCO. *Journal of experimental botany*, **59**, 1543-1554.

Atkins W.M. (2015) Biological messiness vs. biological genius: mechanistic aspects and roles of protein promiscuity. *The Journal of steroid biochemistry and molecular biology*, **151**, 3-11.

Bar-Even A., Milo R., Noor E. & Tawfik D.S. (2015) The moderately efficient enzyme: futile encounters and enzyme floppiness. *Biochemistry*, **54**, 4969-4977.

Bar-Even A., Noor E., Savir Y., Liebermeister W., Davidi D., Tawfik D.S. & Milo R. (2011) The moderately efficient enzyme: evolutionary and physicochemical trends shaping enzyme parameters. *Biochemistry*, **50**, 4402-4410.

Bar-Even A. & Tawfik D.S. (2013) Engineering specialized metabolic pathways—is there a room for enzyme improvements? *Current opinion in biotechnology*, **24**, 310-319.

Bauld N.L. (1997) *Radicals, ion radicals, and triplets*. Wiley-VCH.

Brizendine A.M., Odokonyero D., McMillan A.W., Zhu M., Hull K., Romo D. & Glasner M.E. (2014) Promiscuity of Exiguobacterium sp. AT1b o-succinylbenzoate synthase illustrates evolutionary transitions in the OSBS family. *Biochemical and biophysical research communications*, **450**, 679-684.

Bui S. & Steiner R.A. (2016) New insight into cofactor-free oxygenation from combined experimental and computational approaches. *Current Opinion in Structural Biology*, **41**, 109-118.

Bunik V.I., Schloss J.V., Pinto J.T., Dudareva N. & Cooper A.J. (2011) A survey of oxidative paracatalytic reactions catalyzed by enzymes that generate carbanionic intermediates: implications for ROS production, cancer etiology, and neurodegenerative diseases. *Advances in enzymology and related areas of molecular biology*, **77**, 307.

Busch F.A., Sage R.F. & Farquhar G.D. (2018) Plants increase CO 2 uptake by assimilating nitrogen via the photorespiratory pathway. *Nature plants*, **4**, 46-54.

Cadenas E. (1989) Biochemistry of oxygen toxicity. *Annual review of biochemistry*, **58**, 79-110.

Christeller J. (1981) The effects of bivalent cations on ribulose bisphosphate carboxylase/oxygenase. *Biochemical Journal*, **193**, 839-844.

Christin P.A. & Osborne C.P. (2014) The evolutionary ecology of C4 plants. *New Phytologist*, **204**, 765-781.

Cleland W.W., Andrews T.J., Gutteridge S., Hartman F.C. & Lorimer G.H. (1998) Mechanism of Rubisco: The Carbamate as General Base✗. *Chemical Reviews*, **98**, 549-562.

Cornish-Bowden A. (2006) Putting the systems back into systems biology. *Perspectives in biology and medicine*, **49**, 475-489.

Dey S., North J.A., Sriram J., Evans B.S. & Tabita F.R. (2015) In vivo studies in Rhodospirillum rubrum indicate that ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) catalyzes two obligatorily required and physiologically significant reactions for distinct carbon and sulfur metabolic pathways. *Journal of Biological Chemistry*, **290**, 30658-30668.

Eisenhut M., Bräutigam A., Timm S., Florian A., Tohge T., Fernie A.R., Bauwe H. & Weber A.P. (2017) Photorespiration Is Crucial for Dynamic Response of Photosynthetic Metabolism and Stomatal Movement to Altered CO 2 Availability. *Molecular plant*, **10**, 47-61.

Eisenhut M., Ruth W., Haimovich M., Bauwe H., Kaplan A. & Hagemann M. (2008) The photorespiratory glycolate metabolism is essential for cyanobacteria and might have been conveyed endosymbiontically to plants. *Proceedings of the National Academy of Sciences*, **105**, 17199-17204.

Erb T.J., Jones P.R. & Bar-Even A. (2017) Synthetic metabolism: metabolic engineering meets enzyme design. *Current Opinion in Chemical Biology*, **37**, 56-62.

Fersht A. (1999) *Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding*. Macmillan.

Fetzner S. & Steiner R.A. (2010) Cofactor-independent oxidases and oxygenases. *Applied microbiology and biotechnology*, **86**, 791-804.

Fried S.D. & Boxer S.G. (2017) Electric Fields and Enzyme Catalysis. *Annual Review of Biochemistry*.

Gerlt J.A., Babbitt P.C. & Rayment I. (2005) Divergent evolution in the enolase superfamily: the interplay of mechanism and specificity. *Archives of biochemistry and biophysics*, **433**, 59-70.

Glasner M.E., Gerlt J.A. & Babbitt P.C. (2006) Evolution of enzyme superfamilies. *Current opinion in chemical biology*, **10**, 492-497.

Hall A. & Knowles J.R. (1975) Uncatalyzed rates of enolization of dihydroxyacetone phosphate and of glyceraldehyde 3-phosphate in neutral aqueous solution. Quantitative assessment of the effectiveness of an enzyme catalyst. *Biochemistry*, **14**, 4348-4352.

Hammes G.G. (2002) Multiple conformational changes in enzyme catalysis. *Biochemistry*, **41**, 8221-8228.

Hammes G.G., Benkovic S.J. & Hammes-Schiffer S. (2011) Flexibility, diversity, and cooperativity: pillars of enzyme catalysis. *Biochemistry*, **50**, 10422-10430.

Harris G.C. & Königer M. (1997) The 'high'concentrations of enzymes within the chloroplast. *Photosynthesis research*, **54**, 5-23.

Healy M.J. & Christen P. (1972) Reaction of the carbanionic aldolase-substrate intermediate with tetranitromethane. Identification of the products, hydroxypyruvaldehyde phosphate and D-5-ketofructose 1, 6-diphosphate. *Journal of the American Chemical Society*, **94**, 7911-7916.

Healy M.J. & Christen P. (1973) Mechanistic probes for enzymic reactions. Oxidation-reduction indicators as oxidants of intermediary carbanions (studies with aldolase, aspartate

aminotransferase, pyruvate decarboxylase, and 6-phosphogluconate dehydrogenase). *Biochemistry*, **12**, 35-41.

Heber U. & Krause G. (1980) What is the physiological role of photorespiration? *Trends in Biochemical Sciences*, **5**, 32-34.

Hernández-Ortega A., Quesne M.G., Bui S., Heyes D.J., Steiner R.A., Scrutton N.S. & de Visser S.P. (2015) Catalytic mechanism of cofactor-free dioxygenases and how they circumvent spin-forbidden oxygenation of their substrates. *Journal of the American Chemical Society*, **137**, 7474-7487.

Herschlag D. & Natarajan A. (2013) Fundamental challenges in mechanistic enzymology: progress toward understanding the rate enhancements of enzymes. *Biochemistry*, **52**, 2050-2067.

Hixon M., Sinerius G., Schneider A., Walter C., Fessner W.-D. & Schloss J.V. (1996) Quo vadis photorespiration: a tale of two aldolases. *FEBS letters*, **392**, 281-284.

Hofmeyr J.-H.S. & Cornish-Bowden A. (2000) Regulating the cellular economy of supply and demand. *FEBS letters*, **476**, 47-51.

Hult K. & Berglund P. (2007) Enzyme promiscuity: mechanism and applications. *Trends in biotechnology*, **25**, 231-238.

Jiménez A., Clapés P. & Crehuet R. (2009) Protein Flexibility and Metal Coordination Changes in DHAP-Dependent Aldolases. *Chemistry-a European Journal*, **15**, 1422-1428.

Joerger A.C., Mueller-Dieckmann C. & Schulz G.E. (2000) Structures of L-fuculose-1-phosphate aldolase mutants outlining motions during catalysis. *Journal of molecular biology*, **303**, 531-543.

Kaltenbach M. & Tokuriki N. (2014) Dynamics and constraints of enzyme evolution. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, **322**, 468-487.

Khersonsky O. & Tawfik D.S. (2010) Enzyme promiscuity: a mechanistic and evolutionary perspective. *Annu Rev Biochem*, **79**, 471-505.

Kroemer M., Merkel I. & Schulz G.E. (2003) Structure and Catalytic Mechanism of l-Rhamnulose-1-phosphate Aldolase†. *Biochemistry*, **42**, 10560-10568.

Lee Y.-M. & Lim C. (2011) Factors controlling the reactivity of zinc finger cores. *Journal of the American Chemical Society*, **133**, 8691-8703.

Lorimer G. & Andrews T. (1973) Plant photorespiration—an inevitable consequence of the existence of atmospheric oxygen. *Nature*, **243**, 359-360.

Lorimer G.H. (1981) The carboxylation and oxygenation of ribulose 1, 5-bisphosphate: the primary events in photosynthesis and photorespiration. *Annual Review of Plant Physiology*, **32**, 349-382.

Machovina M.M., Usselman R.J. & DuBois J.L. (2016) Monooxygenase Substrates Mimic Flavin to Catalyze Cofactorless Oxygenations. *J Biol Chem*, **291**, 17816-17828.

Maret W. (2012) New perspectives of zinc coordination environments in proteins. *Journal of inorganic biochemistry*, **111**, 110-116.

Massey V. (1994) Activation of molecular oxygen by flavins and flavoproteins. *Journal of Biological Chemistry*, **269**, 22459-22462.

Mattevi A. (2006) To be or not to be an oxidase: challenging the oxygen reactivity of flavoenzymes. *Trends in biochemical sciences*, **31**, 276-283.

Mohamed M.F. & Hollfelder F. (2013) Efficient, crosswise catalytic promiscuity among enzymes that catalyze phosphoryl transfer. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, **1834**, 417-424.

Morandini P. (2009) Rethinking metabolic control. *Plant Science*, **176**, 441-451.

Morandini P. (2013) Control limits for accumulation of plant metabolites: brute force is no substitute for understanding. *Plant biotechnology journal*, **11**, 253-267.

Morell M.K., Paul K., Kane H. & Andrews T. (1992) Rubisco: maladapted or misunderstood. *Australian journal of botany*, **40**, 431-441.

Northrop D.B. (1998) On the meaning of Km and V/K in enzyme kinetics. *J. Chem. Educ*, **75**, 1153.

Oliva M., Safont V.S., Andrés J. & Tapia O. (2001) Transition structures for D-ribulose-1, 5-bisphosphate carboxylase/oxygenase-catalyzed oxygenation chemistry: role of carbamylated lysine in a model magnesium coordination sphere. *The Journal of Physical Chemistry A*, **105**, 4726-4736.

Pabis A. & Kamerlin S.C.L. (2016) Promiscuity and electrostatic flexibility in the alkaline phosphatase superfamily. *Current opinion in structural biology*, **37**, 14-21.

Paul M.J. & Foyer C.H. (2001) Sink regulation of photosynthesis. *Journal of experimental botany*, **52**, 1383-1400.

Pearce F.G. (2006) Catalytic by-product formation and ligand binding by ribulose bisphosphate carboxylases from different phylogenies. *Biochemical Journal*, **399**, 525-534.

Raines C.A. (2003) The Calvin cycle revisited. *Photosynthesis research*, **75**, 1-10.

Raven J.A. (2009) Contributions of anoxygenic and oxygenic phototrophy and chemolithotrophy to carbon and oxygen fluxes in aquatic environments. *Aquatic Microbial Ecology*, **56**, 177-192.

Rohwer J.M. & Hofmeyr J.-H.S. (2010) Kinetic and thermodynamic aspects of enzyme control and regulation. *The Journal of Physical Chemistry B*, **114**, 16280-16289.

Schloss J.V. (2002) Oxygen toxicity from plants to people. *Planta*, **216**, 38-43.

Schloss J.V. & Hixon M.S. (1998) 22 ENOL CHEMISTRY AND ENZYMOLOGY. *Comprehensive Biological Catalysis: Reactions of nucleophilic*, **2**, 43.

Schoevaart R., van Rantwijk F. & Sheldon R.A. (2000) Stereochemistry of nonnatural aldol reactions catalyzed by DHAP aldolases. *Biotechnology and bioengineering*, **70**, 349-352.

Silva P.J. (2016) Refining the reaction mechanism of O2 towards its co-substrate in cofactor-free dioxygenases. *PeerJ*, **4**, e2805.

Stitt M. & Schulze D. (1994) Does Rubisco control the rate of photosynthesis and plant growth? An exercise in molecular ecophysiology. *Plant, Cell & Environment*, **17**, 465-487.

Studer R.A., Christin P.A., Williams M.A. & Orengo C.A. (2014) Stability-activity tradeoffs constrain the adaptive evolution of RubisCO. *Proc Natl Acad Sci U S A*, **111**, 2223-2228.

Tapia O. & Andres J. (1992) Towards an explanation of carboxylation/oxygenation bifunctionality in Rubisco. Transition structure for the carboxylation reaction of 2, 3, 4-pentanetriol. *Molecular Engineering*, **2**, 37-41.

Tcherkez G. (2013) Modelling the reaction mechanism of ribulose-1,5-bisphosphate carboxylase/oxygenase and consequences for kinetic parameters. *Plant Cell Environ*, **36**, 1586-1596.

Tcherkez G. (2016) The mechanism of Rubisco-catalysed oxygenation. *Plant, cell & environment*, **39**, 983-997.

Tcherkez G.G., Farquhar G.D. & Andrews T.J. (2006) Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. *Proc Natl Acad Sci U S A*, **103**, 7246-7251.

Todd A.E., Orengo C.A. & Thornton J.M. (2001) Evolution of function in protein superfamilies, from a structural perspective. *Journal of molecular biology*, **307**, 1113-1143.

Traut T. (2008) The Limits For Life Define The Limits For Enzymes. *Allosteric Regulatory Enzymes*, 29-48.

Urban M.A., Nelson D.M., Street-Perrott F.A., Verschuren D. & Hu F.S. (2015) A late-Quaternary perspective on atmospheric pCO2, climate, and fire as drivers of C4-grass abundance. *Ecology*, **96**, 642-653.

von Borzyskowski L.S., Rosenthal R.G. & Erb T.J. (2013) Evolutionary history and biotechnological future of carboxylases. *Journal of biotechnology*, **168**, 243-251.

Warren C.R. & Adams M.A. (2004) Evergreen trees do not maximize instantaneous photosynthesis. *Trends in plant science*, **9**, 270-274.

Warshel A., Sharma P.K., Kato M., Xiang Y., Liu H. & Olsson M.H. (2006) Electrostatic basis for enzyme catalysis. *Chem Rev*, **106**, 3210-3235.

Whitney S.M., Houtz R.L. & Alonso H. (2011) Advancing our understanding and capacity to engineer nature's CO 2-sequestering enzyme, Rubisco. *Plant Physiology*, **155**, 27-35.

Williams R.J. (1995) Energised (entatic) states of groups and of secondary structures in proteins and metalloproteins. In: *EJB Reviews*, pp. 257-275. Springer.

Wilson R., Beezer A.E. & Mitchell J.C. (1995) A kinetic study of the oxidation of L-ascorbic acid (vitamin C) in solution using an isothermal microcalorimeter. *Thermochimica Acta*, **264**, 27-40.

Wolfenden R. & Snider M.J. (2001) The depth of chemical time and the power of enzymes as catalysts. *Accounts of chemical research*, **34**, 938-945.

Young J.N., Heureux A.M., Sharwood R.E., Rickaby R.E., Morel F.M. & Whitney S.M. (2016) Large variation in the Rubisco kinetics of diatoms reveals diversity among their carbon-concentrating mechanisms. *Journal of experimental botany*, **67**, 3445-3456.

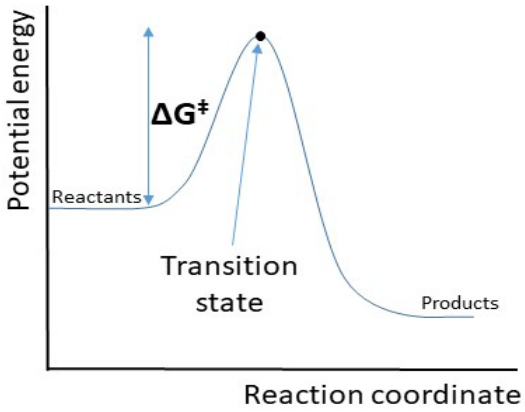
Zelitch I., Schultes N.P., Peterson R.B., Brown P. & Brutnell T.P. (2009) High glycolate oxidase activity is required for survival of maize in normal air. *Plant Physiology*, **149**, 195-204.

Zhan C.-G., Niu S. & Ornstein R.L. (2001) Theoretical studies of nonenzymatic reaction pathways for the three reaction stages of the carboxylation of ribulose-1, 5-bisphosphate. *Journal of the Chemical Society, Perkin Transactions 2*, 23-29.

Box 1. Definitions

Transition state theory

The transition state theory was initially developed by Eyring, Evans and Polanyi, as a physical conceptual framework to chemical reaction rate constants. It assumes that for a bimolecular reaction to occur, reactants have to form an activation complex, the transition state, which is in quasi-equilibrium with the reactants. The transition state sits on the highest point of energy along the reaction coordinates (scheme 1). The Eyring equation relates the rate constant of a chemical



reaction to the pseudo-equilibrium constant ($K^{\neq} = e^{-\frac{\Delta G^{\neq}}{RT}}$) between the reactants and the transition state as follows: $k = \frac{k_B T}{h}\, e^{-\frac{\Delta G^{\neq}}{RT}}$, where k_B and h are the Boltzmann constant and Plank constant respectively, and T is absolute temperature.

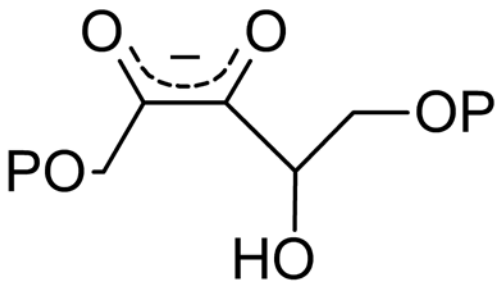
Scheme 1. Schematic energy profile of an exergonic chemical reaction

Rate enhancement

Enzymatic rate enhancements are calculated as the ratio of the catalysed to non-catalysed first order (or pseudo-first order) rate constant for the reaction, k_{cat}/k_{noncat}. Non-catalysed reaction rates are often very slow under ambient conditions, but can be extrapolated from experiments done at high temperature, provided reactants and products are stable enough (this is clearly not the case for RuBP). However, energy barriers (ΔG) for the non-catalysed carboxylation of RuBP have been obtained from computational models (Zhan et al. 2001). These barriers can be converted into rate constants using the Eyring equation described above, which allowed us to calculate estimates of the rate of enhancement achieved by Rubisco (see main text).

Carbanion

A carbanion is an organic molecule with a negative charge at a C-atom position, and can thus be viewed as the conjugate base of an organic acid where proton dissociation occurs at a carbon atom ($C–H \leftrightarrow C^- + H^+$). In most cases, it requires a very strong base or enzymatic catalysis, since acidic carbon atoms are generally very weak acids. This is the case of the C-3 atom involved in the enolization step of Rubisco mechanism. Carbanions are more stable in molecules

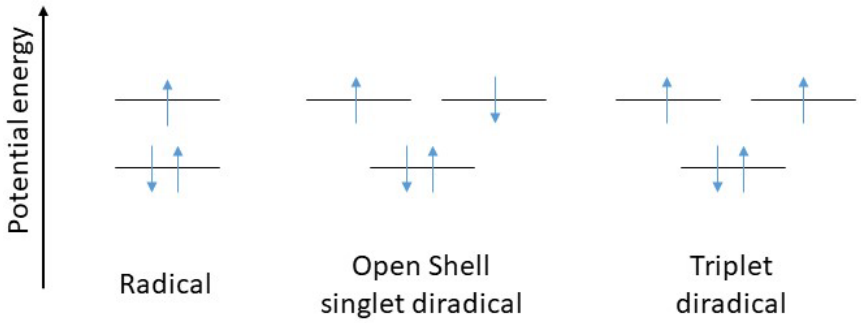


where charge delocalization of is possible, such as enolates (scheme 2).

Scheme 2. Schematic representation of the resonance form of the enolate of RuBP

Radicals and triplets

Radicals can be defined as molecules possessing one unpaired electron in their outer orbital. Molecules that have two unpaired electrons are thus defined as biradicals. The latter can adopt two distinct electronic states: a singlet state (also named open shell singlet), where the two unpaired electrons adopt opposite spins, or a triplet state, where the two unpaired electrons have a parallel spin (scheme 3; Bauld 1997). Carbanion can also adopt (di)radical configurations, especially when electronic delocalization is possible across the molecule.



Scheme 3. Schematic representation of possible spin orientations of electrons in radicals

Figure legends

Figure 1. Kinetic performances of Rubisco relative to other known enzymes. Panel a to c: frequency plot of k_{cat}/K_m, k_{cat} and K_m for all measured unmutated enzymes acting on their natural substrate stored in the BRENDA database (replotted from supplemental data in Bar-Even et al. 2011; curves were smoothed with Originlab software using a moving average filter), together with the range of values measured on the different Rubisco forms (green lines). Panel d is a barplot of rate enhancement values for 18 enzymes as reported in Horvat and Wolfenden 2005.

Figure 2. Scheme illustrating the potential radical character of the delocalized carbanionic enolate of RuBP (One of the possible diradical form of the carbanion is shown in parenthesis). As a triplet ground state species, O_2 can react easily only with radicals/triplets, while CO_2 can react readily with the carbanion, so that the two gaseous substrates could be competing for two different spin states of the enol intermediate (see main text for details).

Table 1. Oxygenase side reactions in enzymes with carbanionic intermediates

Enzyme name	Organism	EC Number	Reaction type	Super family	Metal Cofactor	Organic cofactor	Carbanionic inermediate	O2 side reaction	Ref
L-Rhamnulose 1-phosphate aldolase	E. coli	4.1.2.13	Reversible aldol condensation	Aldolases (class II)	Zn2+ (Ni2+, Cu2+, Mn2+)	-	DHAP Enol(ate)	Yes (oxidase)	Hixon and Shloss 1996
L-Fuculose 1-phosphate aldolase	E. coli	4.1.2.13	Reversible aldol	Aldolases (class II)	Zn2+ (Ni2+, Cu2+,	-	DHAP Enol(ate)	No	Hixon and Shloss 1996

			condensation		Mn2+)				
Fructose 1,6-bisphosphate aldolase	E. coli	4.1.2.13	Reversible aldol condensation	Aldolases (class II)	Zn2+ (Ni2+, Cu2+, Mn2+)	-	DHAP Enol(ate)	No	Hixon and Shloss 1996
Fructose 1,6-bisphosphate aldolase	O. Cuniculus (muscle)	4.1.2.13	Reversible aldol condensation	Aldolases (class I)	-	-	Enamine(ate) (Schiff base)	No	Abell and Schloss 1991
Fructose 1,6-bisphosphate aldolase	S. aureus	4.1.2.13	Reversible aldol condensation	Aldolases (class I)	-	-	Enamine(ate) (Schiff base)	Yes (oxidase)	Abell and Schloss 1991
Acetolactate	S.	4.1.3.18	Reversible	ThDP-	Mg2+	ThDP	(α-	Yes	Abell and

synthase II	typhimurium		Keto acids condensation	dependent enzymes		FAD	hydroxyethyl)-ThDP enanmine(ate)	(monooxigenase)	Schloss 1991
Pyruvate decarboxylase	S. cerevisiae Z. mobilis	4.1.1.1	decarboxylation	ThDP-dependent enzymes	Mg2+	ThDP	(α-hydroxyethyl)-ThDP enanmine(ate	Yes (monooxigenase)	Abell and Schloss 1991
Glutamate decarboxylase	E.coli	4.1.1.15	decarboxylation	PLP-dependent enzymes	-	PLP	Quinonoid	Yes (oxidase)	Abell and Schloss 1991
DOPA	S. scrofa	4.1.1.28	decarboxylation	PLP-dependent	-	PLP	Quinonoid	Yes	Bertoldi et al.

decarboxylase	(kidney)			enzymes				(oxidase)	1999
Ornithine decarboxylase	Lactobacillus	4.1.1.17	decarboxylation	PLP-dependent enzymes	-	PLP	Quinonoid	Yes (oxidase)	Bertoldi et al. 1999
Aspartate aminotransferase	E. coli G. gallus	2.6.1.1	transamination	PLP-dependent enzymes	-	PLP	Quinonoid	No	Toney 2014
Rubisco	S.oleracea R. rubrum	4.1.1.39	Carboxylation	Enolases	Mg2+ (Ni2+, Cu2+, Mn2+)	-	RuBP Enolate	Yes (monooxygenase)	Lorimer 1981
Phophoenolpyruvate	Z. mays	4.1.1.31	Carboxylation	Carboxy-	Mg2+	-	Pyruvate	No	Von

Carboxylase				lyases	(Mn2+, Co2+, Fe2+, Cd2+)		Enolate		Borzyskowski et al. 2013
Acetyl-CoA carboxylase	E.coli C.aurantiacus	6.4.1.2	Carboxylation	Biotin dependent enzymes	-	Biotin, ATP	Acetyl-CoA Enolate	No	Von Borzyskowski et al. 2013

Figure 1

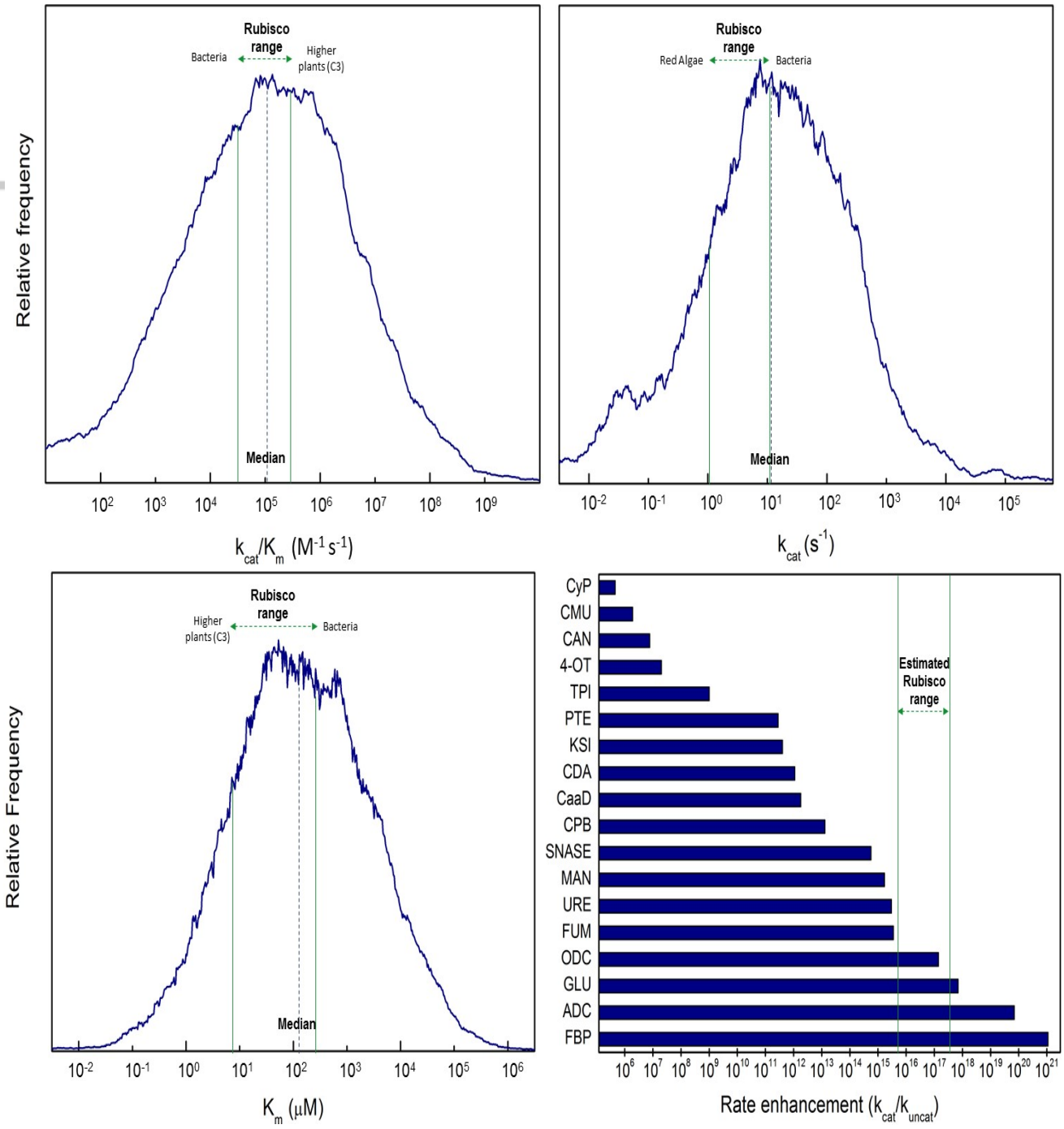


Figure 2