Ancestral *versus* Modern Substrate Scope in Family-1 Glycosidases

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Abstract: Promiscuity, the capability of catalyzing a diversity of chemical reactions, is a desirable feature in many enzymes intended for biotechnological applications. Promiscuous activities, however, are typically depressed in specialized modern enzymes. On the other hand, several ancestral reconstruction studies have reported enzymes with enhanced promiscuity. Glycosidases catalyze the hydrolysis of glycosidic bonds in all living cells but find practical applications in the synthesis of glycoconjugates as catalysts of the reverse reaction. Here, we use a library of ~500 possible substrates to compare the catalytic scope of the modern family-1 glycosidase from *Halothermothrix orenii* with that of a putative ancestral family-1 glycosidase derived from sequence reconstruction at a bacterial-eukaryotic common ancestor. The modern enzyme is the better catalyst for the hydrolysis of small substrates typically used to assess the activity of family-1 glycosidases. We identify, however, a trend for the modern enzyme to become less catalytically efficient with increasing substrate size. Such trend is not apparent in the conformationally-flexible ancestral glycosidase, which is in fact the better catalyst for the hydrolysis of flavonoid glycosides. Our results support that ancestral sequence reconstruction may provide a basis for enzyme engineering for the synthesis of glycoconjugates with large glycosyl acceptors.

Enzymes have been commonly described as highly specific catalysts capable of enhancing the rate of a given chemical reaction by many orders of magnitude. However, it is becoming increasingly clear that most enzymes are moderately efficient[1] and that many enzymes are promiscuous to some extent[2-4]. The concept of enzyme promiscuity, as commonly understood, encompasses the capability to catalyze different but related chemical reactions on a given substrate (*i.e.,* catalytic promiscuity), as well as the capability to catalyze the same chemical transformation on different substrates (*i.e.,* substrate promiscuity or substrate scope). With some obvious exceptions, such as detoxification enzymes,[5] promiscuous activities appear at low level and have no known physiological role. Yet, low-level activities may serve as starting points for the directed laboratory evolution of enzymes that efficiently catalyze the actual chemical transformation that is of biotechnological interest.[6-8] More generally, enzyme promiscuity is likely linked to conformational diversity, a biophysical feature known to contribute to evolvability, *i.e.,* to the capability to generate new functionalities.[9-11] Overall, promiscuity is a desirable feature in enzymes intended for biotechnological applications.[12-13] In fact, the initial surge in the number of publications dealing with enzyme applications to the transformation of non-natural products in the seventies and eighties[14] has been attributed in part to the realization that many enzymes are promiscuous catalysts.[15]

Some 60 years ago, Pauling and Zuckerkandl proposed that plausible approximations to the sequences of proteins belonging to extinct organisms can be derived from comparative analyses of the known sequences of their modern descendants.[16] Many proteins encoded by reconstructed ancestral sequences ("resurrected" ancestral proteins, in jargon) have been prepared and characterized in the last ~25 years.[17-25] They have often been found to display interesting properties from both the evolutionary and biophysical viewpoints. In fact, in a substantial number of cases resurrected ancestral proteins have shown enhanced promiscuity. [22,26-29] This

result may reflect that the most ancient enzymes were generalists[30-32] or simply that ancestral sequence reconstruction often targets pre-duplication phylogenetic nodes in the emergence of new functionalities.[27-28] Regardless of evolutionary interpretations, it emerges that ancestral reconstruction provides a valuable approach to search sequence space for enzyme promiscuity, a feature that is useful in biotechnological application scenarios but that it is accidental in most modern proteins.[27]

Glycosidases are highly efficient enzymes that accelerate the rate of hydrolysis of glycosidic bonds by up to about 17 orders of magnitude.[33] However, they can also catalyze the reverse reaction under conditions that thermodynamically favor synthesis, such as high concentrations of substrates, use of activated substrates or use of cosolvents to reduce water concentration.[15] Glycosidases can thus be used to couple sugars with glycosyl acceptors (*i.e.,* aglycons) to yield glycoconjugates with a diversity of applications.[15]

Glycosidases are widely distributed and are currently classified into 189 evolutionary distinct families.[34] We recently performed an ancestral reconstruction exercise targeting family-1 glycosidases, which share a TIM-barrel structure.[35] The TIM-barrel is a very common protein fold, that provides a framework for a wide diversity of natural enzyme functionalities.[36-38] Remarkably, we found that a putative ancestor of eukaryotic and bacterial family-1 glycosidases displayed much enhanced conformational flexibility, as shown by molecular dynamics simulations, proteolysis experiments, and missing regions in electronic density maps derived from X-ray crystallography.[35] Conformational flexibility is a biophysical feature commonly linked to promiscuity[9-11] and enhanced promiscuity may result in depressed levels of catalysis with some reactions/substrates while leading at the same time to increased levels of catalysis with other reactions/substrates. However, in our previous work [35] we found the ancestral glycosidase to be a less efficient catalysis than a representative modern family-1 glycosydase when tested with substrates commonly used to determine activity of family-1 glycosidases, pNP-glu and pNP-gal (Figure 1), as well with four substrates with larger aglycon moieties, MUB-gal, RES-gal, FMG-gal and RED-gal (Figure 1). Overall, we could not find a glycoside substrate for which the hydrolysis catalyzed by the ancestral glycosidase was faster compared with the representative modern glycosidase.

Here, we report an extensive comparison of the catalysis pattern of our ancestral family-1 glycosidase with that of the modern family-1 glycosidase from *Halothermothrix orenii*. These two enzymes, ancestral and modern, display similar thermophilic stability and share the TIM-barrel structure (Figure 2), but they display comparatively low sequence identity (58%) and clearly different patterns of conformational flexibility (Figure 2), with the ancestral protein being clearly more flexible.[35] Assessing glycosidase promiscuity is challenging, because these enzymes are widely diverse and are known to catalyze many different reactions. The current version of the CAZy database [34] lists 34 activities in family 1 alone and, additionally, the ancestral glycosidase could potentially catalyze some of the many reactions found in other families. Furthermore, glycosidases are already promiscuous to some extent regarding the aglycon moiety of the substrate.[15] We thus performed a chemo-informatics screening of the internal chemical compound library at Astra Zeneca, according to the following criteria: i) all the structures contained a 6-member ring

heterocycle (hexose and others), ii) the connection to the rest of the molecule was always at C1 via oxygen (or rarely sulphur), iii) the rest of the molecule could be either another sugar or something completely different. As a result, ~500 compounds were selected for experimental screening.

The main goal of the experimental screening was to identify "difficult" reactions with low levels of catalysis by the modern enzyme but higher levels of catalysis by the ancestral one. We, therefore, used comparatively high protein concentration (~20 μM) and a long incubation time (24 hours). The extent of reaction was assessed by mass spectrometry (see Experimental Section in Supporting Information for details). The results were as follows. 189 of the targeted substrates could not be detected. For 176 of the substrates significant conversion into products was not observed, neither with the modern enzyme, nor with the ancestral one. 55 of the substrates appeared highly susceptible to glycosidase-catalyzed degradation and their complete conversion into products was observed with both, the modern and the ancestral enzymes. For the remaining 60 of the substrates screened, significant conversion into products was found with at least one of the enzymes but full conversion was not observed with the two enzymes simultaneously. Distributions of molecular weights for these 60 "difficult" substrates (Figure 3) point to some interesting trends. Full conversion with the modern enzyme leads to a distribution peaked at about 450 g/mol, while a flatter molecular-weight distribution is observed with the substrates for which the modern enzyme only achieved partial conversion. This suggests that, although chemical differences are obviously relevant, the modern enzyme has a general preference for comparatively small size, at least for difficult to hydrolyze substrates. Interestingly, such preference is not apparent with the ancestral enzyme (Figure 3). These suggested trends are supported by the more detailed analyses given further below.

For three substrates (narirutin, linarin and hesperidin) complete reaction after 24 hours was observed with the ancestral glycosidase, but not with the modern glycosidase. These three substates are glycosylated flavonoids with similar molecular structures (Figure 1). Interestingly, out of the 34 activities listed for modern family-1 glycosidases in the CAZy database,[34] only 3 appear to correspond unequivocally to the hydrolysis of flavonoid glycosides, specifically the 3 activities involving substrates with anthocyanin or isoflavone as aglycons. We have carried out a more detailed characterization of the degradation of narirutin, linarin and hesperidin as catalyzed by the modern and ancestral glycosidases. Kinetic experiments in a time scale of several hours followed by mass spectrometry confirmed that, in the three cases, hydrolysis leads to the non-glycosilated flavonoid and that the ancestral glycosidase is a better catalyst than the modern glycosidase for these substrates (Figure 4). In fact, complete conversion of substrate to products is observed within hours with the ancestral glycosidase as a catalyst, but not with the modern glycosidase. Also, substantial hydrolysis is achieved in a time scale of minutes with the ancestral glycosidase as catalyst but not with the modern glycosidase (Figure 4).

We further used mass spectrometry to determine profiles of initial rate of reaction versus substrate concentrations (Figure 5a) and determined catalytic parameters from the fitting of the Michaelis-Menten equation to the profiles (Figure 5b). The values of the ancestral and modern catalytic efficiency for the hydrolysis of the flavonoid glycosides are compared in Figure 5c with the values previously

determined [35] for the hydrolysis of pNP-glu, pNP-gal, MUB-gal, RES-gal, FMG-gal and RED-gal (Figure 1). The comparison reveals patterns consistent with those expected for a substrate generalist enzyme (in the case of the ancestral glycosidase) and for a specialized enzyme (in the case of the modern glycosidase). That is, the values for the modern glycosidase span about 6 orders of magnitude and show a clear trend towards decreased catalytic efficiency with increasing substrate molecular weight. This result likely reflects the conformationally rigidity of the modern glycosidase [35] which may hamper the establishment of stabilizing interactions with the transition state of the reaction for the larger substrates. In fact, the pattern observed with the conformationally-flexible ancestral glycosidase is completely different (Figure 5c). Catalytic efficiency values are similar with the ancestral enzyme (up to two orders of magnitude) for the various substrates of Figure 1 and no clear trend with molecular weight is apparent. As a result, while the ancestral glycosidase is a much less efficient catalyst than the modern enzyme with typical family-1 glycosidase substrates, such as pNP-glu, pNP-gal, the two enzymes display similar levels of catalysis with the larger substrates among those in Figure 1, as it is visually apparent in Figure 5c. Actually, the ancestral glycosidase is the better catalyst with flavonoid glycosides as substrates (Figure 5d), a result which may plausibly reflect a specific preference for flavonoid aglycons or rutinose moieties but also a capability of the conformationally-flexible ancestral glycosidase to accommodate large substrates.

The enhanced ancestral catalysis with flavonoid glycosides poses intriguing questions in an evolutionary context. Flavonoids are highly diverse secondary metabolites produced by fungi and land plants, mostly as glycoside derivatives. Fungi diverged roughly 1.3 billion years ago, while land plants evolved from algae about 1 billion years ago.[39] On the other hand, our putative ancestral glycosidase derives from sequence reconstruction targeting a common ancestor of bacteria and eukaryotes, *i.e.*, a very ancient phylogenetic node (about 4 billion years). Under the, admittedly speculative, assumption that our putative ancestral glycosidase reproduces to some extent the properties of glycosidases that existed ~4 billion years ago, it is somewhat puzzling that it hydrolyzes compounds that are commonly found in organisms that evolved much later. Of course, we cannot rule out that flavonoid glycosides had roles in early life and were already produced ~4 billion years ago. Another possibility is simply that the ancestral glycosidases were generalists, [30-32] capable to hydrolyze a diversity of glycoside substrates and that flavonoid glycosides simply resemble some of the actual ancestral substrates.

Most natural flavonoids are glycosylated, and their de-glycosylation may be of practical interest in some cases.[40] An example is the enzymatic de-glycosylation of naringin, a substance contributing to bitter taste of oranges and grapefruits, to improve juice flavor. Furthermore, de-glycosylation may allow the subsequent link of a different sugar to the flavonoid aglycon to optimize glycosylation pattern for a variety of applications. Our ancestral glycosidase could potentially serve as starting point for the directed evolution of de-glycosylation enzymes useful in the context of the biotechnological applications of flavonoids. However, our results have general implications beyond this specific possibility. Glycosidases can be used to catalyze the coupling of sugars with aglycons to yield glycoconjugates, which have a diversity of applications. In several cases, glycoconjugates of interest bear large aglycons. For instance, cardiac glycosides are derived from steroid aglycones, and linking sugars to

proteins has been used to increase half-life and in vaccine development.[15] In a substantial number of cases resurrected ancestral proteins have shown enhanced promiscuity, [22,26-29] a catalysis pattern linked to conformational flexibility.[9-11] Furthermore, there is experimental evidence that conformationally-flexible resurrected ancestral proteins may be suitable scaffolds for the generation and directed evolution of new enzyme functionalities.[41-42] Against this backdrop, our results support ancestral reconstruction as a tool to systematically search sequence space for glycosidases with enhanced conformational flexibility and a capability to accommodate and process large substrates, which may provide a basis for enzyme engineering for the synthesis of glycoconjugates with large glycosyl acceptors.

Supporting Information

The authors give information on experimental procedures, as well as the sequences of the modern and the ancestral glycosidases.

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Conflict of interest

Leonardo De Maria, Martin Hayes and Francesco Falcioni are current employees and/or shareholders of AstraZeneca. The authors declare no competing interest.

Data availability statement

The data that support the conclusions of this study are available from the authors upon reasonable request.

Keywords: ancestral sequence reconstruction – conformational flexibility – enzyme promiscuity – substrate scope: glycosidases – TIM-barrel fold

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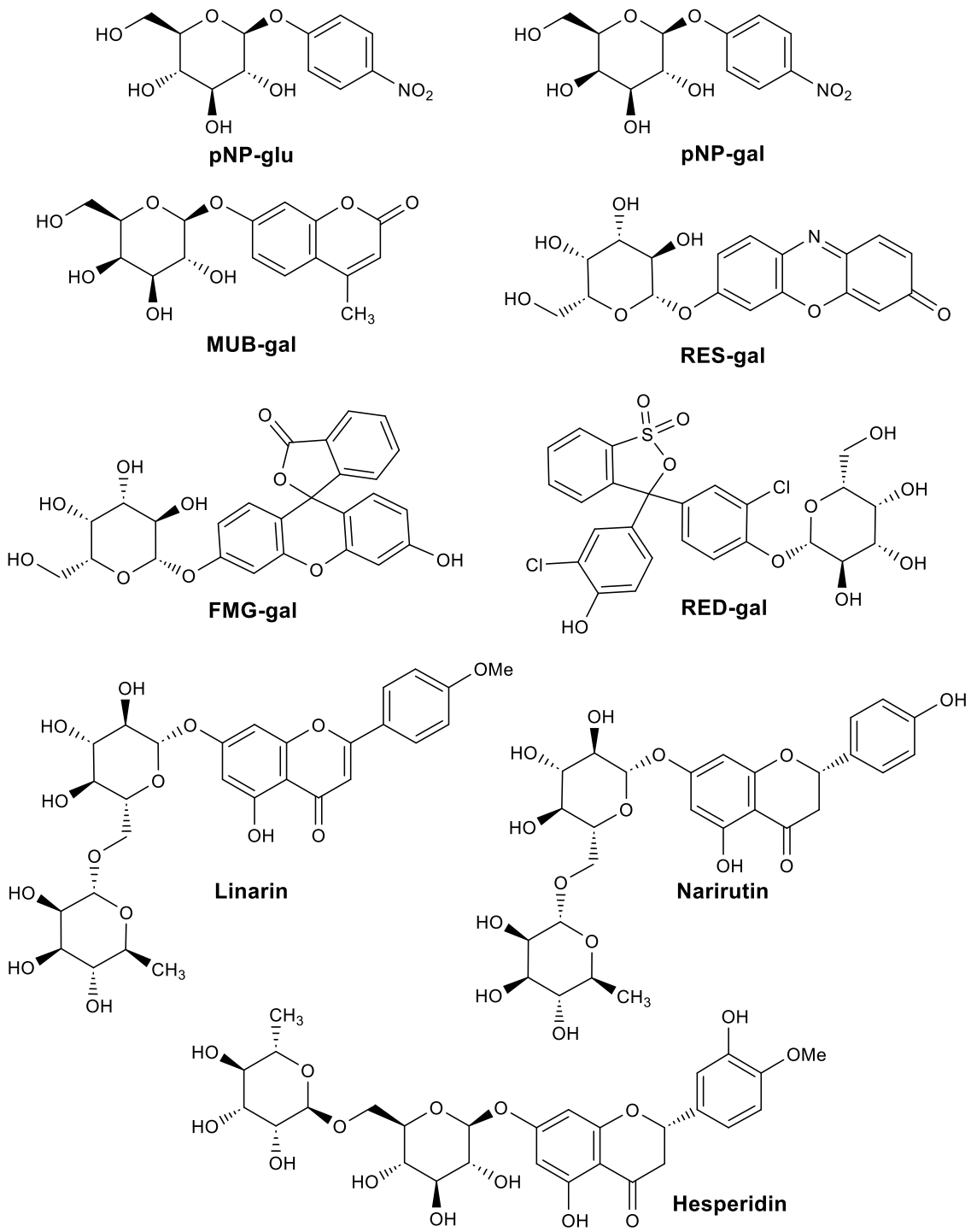

Figure 1. Glycosidase substrates discussed in detail in this work. pNP-glu: 4-nitrophenyl-β-D-glucopyranoside. pNP-gal: 4-nitrophenyl-β-D-galactopyranoside. MUB-gal: 4-Methylumbelliferyl β-D-galactopyranoside. RES-gal: Resorufin β-D-glucopyranoside. FMG-gal: Fluorescein mono-β-D-galactopyranoside. RED-gal: Chlorophenol Red-β-D-galactopyranoside. Glycoside flavonoids: linarin, hesperidin and narirutin.

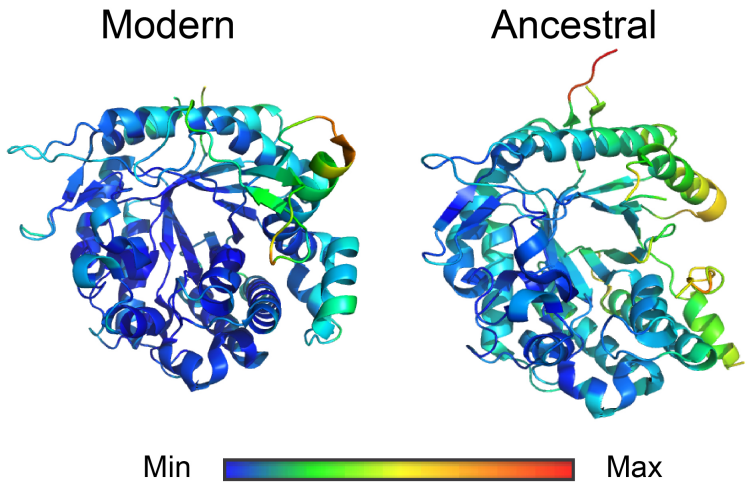

Figure 2. X-ray crystallographic structures of a putative ancestor of eukaryotic and bacterial family-1 glycosidases (PDB ID: 6Z1H) and the modern family-1 glycosidase from *Halothermothrix orenii* (PDB ID: 4PTV). For illustration, structures have been colored according to normalized crystallographic B-factor values. The enhanced conformational flexibility of the ancestral proteins is actually supported by proteolysis experiments and by molecular dynamics simulations.[35] Enhanced conformational flexibility in the ancestral protein is also revealed by missing regions in electronic density maps and, consequently, in the 3D-structure, as it is visually apparent.

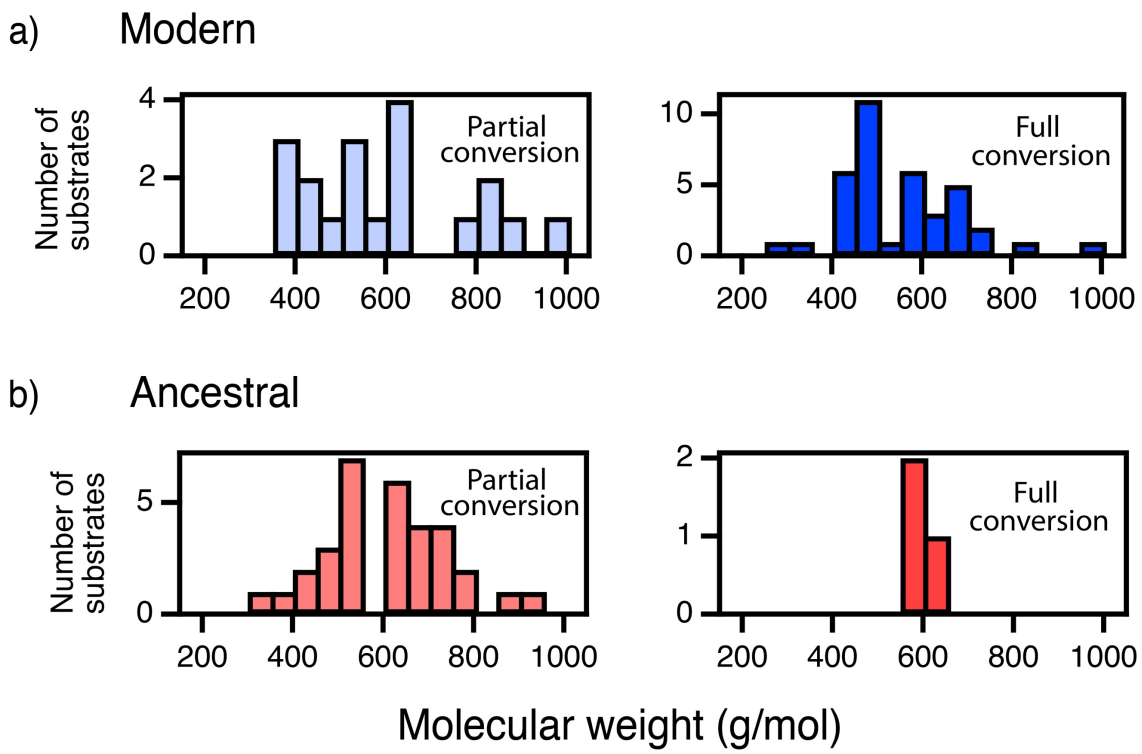

Figure 3. Molecular weight distributions for the substrates that showed significant conversion into products with at least one of the enzymes but for which full conversion was not observed with the two enzymes, modern and ancestral, simultaneously. Distributions are shown for four overlapping substrate sets: substrates displaying partial and full conversion with the modern glycosidase (a) and substrates displaying partial and full conversion with the ancestral glycosidase (b). In all cases, bins of 50 g/mol are used. These distributions suggest that the modern enzyme has a general preference for comparatively small substrates, while such preference is not apparent with the ancestral enzyme.

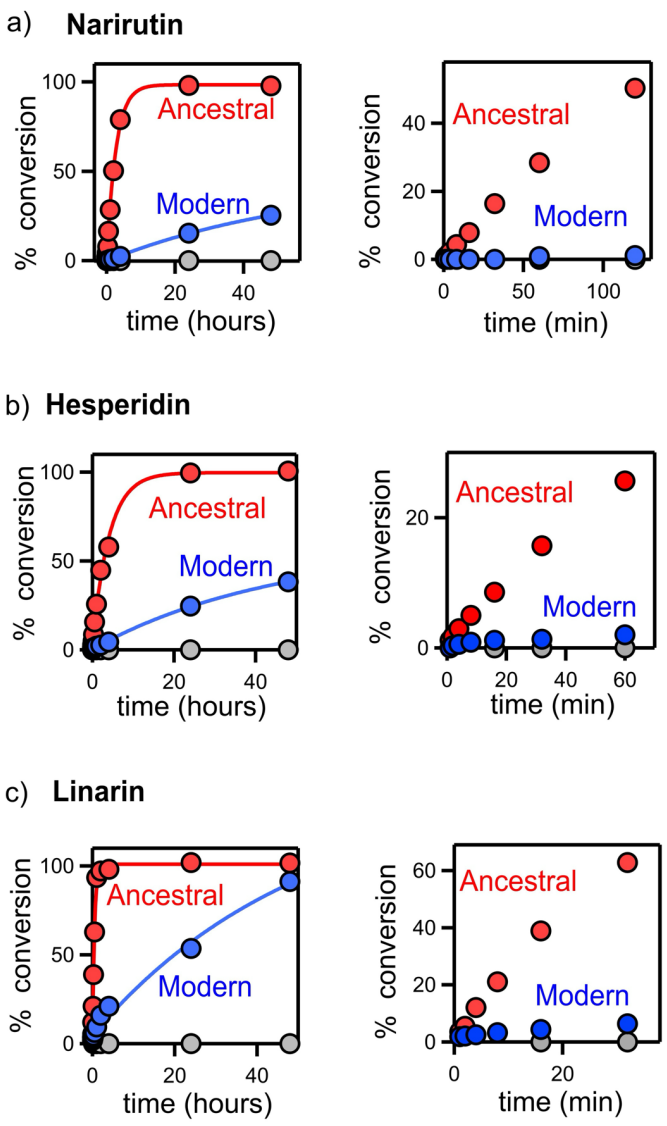

Figure 4. Kinetic of hydrolysis of flavonoid glycosides catalyzed by the ancestral glycosidase (red) and the modern glycosidase (blue). Blank experiments in the absence of enzyme are also shown (grey). Hydrolysis kinetics were followed based on the concentration of the flavonoid product, as determined by mass spectrometry. Note that the rate of the uncatalyzed reaction is negligible, in agreement with the known fact that glycosidic bonds display an extremely low rate of spontaneous hydrolysis.[43] The panels on the left show that, in all cases, full conversion into products is achieved in a time scale of hours with the ancestral enzyme as catalyst, but not with the modern enzyme. The panels on the right are blow-ups of the low time region and show that that significant hydrolysis is achieved in a time scale of minutes with the ancestral glycosidase as catalyst but not with the modern glycosidase.

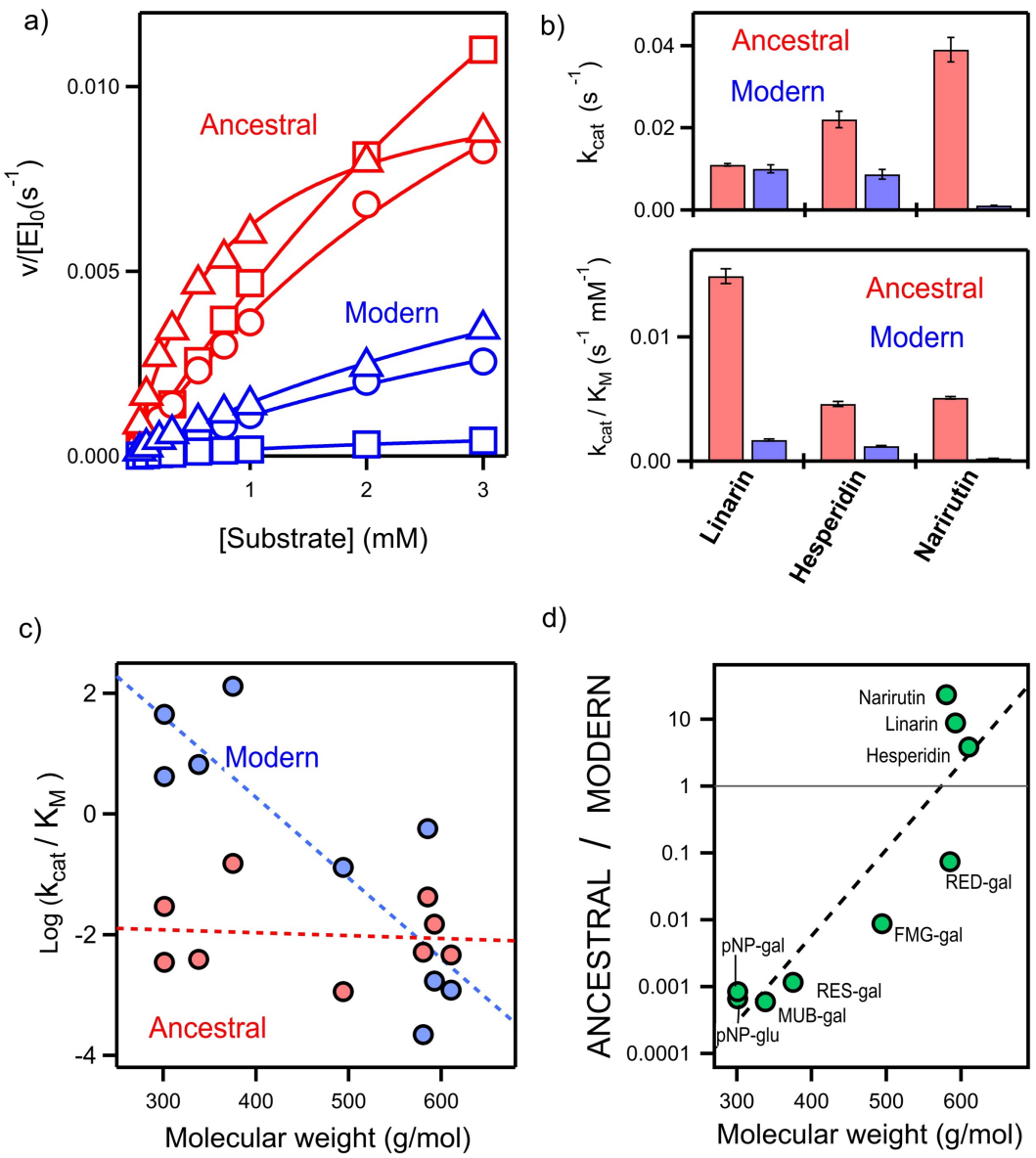

Figure 5. Catalytic parameters for glycoside hydrolysis. (a) Profiles of rate of hydrolysis *versus* substrate concentration for the hydrolysis of the flavonoid glycosides linarin (triangles), hesperidin (circles) and narirutin (squares), as catalyzed by the ancestral glycosidase (red) and the modern glycosidase (blue). $[E]_0$ and v stand for the total enzyme concentration and the initial reaction rate. Continuous lines represent the best fits of the Michaelis-Menten equation to the experimental data. (b) Values of the catalytic rate constant (k_{cat}) and catalytic efficiency (k_{cat}/K_M) for the hydrolysis of flavonoid glycosides derived from the fittings shown in (a). Error bars represent standard deviations from the fittings. (c) Plot of logarithm of catalytic efficiency *versus* substrate molecular weight for the hydrolysis of all the substances shown in Figure 1, as catalyzed by the ancestral glycosidase (red) and the modern glycosidase (blue). The dashed lines are meant to guide the eye to the general trends. (d) Plot of the ancestral over modern catalytic efficiency ratio for all the substrates shown in Figure 1. The dashed line is meant to guide the eye to the general trend.