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Evolutionary and mechanistic relationships between glycosidases acting on α- and β-bonds☆

Mark R. Stam, Eric Blanc, Pedro M. Coutinho and Bernard Henrissat*

Architecture et Fonction des Macromolécules Biologiques, UMR6098, Centre National de la Recherche Scientifique, Universités d'Aix-Marseille I & II, Case 932, 163 Avenue de Luminy, 13288 Marseille cedex 9, France

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Abstract—Because of the fast accumulation of sequences derived from genome sequencing efforts, the sampling of the sequence space in glycosidase and related enzyme families is such that sensitive sequence similarity detection methods like PSI-BLAST are now able to reveal distant, but clear, structural and evolutionary relations between glycosidases acting on α- and β-bonds. We have observed this trend within groups of glycosidases with completely different folds. We postulate that the evolutionary interconversion between α- and β-acting glycosidases was greatly facilitated by the fact that both types share a similar axial orientation of the glycosidic bond in the reactive bound substrate. Glycosides in the β anomeric configuration, require a sugar ring distortion, resulting in an axial orientation of the glycosidic bond, equivalent to that of an α glycosidic bond, prior to displacement by nucleophilic substitution.

Keywords: Glycosidase; Phosphorylase; Molecular mechanism; Evolution; Sugar ring distortion

1. Introduction

Glycosidases, the enzymes that cleave the glycosidic bonds in glycoconjugates, oligo- and poly-saccharides, are a group of enzymes that act on one of the most structurally diverse range of substrates in nature. Due to their central role in the degradation of complex carbohydrates, these enzymes have been the subject of many classical studies in biology. The first enzyme whose structure was solved was lysozyme, the enzyme that cleaves glycosidic bonds in peptidoglycan.[1] Over 50 years ago, Koshland had described accurately the two major molecular mechanisms of glycosidases,[2] which lead to either retention or inversion of the anomeric configuration at the site of cleavage, through double or single nucleophilic displacements, respectively. Much later, glycosidases have been classified in families based on amino acid sequence similarities.[3–5] This classification system, which presently includes over 100 families, is available in the continuously updated carbohydrate-active enzyme database (CAZy) at http://afmb.cnrs-mrs.fr/CAZY/.[6] The CAZy families usually do not coincide with substrate specificity, but they correlate with the 3-D structures and molecular mechanism of glycosidases.[7–9] Because the 3-D structure of proteins is better conserved than their sequences, several families have been found to share structural and mechanistic similarity. These similarities have been used to define a hierarchical grouping, higher than the families, and termed 'clan'.[5] Clans group together glycosidase families that have a similar 3-D structure and an identical catalytic machinery, and that operate on stereochemically related substrates.[8,10] However, with about 50 families with known structures, it is now apparent that a given fold can be found for both 'inverting' and 'retaining' glycosidases and this raises the question of whether this is the result of convergent evolution or the result of mechanistic evolution.

In the early 1990's, glycosidase families were defined from a somewhat limited sample (historically the first

☆ Throughout this manuscript α- and β- assume a D-*gluco* configuration for the sugar.

* Corresponding author. E-mail: Bernard.Henrissat@afmb.univ-mrs.fr

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35 families of glycosidases were derived from analysis of the 300 sequences available in 1991[3]). At that time, biocomputing tools were not as powerful as today and, perhaps more significantly, the sample of the sequences available was not large enough to derive interfamily relationships without structural data. The 59-fold increase in sequence data in the last decade (there were over 16,000 sequences of glycosidases and related proteins in the CAZy database in May 2005) generates a much better sampling of diversity than when the families were first defined, and sensitive sequence comparison methods can now reveal relationships that could not be detected earlier [see, e.g., Ref. 11].

3-D Structure comparisons and careful amino acid sequence analyses have already suggested possible evolutionary relationships between retaining and inverting β-glycosidases. Examples include (i) retaining GH22 lysozymes and inverting GH19 chitinases[12] and (ii) β-furanosidases from family GH43 (inverting mechanism) and families GH32 and GH68 (retaining mechanism).[13–15] Here, we wanted to examine the possible evolutionary relationships between glycosidases cleaving glycosidic bonds of *opposite* orientation (namely, α vs β). Such an interconversion might be hard to conceive, but the limited number of sugar ring distortions observed in the conformational paths of glycosidases (for reviews, see: Refs. 16 and 17) suggest that the differences between α- and β-glycosidases might be less than thought.

2. Materials and methods

Our past experience with PSI-BLAST analyses of glycosidase sequences using the non-redundant database of the NCBI (hereafter 'NR') is that the results can be polluted by the modular structure of many of these enzymes (for a review on the modular structure of glycosidases, see: Ref. 18). To maximize the sensitivity of our sequence comparisons and to minimize such 'contaminations', we used PSI-BLAST[19] against a laboratory-made library of sequences containing the catalytic modules of glycosidases derived from CAZy, namely, where the signal peptides, linkers, propeptides, carbohydrate-binding, protein-binding, and other noncatalytic modules of carbohydrate-active enzymes have been removed.[6] The size of this library (hereafter called 'CAZyModO'), only about 1% of that of NR, has the additional advantage of reducing computing time dramatically. To test the validity of this approach and for comparison purposes, we have also downloaded NR and used it for PSI-BLAST analyses in the early stages of our work. For both sequence libraries, the database-size parameter used for calculation was set to that of NR (467,799,957 letters on 19 March 2004)[20] in order to obtain data that can be compared and to use the same commonly accepted threshold for inclusion in a subsequent iteration (*E*-value of 10^{-3}). Sequence alignments were done using MUSCLE[21] and structural alignments with T-Coffee.[22] In several instances we made use of the 3D-PSSM server.[23]

3. Results

To ensure the best enzymatic significance, we have used the sequences of well characterized glycosidases—whenever possible with a solved 3-D structure—for our biocomputing searches. Using the sequence of the α-L-rhamnosidase of *Aspergillus aculeatus*[24] (GenBank AAG13964.1; family GH78; EC 3.2.1.40; inverting mechanism) as the query and NR as the library for PSI-BLAST searches, several inverting cellobiose and chitobiose phosphorylases (family GH94), α-glucosidases of family GH63 (undetermined catalytic mechanism), inverting glucoamylases (family GH15), and inverting α,α-trehalases (family GH37) were retrieved already at the second iteration. Inverting α-(1→2)-L-fucosidases (GH95) along with members of the preceding families were progressively retrieved upon further iterations. Several members of family GH65 were also picked after several iterations. During the search, several other glycosidase sequences appeared, such as α-(1→2)-mannosidases of family GH92 or cellulases from families GH9, GH8, and GH48; however these remained below the threshold of inclusion.

Using the same family GH78 α-L-rhamnosidase sequence as the query and CAZyModO as the search database, the same families (GH94, GH15, GH63, GH37, GH95, and GH65) were also progressively retrieved, but using only a fraction of the computing time necessary for searching NR. The much shorter computing time allowed performing PSI-BLAST searches with a number of different query sequences from different families for increased reliability. Depending on the starting sequence, variations were observed in the order of appearance of the various sequences. For instance, family GH65 was found only when starting from members of family GH15 or GH78. On the other hand, our analyses consistently grouped together families GH78, GH94, GH15, GH63, GH37 and GH95. Given the foregoing results, it was decided to continue our analyses only using CAZyModO as the search database.

When inverting polygalacturonase 2 from *Erwinia carotovora* (SwissProt P26509; family GH28; EC 3.2.1.15) was used as the query, pectate lyases of family PL1 were retrieved after two PSI-BLAST iterations, together with inverting dextranases of family GH49. All of these enzymes cleave α-glycosidic bonds. One additional iteration was sufficient to retrieve family GH87 mycodextranases (acting on α-glycosidic bonds; unknown stereochemical outcome) and inverting ι-carrageenases of family GH82 (acting on β-linked substrates).

In a last series of PSI-BLAST analyses, the catalytic domain of inverting β-amylase from *Bacillus cereus* (SwissProt P36924; family GH14; EC 3.2.1.2) was used as the query. Retaining β-galactosidases of families GH42 and GH35 were retrieved after one and two iterations, respectively. Further iterations then progressively retrieved other families which make up 'clan' GH-A. This clan groups together over 2800 sequences from at least 16 families (families GH1, GH2, GH5, GH10, GH17, GH26, GH35, GH39, GH42, GH50, GH51, GH53, GH59, GH72, GH79, and GH86), which operate with a retaining mechanism on equatorial glycosidic bonds.

4. Discussion

4.1. Relationships between inverting glycosidases acting on α- and β-bonds

The molecular mechanism of both retaining and inverting glycosidases involves one of four possible oxocarbenium ion-like transition states.[16,25] Among β-retaining glycosidases, the transition state-like ring conformers $^{4}H_{3}$, $^{2,5}B$, and $B_{2,5}$ were described for families GH5 and GH7,[26,27] GH11[28] and GH26[17], respectively. For β-inverting families the $^{2,5}B$ transition state-like conformer has been observed in families GH6, GH8, and GH48.[27,29,30] All these conformations have in common a coplanar position for C-5, O-5, C-1, and C-2 of the sugar ring.[16,25]

Other intermediate forms corresponding to the Michaelis complex that precedes the transition state, have also been observed, permitting the unambiguous identification of the different steps of the reaction pathway.[16,25] In β-acting enzymes, these observed Michaelis complexes correspond to significant ring distortions required to place the glycosidic bond into a pseudo-axial orientation, which allows a favorable in-line nucleophilic attack.[26,31] It should be noted that significant ring distortions accompanying the formation of the Michaelis complex are unnecessary for family GH15 glucoamylases and related α-inverting enzymes, since the glycosidic bond of the substrate is naturally in the favorable axial conformation for nucleophilic attack.[32] In fact, the molecular mechanism of inverting glycosidases acting on α-bonds involves the attack of a nucleophilic water molecule (suitably activated by an enzymatic base) onto the anomeric carbon of the non-distorted glycoside with concomitant departure of the axial glycosidic linkage (suitably activated by protonation of the glycosidic oxygen) (Fig. 2A; for reviews, see: Refs. 25, 33 and 34).

PSI-BLAST analyses conducted with the set of sequences available in the CAZy database (May 2005) clearly point to a common evolutionary origin for families GH37, GH63, GH15, GH65, GH78, and GH94. This is further substantiated by the clear similarity of the X-ray structures available for families GH15,[35] GH65,[36] and GH94,[37] and our analysis suggests that family GH37 α,α-trehalases and family GH78 α-L-rhamnosidases have a similar $(\alpha/\alpha)_6$-barrel structure. It is likely that this structure also prevails in family GH95 α-(1→2)-L-fucosidases and in family GH92 α-(1→2)-mannosidases. To analyze the significance of this grouping at the structural level, we have produced a structural alignment of representatives of families GH15, GH65, and GH94. The location of the catalytic residues (proton donor and base, Fig. 1A) was examined and compared to that deduced for families GH37, GH63, GH78, GH92, and GH95 after multiple sequence alignment in each family and 3D-PSSM threading analyses. The results are summarized in Figure 1B, which shows that all the α-helices constituting the $(\alpha,\alpha)_6$ barrel are aligned with conservation of the position of the catalytic residues (note that the base is replaced by phosphate in the phosphorylases), further pointing toward a common evolutionary origin for these enzymes. However, while enzymes of families GH15, GH37, GH63, GH65, GH78, GH92, and GH95 operate on α-glycosidic bonds, members of the recently created family GH94 (containing cellobiose, cellodextrin and chitobiose phosphorylases) cleave chitobiose or cellobiose, namely, β-linked sugars.

In family GH65, which contains both hydrolases and phosphorylases, a variation of the canonical mechanism of the inverting α-glycosidases of family GH15 is employed by the phosphorylases where the nucleophilic water is replaced by a phosphate (Fig. 2B).[36] Can this mechanism be transposed to β-cleaving enzymes such as chitobiose phosphorylase of family GH94? In the latter, a ground-state $^{4}C_{1}$ chair conformation of the substrate would place the β-glycosidic bond in an equatorial orientation unsuitable for an in-line attack by the nucleophile. For catalysis to take place, it is therefore necessary that the substrate undergoes a distortion from the $^{4}C_{1}$ chair to a conformation that places the β-glycosidic bond in an axial orientation,[26,31] suitable for nucleophilic substitution by phosphate.[37] In fact the reactive, distorted, β-glycosidic bond is topographically and chemically similar to an α-glycosidic bond (Fig. 2C). We note that the mechanistic resemblance between the α-acting hydrolases (Fig. 2A), α-acting phosphorylases (Fig. 2B), and β-acting phosphorylases (Fig. 2C) can be extended to β-acting hydrolases (Fig. 2D) so that the pseudo-axial orientation of the glycosidic bond, required for the formation of the Michaelis complex and therefore required for catalysis, is achieved in both classes of β-acting enzymes.

In fact, three glycosidase families, namely GH8, GH9, and GH48, contain enzymes that cleave β-glycosidic bonds with inversion of configuration and with similar $(\alpha/\alpha)_6$-barrel structures.[38–40] These inverting cellulase

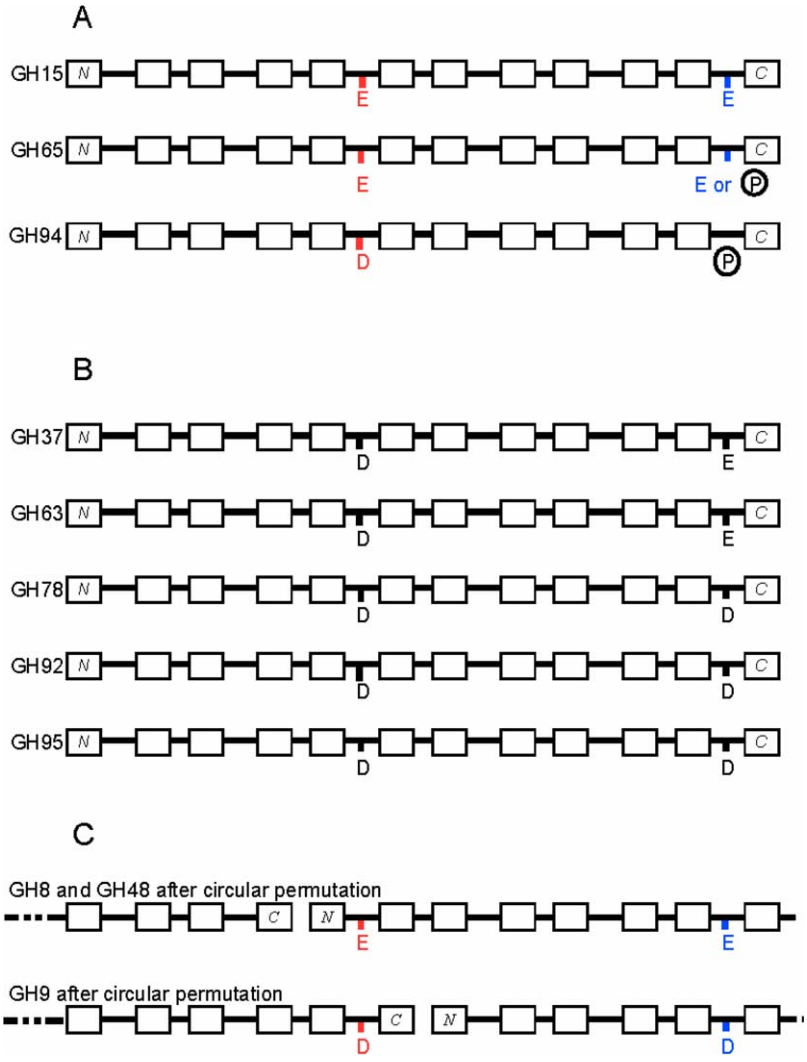

Figure 1. Schematic structural alignment of (α/α)6-barrel glycosidases. The α-helices are represented by boxes. *N* and *C* denote the helices situated close to the *N*- and *C*-termini of the proteins. Where known, the catalytic acid is shown in red and the catalytic base in blue (P indicates where phosphate is the nucleophile). (A) Structural alignment of families GH15, GH65 and GH94 based on superimposition of 3-D structures. (B) Structural alignment based on multiple alignments mapped with secondary structure prediction for related families GH37, GH63, GH78, GH92 and GH95. The position of the conserved candidate catalytic residues is shown in black. (C) Circular permutations yield a similar positioning of the experimentally determined catalytic residues in families GH8, GH48 and GH9 (3-D structures are available for each of these three families).

have been classified in the same SCOP superfamily (named 'Six-hairpin glycosidases')[41] as all the previously mentioned inverting glycosidases families with $(\alpha/\alpha)_6$-barrel folds. However, the cellulase families were not retrieved by our PSI-BLAST analyses. Some of these cellulases did appear in the PSI-BLAST searches, but they never achieved scores that brought them above the threshold of inclusion in the next iteration. This result provided an excellent control that the grouping of families GH15, GH37, GH63, GH65, GH78, GH92, GH94, and GH95 by PSI-BLAST was not just dictated by a similar 3-D structure. At the structural level though, only cellulases of family GH9 have a positioning of their catalytic residues similar to that of the enzymes shown in Fig. 1C. However, we noted that a circular permutation within the sequences of families GH8 and GH48 would be sufficient to place their catalytic residues in a position (Fig. 1C) similar to that of the other enzymes.

We believe that all the enzymes in families GH15, GH37, GH63, GH65, GH78, GH92, GH94, and GH95, and possibly GH8, GH9, and GH48, share a common ancestry and that the evolutionary interconversion between α- and β-linkage specificity was greatly facilitated by the fact that both α- and β-acting enzymes share a very similar orientation of the glycosidic bond in the reactive bound substrate. Because the similarity in the glycosidic bond orientation is dictated solely by chemistry and not by the fold of the enzymes, we wished to examine if a similar sequence-based grouping of inverting enzymes acting on α- and β-glycosidic linkages could be found for folds other than the $(\alpha/\alpha)_6$-barrel

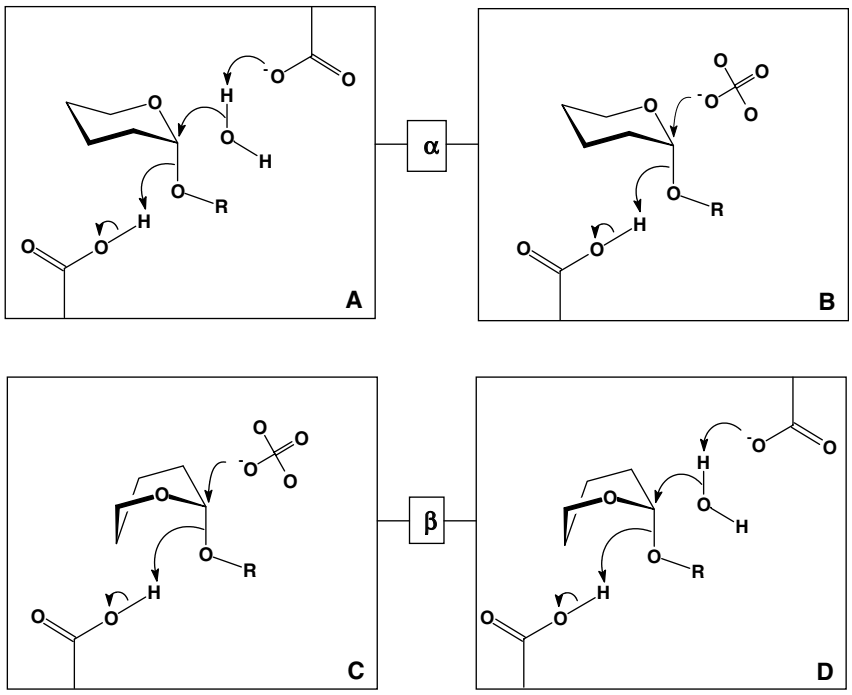

Figure 2. Mechanistic similarity in various inverting families of glycosidases and related enzymes sharing an $(\alpha/\alpha)_6$-barrel structure. (A) Mechanism of α-acting glycoside hydrolases from families GH15, GH37, GH63, GH65, GH78, GH92, and GH95. (B) Mechanism of α-acting disaccharide phosphorylases from family GH65. (C) Mechanism of β-acting disaccharide phosphorylases from family GH94. (D) Mechanism of β-acting glycoside hydrolases from families GH8, GH9, and GH48.

examined above. For this purpose, we have subjected to PSI-BLAST searches a polygalacturonase from family GH28. The enzymes of this family degrade α-(1→4)-linked polygalacturonic acid with overall inversion of anomeric configuration.[42] The three-dimensional structure has been solved for several members of this family and is a typical β-helix.[43,44] Starting from polygalacturonase 2 from *Erwinia carotovora*, PSI-BLAST rapidly retrieved pectate lyases (CAZy family PL1) and inverting dextranases of families GH49 and GH87. Like polygalacturonases, pectate lyases and dextranases of family GH49 act on α-linked glycosidic bonds and have a β-helical fold.[45,46] One more PSI-BLAST iteration was sufficient to pick inverting ι-carrageenases of family GH82, which also have a β-helix structure[47] but act on β-linked substrates. If this result is not dictated solely by the structural resemblance, then this would indicate that the evolutionary interconversion between inverting α- and β-hydrolases has occurred independently in at least two totally unrelated glycosidase ancestors.

4.2. Relationships between α- and β-glycosidases of *opposite* mechanisms

Similarly to the foregoing, the mechanistic requirement of a distortion of the substrate in the active site of a β-acting retaining glycosidase places the glycosidic bond in an axial orientation (Fig. 3A) corresponding to that

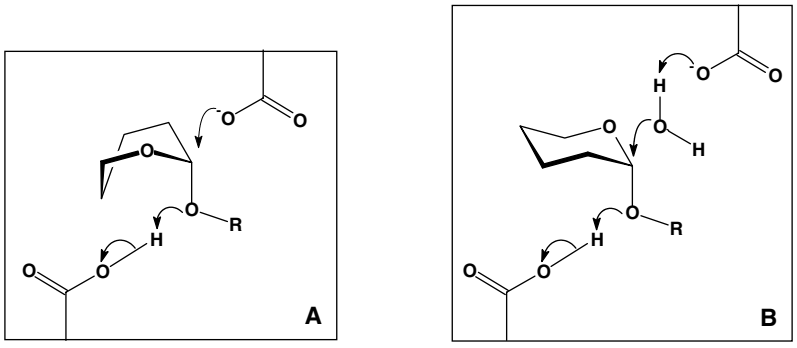

Figure 3. Similarity between the glycosylation step of retaining glycosidases from clan GH-A (A) and the single displacement mechanism of inverting β-amylases (B).

of the α-linkage in an inverting enzyme (Fig. 3B). However, it is important to stress that the transformation from a retaining β-acting enzyme into an inverting α-glycosidase also requires the suitable positioning of a nucleophilic water molecule in the latter. Despite this additional constraint, a PSI-BLAST analysis of the sequences present in the CAZy database, starting from the inverting β-amylase from *Bacillus cereus* (a member of family GH14 with a $(\beta/\alpha)_8$-barrel structure)[48] readily retrieved retaining β-galactosidases of families GH42 and GH35 and then the other families which make up clan GH-A. Clan GH-A groups together well over 3000 sequences from at least 16 families (families GH1, GH2, GH5, GH10, GH17, GH26, GH35, GH39, GH42, GH50, GH51, GH53, GH59, GH72, GH79, and GH86) with great variation of substrate specificity, and therefore offers an excellent sampling of the sequence space for PSI-BLAST. Interestingly, the structural similarity between family GH14 β-amylases and clan GH-A glycosidases had been noted 10 years ago in an elegant and insightful comparison of 3-D structures.[49]

Enzyme active sites are known for their plasticity. Within structural superfamilies where only the fold is maintained, a number of mechanisms have been suggested for the development of distinct activities. Cycles of point mutation leading to either functional optimization or to new activities, together with other events like speciation, duplication, and divergence, and even circular permutation, are likely to have led to present-day enzymes.[50] As catalytic sites are gradually adapted, structural aspects from the 'parent' enzyme are retained, meaning that active site location and/or substrate type are usually retained. In the $(\alpha/\alpha)_6$-barrel structural superfamily, we observe that this extends to the retention of overall aspects of the catalytic mechanism of glycosidases.

4.3. Significance

It has been already shown that nature has been able to change retaining into inverting β-glycosidases.[12–15] Such modifications have been performed in a couple of occasions by site-directed mutagenesis.[51,52] Here, we wanted to examine if nature has been able to transform enzymes able to cleave β-glycosidic bonds into α-cleavers, or if α- and β-glycosidases have evolved independently. The results of sequence and structural comparisons coupled to mechanistic considerations suggest that nature has

- invented glycosidases several times from completely different protein scaffold ($(\alpha/\alpha)_6$-barrels, $(\alpha/\beta)_8$-barrels, β-helices, etc.),
- used existing carbohydrate-acting enzymes as scaffold to evolve new activities when opportune,[53–56]
- taken advantage of the requirement for axial orientation of the glycosidic bond to evolve (sometimes but not always) interconversion between α- and β-acting glycosidases (and related phosphorylases and polysaccharide lyases).

This work has focused on the evolution of glycosidase mechanisms within structural families. However interesting in an evolutionary perspective, the exploration of this ancient past only helps very indirectly the functional prediction for the thousands (and ever growing number) of glycosidase-related genes that result from genome sequencing efforts worldwide by placing a strong warning. The fact that modern comparison algorithms coupled to the large sequence sample now available are able to group together glycosidases that operate on α- and β-bonds (with either retaining or inverting mechanism) clearly shows that the detection of distant relationships (by PSI-BLAST or other algorithms such as fold recognition) is wholly *inappropriate* for automatic glycosidase function prediction in genomes. We also note that alternative reactions have sometimes been selected (phosphorylases, lyases) in the course of the evolution of glycosidases, and these will also affect adversely function prediction based on distant similarities.

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