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Reassessment of Acarbose as a Transition State Analogue Inhibitor of Cyclodextrin Glycosyltransferase†

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ABSTRACT: The binding of several different active site mutants of Bacillus circulans cyclodextrin glycosyltransferase to the inhibitor acarbose has been investigated through measurement of Kᵢ values. The mutations represent several key amino acid positions, most of which are believed to play important roles in governing the product specificity of cyclodextrin glycosyltransferase. Michaelis–Menten parameters for the substrates α-maltotriosyl fluoride (αG3F) and α-glucosyl fluoride (αGF) with each mutant have been determined by following the enzyme-catalyzed release of fluoride with an ion-selective fluoride electrode. In both cases, reasonable correlations are observed in logarithmic plots relating the Kᵢ value for acarbose with each mutant and both kcat/Km and Kᵢ for the hydrolysis of either substrate by the corresponding mutants. This indicates that acarbose, as an inhibitor, is mimicking aspects of both the ground state and the transition state. A better correlation is observed for αGF (r = 0.98) than αG3F (r = 0.90), which can be explained in terms of the modes of binding of these substrates and acarbose. Re-refinement of the previously determined crystal structure of wild-type CGTase complexed with acarbose [Strookopytov, B., Penninga, D., Rozeboom, H. J., Kalk, K. H., Dijhuizen, L., and Dijkstra, B. W. (1995) Biochemistry 34, 2234–2240] reveals a binding mode consistent with the transition state analogue character of this inhibitor.

Transition state analogues have long provided a valuable tool for probing enzymatic mechanisms as well as providing a basis for the design of tight binding enzyme inhibitors. Their design is based on the principle that extra binding interactions develop between the enzyme and substrate in the transition state complex conferring reaction specificity and rate acceleration. Thus, analogues which mimic the substrate transition state structure should bind tightly. This transition state analogue, which was first proposed by Pauling in 1946 (2) and then further evolved by Lienhard (3) and Wolfenden (4), has provided the framework for the design of a wide variety of tight binding, reversible enzyme inhibitors. Key characteristics of sugar-based transition state analogue inhibitors for glycosidases are a positive charge, a trigonal anomeric center, and a half-chair-like conformation (5–8).

A useful way of determining whether a particular inhibitor is truly a transition state analogue or is just a fortuitously tight binding inhibitor involves probing the effects on the Kᵢ value of modifications to the interactions between the enzyme and inhibitor and then probing the effects of equivalent modifications to the enzyme/substrate complex at the transition state, as revealed in kcat/Km values. If the effects on the inhibitor Kᵢ value correlate well with the effects on substrate kcat/Km, then it can be concluded that the inhibitor is indeed a transition state analogue. This approach has been reviewed recently (9). Such modifications can be effected in two ways. One way involves the synthesis and kinetic evaluation of a series of modified substrates with the wild-type enzyme, along with measurement of Kᵢ values for the identically modified inhibitors. The other approach involves the construction of a series of mutant enzymes, preferably mutated at sites thought to play a role in substrate recognition and catalysis, and then measurement of kcat/Km and Kᵢ values with each mutant for a single substrate and transition state analogue inhibitor, respectively. The choice of approach depends primarily upon whether it is easier to generate mutant enzymes or modified substrates and inhibitors. The former approach has been used to demonstrate that a series of phosphonamidate peptide derivatives are indeed true transition state analogue inhibitors for zinc peptidases and thermolysin (10–12). Similarly, nojiritetrazoles were shown by this method to be true transition state analogues for a range of glycosidases (7). Using the same approach, 1-deoxynojirimycin and castanospermine were shown not to be transition state analogues for Agrobacterium sp. β-glucosidase but rather, tight binding fortuitous inhibitors (13). The latter approach has been applied to rat carboxypeptidase A (12, 14) and glucoamylase (15). It seemed possible that such an approach could be applied to interactions of the

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Acarbose as a Transition State Mimic of CGTase

CGTases are glycosyl transferases that degrade starch into linear or cyclic oligosaccharides (cyclodextrins). Much attention has been paid to these enzymes for their value in producing cyclodextrins, which have wide industrial applications (17). However, most CGTases suffer from a low product specificity, resulting in mixtures of most commonly α-, β-, and γ-cyclodextrins which then require costly crystallization procedures for purification (18). Therefore, a great deal of effort has been expended to design CGTases with unique product specificities by generation of a number of mutants modified in the active site (19, 20). These enzymes belong to the same sequence- and structure-related family [Family 13; a (ββα)8 barrel] as do α-amylases and many α-glucosidases. As such, they are inhibited by the natural product acarbose, a pseudotetrasaccharide isolated from Actinoplanes, which has long been known as a potent reversible inhibitor of many Family 13 enzymes including the pig pancreatic α-amylase ($K_i = 10 \mu M$) (21), as well as of the Family 15 glucoamylase ($K_i = 0.1 \text{nM}$) (22) (Figure 1). Indeed, it is currently used as a drug for the control of blood glucose levels in diabetics (1). Indeed, it is currently used as a drug for the control of blood glucose levels in diabetics (1).

In an effort to investigate the interactions of acarbose with CGTase, we have screened a range of active site mutants of acarbose to further understand the binding mode of acarbose to CGTase observed crystallographically (16).

Complexes of acarbose with other α-maltotriosyl fluoride (αG3F) and α-glucosyl fluoride (αGF), were used in this analysis, allowing the determination of accurate kinetic parameters in a direct assay monitoring fluoride ion release (31). By correlating the kinetic parameters so determined with $K_i$ values for acarbose, insights have been obtained into the nature of the strong inhibition afforded by this naturally occurring and therapeutically important inhibitor. In addition, to investigate whether an alternative binding mode of acarbose would be compatible with the original data set, the structure was re-refined.

**MATERIALS AND METHODS**

**General.** All mutant CGTases were generated and purified as previously reported (32). αG3F and αGF were synthesized by the method of Junnemann et al. (33). All other chemicals were purchased from Sigma Chemical Co. with the exception of acarbose which was generously provided by Miles Inc. (now Bayer).

**Kinetic Analysis. (A) Glycosyl Fluorides.** The enzymecatalyzed reactions were followed by monitoring the release of fluoride using an Orion fluoride-selective electrode interfaced to a pH/ion selective meter (Fischer Scientific). Data were collected using the program Terminal and analyzed using the program Grafit (34). In a typical experiment, αG3F or αGF was preincubated in 100 mM citrate buffer, pH 6.0, at 30 °C, for 10 min. The reaction was initiated by adding an appropriately diluted aliquot of enzyme in citrate buffer [final enzyme concentration with αG3F as substrate: wild-type (0.25 μg/mL); S145E (1.1 μg/
mL); S146P (13.4 μg/mL); D371N (1.7 μg/mL); N193G (1.5 μg/mL); N326Q (3.2 μg/mL); Y195G (3.2 μg/mL); Y195F (1.1 μg/mL); Y195L (1.1 μg/mL); with αGF as substrate: wild-type (0.25 μg/mL); S145E (19.3 μg/mL); S146P (416 μg/mL); D371N (97.3 μg/mL); N193G (65 μg/mL); N326Q (254 μg/mL); Y195G (276 μg/mL); Y195F (16.6 μg/mL); Y195L (28.1 μg/mL)] and the progress of the reaction monitored for approximately 5–10 min or until a maximum of 10% of substrate was consumed. For each enzyme, a total of seven substrate concentrations were analyzed, ranging from 0.2 to 5 times Km. In almost all cases the kinetic constants K_m, V_max and k_cat were calculated from a fit to the Michaelis–Menten equation using the program GraFit (34). For those mutants with very high K_m values (Y195G and N326Q), thus for which saturation behavior could not be observed, an accurate value of k_cat/K_m was obtained from the slope of the Lineweaver–Burk plot, and very approximate estimates of the individual parameters were obtained from the intercepts.

(B) Evaluation of Oligosaccharides as Acceptors. Differences in rate upon the addition of various maltoligosaccharide acceptors were determined by measuring the initial rate of D371N CGTase (10 μL aliquot, final concentration was 97.3 μg/mL) catalyzed fluoride release using a single concentration of αGF (40 μL aliquot, final concentration was 28 mM) in 100 mM citrate buffer, pH 6.0 (final volume before the addition of oligosaccharide was 260 μL). After following the reaction for 2 min, a 20 μL aliquot of the appropriate acceptor was added and the progress of the reaction monitored. Final concentrations of G to G6 were 30 mM.

(C) Inhibition of CGTases by Acarbose. Approximate K_i values for acarbose with each mutant were first determined by measuring the rate of enzyme-catalyzed fluoride release for a single concentration of αGF or αGF at each of a series of concentrations of acarbose (6–8 concentrations), bracketing the K_i value so determined. The enzyme concentrations were identical to those used above for the respective substrate. The observed rates were plotted in the form of a Dixon plot (1/V vs [acarbose]), and the K_i value was determined (assuming competitive inhibition) from the intercept of this line with the horizontal line drawn through 1/V max. In our experience, this approach consistently provides a K_i value close (within 20%) to that ultimately measured by a full analysis. Full K_i determinations were performed by measurements of V_max and K_m values at a series of inhibitor concentrations (typically five concentrations) which bracket the K_i value to be determined. The value of K_i and the mode of inhibition were determined through a direct fit of the data to expressions for each inhibition mode using the program GraFit (34).

Crystallographic Refinement. The CGTase–acarbose structure (cgcta, PDB id code: 1cxg) was re-refined against the old data. To guard against overfitting, the free R-factor technique (35) was used, which was not available at the time the initial structure was refined. The structure of the CGTase mutant N193G (J. Uitdehaag, unpublished), without any ligands or waters, was taken as the starting model, because its cell parameters best resembled those of the cgcta data set (16), with the same indices as the free R-factor set used for the N193G refinement, comprising 2248 reflections out of the total of 23 325.

For the actual refinement, the program TNT (36) was used in combination with O (37). The overall procedure for CGTase has been outlined previously (30). The number of refinable parameters was reduced by including only a few solvent oxygens, and keeping very tight restraints on the stereochemistry, which limits the degrees of freedom of bound atoms. At the beginning, the new initial structure had an R-factor of 34.0% and a free R-factor of 34.4%. In the course of refinement, and after model rebuilding (see below), this decreased to a final R-factor of 18.2% and a final free R-factor of 23.7%. This new R-factor is higher than in the old structure, but more in line with what is expected from 2.5 Å data (35). The new structure was analyzed with PROCHECK (38) and WHATCHECK (39), and had improved stereochemistry compared to the old model (see Table 1).

<table>
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<th>Table 1: Quality of the Old and Re-refined CGTase + Acarbose Models</th>
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<td>neighboring atom B factor correlations (Å^2)</td>
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* R-factor = Σ||Fo - Fc||/ΣFo, where Fo and Fc are the observed and calculated structure factor amplitudes of reflection h, respectively. Reflections between 8.0 and 2.5 Å were used, with bulk solvent correction as implemented in TNT (36). The low R-factor of the old structure might indicate over refinement (35). The free R factor is calculated as the R factor, using Fc that were excluded from the refinement.

Including all atoms. The error models are R factor based, which could explain the higher errors in the cross-validated structure. A high value of the G factor indicates good overall stereochemistry.

RESULTS AND DISCUSSION

Kinetic Analysis of Active Site Mutants of CGTase Using Glycosyl Fluorides as Substrates. As reported previously, both αGF and αGF are convenient substrates for CGTase since the enzyme-catalyzed release of fluoride can be easily assayed using an ion-selective fluoride electrode (31). Even though CGTase otherwise prefers longer oligosaccharides, a short one, such as αGF, can be used if a good leaving group, such as fluoride, is provided. When the release of fluoride from αGF is followed using the fluoride electrode, a low reaction rate is observed over a lengthy induction period, but this rate gradually increases with time in an apparently sigmoidal manner, as is shown in Figure 3. It seemed likely that this behavior was a consequence of a transglycosylation reaction between two molecules of αGF.
Acarbose as a Transition State Mimic of CGTase

Biochemistry, Vol. 37, No. 49, 1998

FIGURE 3: Reaction of D371N CGTase with αGF (28 mM) and various acceptors showing fluoride release over time. Concentrations of acceptors used were: ○ = no added acceptor; ● = 30 mM G3; □ = 30 mM G4; ■ = 30 mM G5; ▼ = 30 mM G6. Inset graph (expansion of main figure): ○ = no added acceptor; ▲ = 30 mM G3; △ = 30 mM G2. The data for G and G2 have been displaced 0.36 mM above the actual initial concentration of fluoride to allow for easier visualization of the results.

producing maltosyl fluoride which acts as a better donor or acceptor, thereby more rapidly reacting to give αGF and so on. The lack of such an induction period when αGF was used as substrate (data not shown) certainly supports this hypothesis. Further evidence that longer oligosaccharides serve as better acceptors was obtained by monitoring the reaction with αGF in the presence of different maltosaccharides. As seen in Figure 3, inclusion of maltotriose considerably shortens the lag period while maltotetrose shorts it yet further. When maltopentaose or maltohexaose was added, no lag phase at all was observed. Since, in this study, we were interested in the rates of reaction of αGF, only the initial slope was used in our kinetic evaluation.

As shown in Table 2, a wide variety of mutations within the active site has been generated in an effort to determine the effect that a particular alteration has on the enzyme-catalyzed reaction. All of the mutants tested were able to utilize αGF as a substrate, albeit to varying extents, with $k_{cat}$ values ranging from 275 to 0.0093 s$^{-1}$, a spread of almost $(3 \times 10^3)$-fold. Interestingly, $K_m$ values for all the mutants studied were greater than that for the wild-type enzyme, indicating varying degrees of disruption of binding interactions. Consequently, $k_{cat}/K_m$ values varied over an even larger range with a spread of $(4.8 \times 10^3)$-fold. The degree of weakening of binding ranged from very small (2-fold) in the cases of the Y195F and S145E mutants to very large (80-fold) in the case of the Y195G mutant. This very small effect with the S145E mutant is consistent with the observation that this residue interacts only with the sugar residue in the $-1$ site, while the aromatic ring provides important interactions with the rest of the substrate, especially the sugar in the $-1$ site (26). Since αGF does not occupy the $+1$ site, minor consequences of removing that hydrogen bond are reasonable, yet complete removal of the side chain is clearly highly deleterious.

Mutations within the CGTase system produced an even larger range of effects on the enzyme-catalyzed reaction of the shorter, monosaccharide substrate αGF; the $k_{cat}$ values spanned from 330 to 0.0002 s$^{-1}$, a range of $(1.6 \times 10^3)$-fold (Table 2). Effects on $K_m$ ranged from a small disruption in binding (<1.5-fold) for the Y195F mutant to significant changes in binding (25-fold) for the Y195G mutant. Effects on $k_{cat}/K_m$ consequently ranged over $(4.1 \times 10^3)$-fold. Similar structure/activity correlations to those observed with αGF are apparent. That is, the proximity of Y195 and N326 to the $-1$ subsite make them important residues for optimal binding of the substrate. N326 is known to sit in a key position in the active site, and kinetic analysis on mutants modified at the equivalent residue in a thermostable CGTase showed drastic changes in pH optima upon mutation (30).

Therefore, mutations at these positions result in the largest disruptions in binding affinity and rate reductions.

Since this enzyme is a transferase, therefore ultimately requiring interactions in both the glycone and aglycone sites, care must be exercised in the interpretation of the kinetic parameters of these various mutants in terms of localized binding interactions. This is particularly true when the rate-determining step for each substrate/mutant combination is not known. If formation of the glycosyl enzyme is rate-limiting, then interactions in the negative ($-1$, $-2$, etc.) sites will likely be the most important. If, however, the second, transglycosylation step is rate-limiting, then interaction with the positive (+1, +2, etc.) sites could be equally important. This uncertainty is largely sidestepped if values of $k_{cat}/K_m$ are interpreted since this second-order rate constant, known as the specificity constant, reflects only the first irreversible step. For CGTase utilizing glycosyl fluoride substrates, as shown with various glycosidases (40–42), it is reasonable to assume that this is the first, glycosylation step; thus, changes in $k_{cat}/K_m$ reflect changes in interactions in the negative sites.

Inhibition of CGTase Mutants by Acarbose. Inhibition constants for acarbose were measured with each of the CGTase mutants, and these are also presented in Table 2. In three cases, including the weakest and strongest binding examples, the $K_i$ value was determined from a full analysis using a range of substrate and inhibitor concentrations as is shown for Y195G CGTase in Figure 4. Inhibition was strictly competitive in each case. In the other cases, $K_i$ values were determined from data obtained at a series of inhibitor concentrations using a fixed concentration of substrate as described under Materials and Methods. The mutant enzymes all bind to acarbose, but less tightly than does wild-type CGTase, with widely varying affinities ranging from a $K_i$ of 0.2 μM for wild type to 341 μM for Y195G CGTase. This large loss of affinity upon removal of the Y195 side chain is again consistent with the key role played by this residue.
Not surprisingly, there is also some mimicry of ground-state changes in transition state binding interactions with substrate. Interactions to the inhibitor therefore correlate quite well with a substantial degree of mimicry. Changes in binding slope of 1.61 (solid line, all data points), clearly indicating a respectable correlation (r² = 0.90, Figure 6A).

A correlation coefficient of r = 0.90 is again observed, but the slope, 0.47, is approximately 3 times smaller than that with log (Kₐ/Kₐcat).

Interestingly, the correlation seen between Kᵢ for acarbose and Kₐ/Kₐcat for αGF is considerably better, with r = 0.98 and a slope of 2.2, as shown in Figure 5B. This very strong correlation clearly indicates that acarbose has properties of a transition state analogue. Again, some ground-state mimicry was observed, the plot of log Kₐ versus log Kᵢ for acarbose having a slope of 0.4 and r = 0.93 (Figure 6B). A better indication of the relative qualities of these two fits is obtained from the coefficient of determination, r². For the Kₐ/Kₐcat plot, the r² value is 0.96, meaning that 96% of the points are well described by the line, whereas for the Kₐ plot the r² value is 0.86, meaning that only 86% of the points are well fit. The better correlation observed for αGF than αG3F is completely consistent with the binding modes of these substrates and of acarbose, as shown in Figure 1. The only occupied subsite common to the substrates and to acarbose is, in fact, the −1 subsite, assuming that acarbose binds in that mode. Thus, the effects of mutations upon interactions at that site will dominate the correlation. Such effects will be particularly large if the mutation directly removes interactions at that site. In addition, changes in interactions at that site resulting from more remote mutations will also be sensed. However, mutations at more remote sites which do not have an effect upon interactions at that site will not be sensed. Thus, mutations affecting only interactions at the +1, +2, and +3 sites will affect the binding of acarbose but will not necessarily affect Kₐ/Kₐcat.
values for the two substrates. This will result in scatter in the plots shown (Figure 5A). Similarly, mutations affecting interactions in the −2 and −3 sites will affect $K_{m}/k_{cat}$ values for αG3F, but not $K_i$ values for acarbose, thus adding additional scatter to that correlation. The −2 and −3 sites are not directly probed by αGF, hence the improved correlation.

Another measure of the importance of probing equivalent interactions with the substrate and with the inhibitor is obtained by selecting data only for mutants in which interactions at the common (−1) subsite are directly affected. The only amino acid residue mutated which fully fits that requirement is Tyr195. Indeed, as shown with the filled circles in Figure 5A, when only such data are considered a much better correlation ($r = 0.97$, slope = 1.55, linear fit displayed as a dashed line) is observed, consistent with the interactions observed crystallographically.

It is instructive to consider the true meaning of the slopes of these linear free energy relationships, which compare inhibitor binding free energies with activation free energies for the reaction, mutant by mutant. A slope of 1.0 would indicate optimal transition state mimicry since this would indicate that mutations cause equal changes in the binding of the transition state analogue and the transition state itself. Any slope greater or smaller than this would indicate a lesser degree of mimicry. It is not, however, clear that a slope of 1.0 is required to indicate any degree of mimicry as has been suggested (9), a conclusion that was developed on the assumption of direct proportionality of rate constants and equilibrium constants themselves. The slopes observed for the $K_{m}/k_{cat}$ plots (1.6−2.2) are therefore approximately equally dissimilar to those observed for the $K_m$ plots (a factor of 2 in each case), perhaps indicating equal mimicry of the ground state and transition state. However it is also quite possible that the large slope in the $K_{m}/k_{cat}$ plot reflects the fact that acarbose has three sugar residues bound in the +1, +2, and +3 sites which are not occupied in the substrate case. This may well have the effect of immobilizing the valienamine moiety at the active site to a greater extent than is the case for the monosaccharide transition state. A consequence of this could be a greater effect of specific mutations on the binding of this valienamine moiety than on the stabilization of the transition state for glucosyl transfer, hence a slope greater than 1.

**Crystallographic Re-refinement.** In light of the results presented here which confirm that acarbose has significant transition state analogue character, the binding mode of acarbose to CGTase was reexamined using the original data set as described under Materials and Methods. This is particularly important since, at the time of publication of the first structure (16), it was not realized that acarbose may undergo a transglycosylation reaction in the crystal, adding a glucose residue to the valienamine (26). The refined structure presented contains all 686 amino acids, 2 Ca$^{2+}$ ions, 1 rearranged acarbose inhibitor, and maltose residues at binding sites (MBS) 1 and 3. At MBS2, originally a maltose was modeled in this position (16), but upon refinement, electron density corresponding to four glucose residues was noted. The coordinates of this structure (2cxg) will be submitted to the PDB. The coordinates of an acarbose-derived maltononaose inhibitor bound to CGTase (1djj) (26) will also be resubmitted (2dij), since in this instance the valienamine is also modeled at subsite −2 and the 6-deoxyglucose at subsite −1.

To reexamine the binding mode of acarbose, the electron density maps were calculated using structures refined with the carbohydrate O6 atoms set to zero occupancy. The OMIT (43) and SIGMAA (44) procedures were used to minimize model bias. The overall statistics (Table 1) show that the old and new structures have a comparable fit to the crystallographic data. However, the electron density calculated after minimizing the refinement bias clearly showed sugar 6-hydroxy groups at subsites −2, −1, and +2, contradicting the presence of a 6-deoxynucleoside at the −1 site, as originally modeled (Figure 7) (16). At subsite +1, electron density for a 6-hydroxy group is absent. This conclusion was further supported by a 2.2 Å resolution data set from a CGTase II 145 mutant complexed to acarbose in which the valienamine moiety was also observed to bind at the −1 subsite (R. Ruiterkamp, unpublished results). Therefore, the 6-deoxynucleoside unit of acarbose was modeled into the +1 subsite, and the valienamine unit (which has a 6-hydroxy group) was modeled into subsite −1, corresponding to the orientation found in α-amylases and other CGTases.

On the basis of this re-refined structure, it is of interest to consider the interactions between the protein and the valienamine moiety in the −1 site that might be responsible for this strong correlation, hence for stabilization of the normal transition state. Probably of primary importance are the hydrogen bonding interactions (3.3 and 2.9 Å, respectively) between OE1 and OE2 of Glu257, the acid/base catalyst, and the NH group of the valienamine. Also of importance, especially given the ring flattening that must occur during catalysis, are the interactions between Asp328 (OD2) and O2 of the valienamine (2.7 Å) as well as between Asp328 (OD1) and O3 of the valienamine (3.2 Å). Other interactions are between His327 (NE2) and both O2 and O3 of the valienamine (3.1 and 3.0 Å, respectively), and between His140 and O6. Interesting stacking interactions also appear to exist between the double bond of the valienamine moiety and the aromatic ring of Tyr100. This substantial network of interactions presumably provides the stabilization needed at the transition state and for binding of this analogue.

**Conclusions.** The excellent correlation seen between log ($K_{m}/k_{cat}$) for hydrolysis of αGF by a series of CGTase...
mutants and log $K_i$ for acarbose for each of these same mutants provides good evidence that acarbose functions, at least in part, as a transition state analogue inhibitor for this enzyme. It seems probable that the majority of the binding interactions providing this high affinity are associated with the valienamine moiety. Since acarbose also contains a trisaccharide portion, it is not surprising that it also exhibits ground-state mimicry as revealed in the plot of log $K_a$ versus log $K_i$. These results are consistent with expectations regarding the structure of the transition state for this retaining transferase. It is also of importance to note that a similar correlation was recently observed for the inverting $\alpha$-glucosidase, glucoamylase (15). These results therefore provide further support for the notion that transition states for inverting and retaining glycosidases are quite similar in character (45, 46). This study also provides a clear example of how detailed kinetic analysis can provide a critical assessment of published crystallographic data resulting in reevaluation of the structural results.

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