Determination of the Catalytic Base in Family 48 Glycosyl Hydrolases

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The catalytic base in family 48 glycosyl hydrolases has not been previously established experimentally. Based on structural and modeling data published to date, we used site-directed mutagenesis and azide rescue activity assays to show definitively that the catalytic base in Thermobifida fusca Cel48A is aspartic acid 225. Of the tested mutants, only Cel48A with the D225E mutation retained partial activity on soluble and insoluble substrates. In azide rescue experiments, only the D225G mutation, in the smallest residue tested, showed an increase in activity with added azide.

Cellulosic biomass has the potential to form a significant component of a sustainable energy system (11). Carbohydrates obtained from the breakdown of cellulose can be utilized by various microorganisms to produce alcohols or combustible hydrocarbons for use as transportation fuels. One of the limiting steps in the development of an efficient and economically feasible system to produce biomass fuels is the enzymatic hydrolysis of cellulose (6, 16). It is important to generate an extensive knowledge base of the cellulases found in nature to establish the catalytic base, on the other hand, has not been experimentally established to date because there is no defined sugar residue in the −1 subsite in any of the obtained structures. Combined structural analyses (8, 9) and molecular simulations (13) suggest an aspartic acid residue (D230 in Cel48F, D255 in Cel48S, and D225 in Cel48A) to be the most likely candidate to carry out the catalytic base function in Cel48 enzymes. Here, we present strong experimental evidence that D225 is indeed the catalytic base in T. fusca Cel48A.

The catalytic domain of T. fusca Cel48A has been previously cloned and expressed in Escherichia coli by our group (3). Using the QuickChange II-XL site-directed mutagenesis kit (Agilent Technologies), we prepared four mutant versions of the protein, containing mutations D225N, D225E, D225A, and D225G. The reasons for the chosen mutations are as follows. Asparagine is the closest structural analog of aspartate that is unable to catalyze hydrolysis. Glutamate is the closest structural and functional analog of aspartate that might retain some catalytic activity. Alanine and glycine are the two smallest amino acids, which would provide more flexibility within the active site, needed for the azide rescue experiments described below. All mutants were expressed and purified as previously described for the native enzyme (3). Activity of the enzymes was measured on insoluble swollen cellulose (SC) and on 1,4-β-d-cellulohexaose (G6), the longest soluble cellooligosaccharide. SC was prepared by treating crystalline cellulose (Sigma-cell 101; Sigma-Aldrich) with phosphoric acid, as described in reference 18, to reduce its crystallinity for easier digestion. Pure G6 was obtained from Megazyme International. All reaction mixtures were prepared by combining Cel48A with 1.7 mg/ml SC or 17 μM G6 in 600 μl of 50 mM sodium acetate buffer, pH 5.5. The enzyme concentrations were 1 μM on SC and 0.17 μM on G6. Reaction mixtures were incubated at 50°C for 4 (G6) or 18 (SC) hours, and soluble products were analyzed via high-performance liquid chromatography (HPLC) using an Aminex HPX-87P column (Bio-Rad) and water as a mobile phase. As expected, the main products observed were...
cellobiose and cellotriose. Trace amounts of cellotetraose were also present in some cases. For SC, percent digestion was calculated from the total measured amount of glucose units released from the bulk substrate. For G6, percent digestion was calculated from the amount of G6 left in the reaction buffer at the end of the reaction. Presented data are averages and standard deviations of triplicate measurements.

Figure 1 shows the activities of the wild-type (WT) and mutant enzymes on SC and G6. On both soluble and insoluble substrates, the replacement of aspartate with glutamate (D225E) resulted in significant partial retention of WT activity. All three other mutants showed only residual activity, which is consistent with the presumed catalytic base role of D225. It is interesting to note that the decrease in activity of the D225E mutant relative to that of the WT was much greater on G6 than on SC. For cellulases acting on insoluble substrates, it is generally considered that access to the substrate, not hydrolysis, is the rate-limiting step. This is evidenced by the cellulase activities on soluble cellobio-oligosaccharides being much higher than those on insoluble cellulose. As a mutation of the catalytic base, D225E should only increase the activation barrier of the hydrolysis step without an effect on the ability of Cel48A to bind cellulose. Hence, it is likely that the effect of the glutamate mutation is partially masked on SC by the high activation energy required to bind an individual cellulose chain in the active site but is fully revealed on a soluble substrate, for which hydrolysis is the rate-limiting step.

To further confirm that Cel48A D225 is the catalytic base and not just an important residue in the catalytic site, we tested the ability of azide to rescue the activity of the mutated versions of the protein (Fig. 2) (5). For this test, activity assays were carried out as described above but with the indicated amount of sodium azide added to the reactions. Of the four mutant enzymes, only the D225G protein showed an increase in activity on SC with added azide. The position of the activated water molecule that attacks the anomeric carbon during hydrolysis is critical. We thus presume that the reason for restored activity in the D225G mutant only is due to the greater flexibility of its active site in comparison to those of the other mutants, which all have more sterically hindering functional groups. The ability of azide to partially restore enzyme activity when D225 is mutated is consistent with its presumed role as the catalytic base in Cel48A.

In summary, by using site-directed mutagenesis and azide rescue hydrolysis assays, we have shown that the T. fusca Cel48A catalytic base is D225. This residue is equivalent to D230 of C. cellulolyticum Cel48F and D255 of C. thermocellum Cel48S. To our knowledge, this is the first time that the catalytic base in family 48 glycosyl hydrolases has been experimentally established.

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REFERENCES


