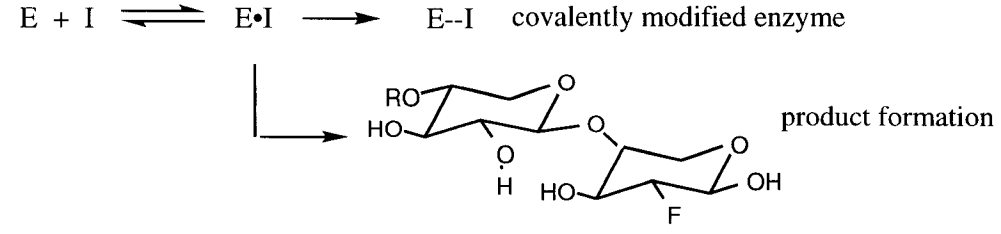
Answers to PS 3, Chemistry 5.50

1. This reaction is analogous to a hydrolysis of an acetal, which can serve as a starting point for thinking about the mechanism. This reaction in solution requires specific acid catalysis and goes through oxocarbenium type transition states. The information that you have been given provides mechanistic insight about xylanase.

First, presteady state kinetics experiments using stopped flow Vis spectroscopy and rapid chemical quench give you some mechanistic insight. In fact the results are strikingly similar to those we examined in class for chymotrypsin. The release of dinitrophenol shows a burst phase, followed by a linear phase. The size of the burst is stoichiometric with enzyme. This result suggests on the first turnover that the dinitrophenol is released more rapidly than all subsequent turnovers. This result requires that some step subsequent to its release is rate limiting. A number of explanations are possible and cannot be distinguished between based on this data. The enzyme could form a covalent intermediate and breakdown of the intermediate could be rate limiting for subsequent turnovers. A conformational change or product release could also be rate limiting in all subsequent turnovers. The rapid chemical quench experiment gives you further information about which explanation may be correct. The lag phase for product formation suggests again that the first turnover is not accompanied by product release (first 100 ms). This suggests that the product is covalently bound and is not released under the quenching conditions. Finding such conditions is usually challenging as often times covalent intermediates break down under the quench conditions. In the case of this intermediate the reaction, could not be quenched with acid as it would breakdown (the covalent intermediate, as you will see subsequently involves attachment of C-1 to a carboxylate). This covalent attachment is further verified, as the substrate labeled with a radio isotope is covalently bound in a ratio of about one substrate per active site. In the case of xylanase, I made up the kinetic pre-steady state results instead of taking them from the literature as I did with the rest of the data. It would be experimentally very difficult to find quenching conditions under which the covalent intermediate would not breakdown. If it broke down, then you would see product generated with the same profile as is observed in the stopped-flow data.

Second, time dependent inactivation by DNP2FXb, suggests that the activity of the enzyme is being lost as a function of time. Higher concentrations of the inhibitor (substrate) result in faster rates of inactivation. One interpretation of the data in Figure 3 is that xylanase partitions between product formation and covalent modification of the enzyme.



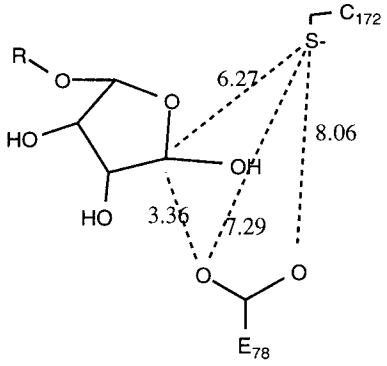
Further evidence in support of covalent modification comes from the mass spectrometric data. The differences in the mass between the inhibited and the uninhibited enzyme is (20,666 Da - 20, 398 Da = 268 ± 5). (see Biochemistry (1994) 33, 7027-32) This mass spectroscopic method allows a distinction to be made between a covalently bound derivative of the substrate and a tightly bound substrate or product. The mass of the latter would contain DNP in addition to the mass associated with the disaccharide. The peptide isolated is consistent with the given sequence and the DNP2FXb attached to some residue within the peptide, without the DNP leaving group. One cannot distinguish between Y, T, S or E as possible sites of labeling given the available information. All of these residues are capable of covalent catalysis.

The pH titration in Fig 2A suggests that two ionizable groups with apparent pKas of 4.7 and 6.7 are involved in enzyme function. In the former case, the group needs to be deprotonated for catalysis and in the latter case it needs to be protonated. A pKa between 4-5 suggests a carboxylate and a pKa around 7 suggests an imidazole. We all know however from the chymotrypsin case that pKas can be perturbed. There is a E in the peptide labeled with DNP2FXb suggesting that E78 might play a role in catalysis.

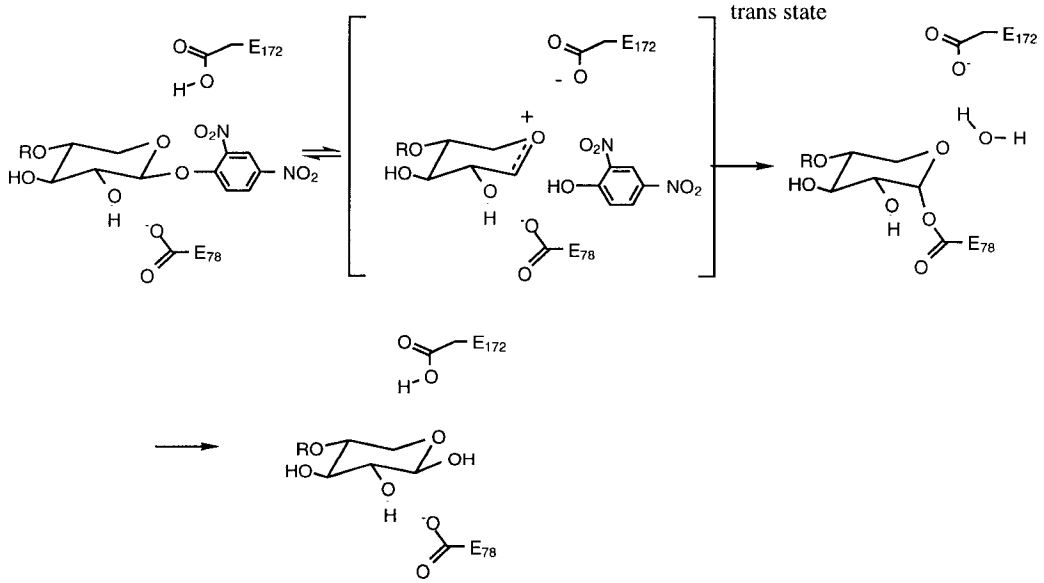
The NMR titration of xylanase containing the labeled ^{13}C-glutamate shows a change in the chemical shift of certain residues as a function of pH. This data reveals pKas similar to those determined in Fig 2A in which k_{cat}/K_m was plotted as a function of pH. The E78 shows a pKa of 4.6 to 4.7 and the E172 shows a pKa between 6.7 and 7.0. [An upfield carbon chemical shift is observed. The carbon chemical shift is related to hybridization of the carbon and sp^3 hybridized carbons are farther upfield than sp^2 hybridized carbons. Protonation alters the carbon hybridization to shift it slightly upfield.] In order to interpret the data in Figures 2B and C, the chemical shifts of each of the labeled glutamates must be assigned. Several Qs also show altered chemical shifts as a function of pH. Qs are unable to ionize under physiological conditions and therefore the differences in chemical shifts observed suggest that the C chemical shift is a sensitive indicator of altered environment.

The inactivated xylanase shows a pKa for only E172 . It is now perturbed to a more normal value for carboxylates, 4.5. This result suggests, in conjunction with the peptide mapping, that the adduct responsible for the time dependent inactivation shown in Fig. 3 is probably E78. Furthermore it suggests that E172 might be changing its function during catalysis from general acid catalysis to general base catalysis. This change is unmasked by trapping the reaction in mid stream.

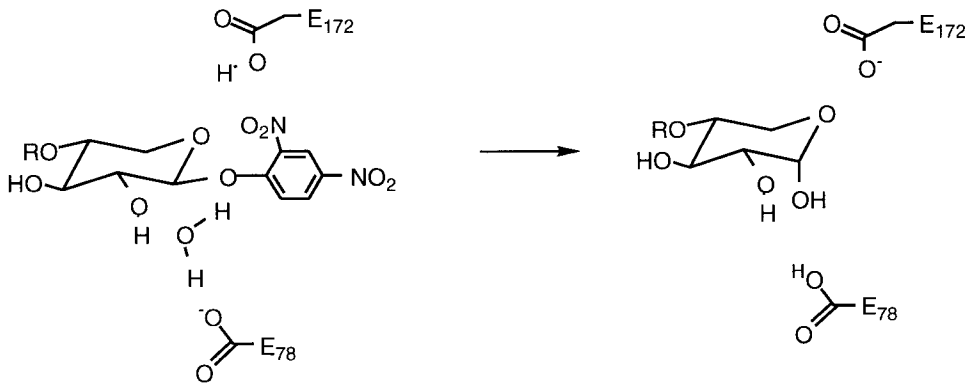
If you look up the structure of xylanase, E78 is in an appropriate position for chemical reaction. The available structure has E172 replaced with a cysteine to prevent catalysis. C172 woulf need to be extended by a carboxylate to mimic the wt protein. This extension will put it in the distance range so that it could be involved in catalysis. The structure is thus consistent with the postulated mechanisms below. Others have suggested additional residues are close and could play an important role. In general the pKas would have to be very much perturbed to account for the available data in Figure 2.



Proposed mechanisms: For retention of configuration, the simplest explanation is two inversions of configuration through a covalent intermediate. Inversion of configuration **does not require** an SN2 mechanism as you learned in organic chemistry. The active site can use steric constraints to effect stereochemical outcomes. This mechanism involves nucleophilic and acid/base catalysis. One can propose a transition state with oxocarbonium ion character based on your knowledge of acetal hydrolysis. The life-time of such an intermediate would be very short and it is perhaps better described as a transition state. Jencks has studied these reactions in great mechanistic detail in model systems.



Alternatively a direct displacement would give inversion. This reaction would likewise go through an oxocarbenium ion transtion state as above. Both types of reactions actually have been observed in Nature.



An oxocarbenium type ts as described above is also expected.

The inhibition studies with DNP2FXb strongly favor the first mechanism. Fluorine adjacent to an oxocarbenium ion slows down both half reactions (formation and hydrolysis of the covalent intermediate). The rate is decreased many thousand fold. Fluorine is electronegative and destabilizes the oxocarbenium ion transition state. The presence of F slows down the second step more dramatically than the first, leading to isolation of the covalent adduct. The reason for the difference in rates between these steps is not completely clear. The more dramatic perturbation of the second step relative to the first, may relate to the relative rates of these two steps in the normal reaction. It also is probably related to the fact that DNP is a better leaving group than an alcohol and one does not need to catalyze the first half reaction to as great an extent as the second half reaction. Placing a fluoro substituent adjacent to the sight of hydrolysis has proven to be a general method to covalently label glycosidases whose mechanism involves covalent catalysis. This method was developed by S. Whithers at the U. of British Columbia.

In this favored mechanism, E78 acts as a nucleophile and E172 must remain protonated to assist in catalyzing the first step, formation of a covalent bond between the enzyme and the substrate. The pKa of E172 is thus perturbed to assist in this chemistry. Once this bond is formed, E172 is deprotonated and its pKa is lowered back into the normal range observed for carboxylates. It can function as a general base catalyst in the second step to activate water for nucleophilic attack.

Note: Some of you may have considered epoxide formation by the C-2 hydoxyl to accomodate the differences between fluorine and hydroxyl at C-2. There is no evidence in the enzymatic systems for this type of reaction. It is however, chemically possible.