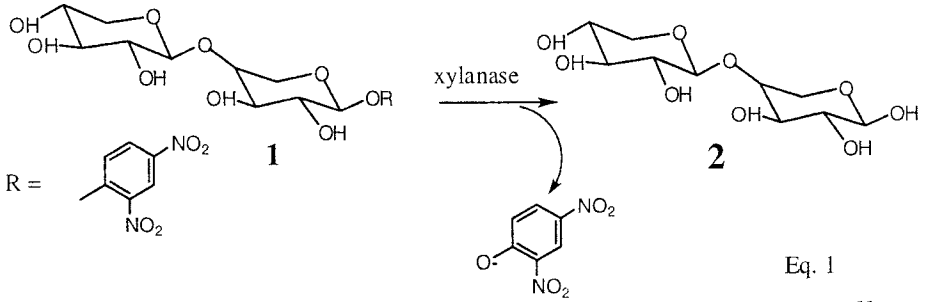
Problem Set 3 Sept 30 due Oct 7

Acid/Base and/or Nucleophilic Catalysis: Looking for intermediates and groups involved in Catalysis using Mechanism Based Inhibitors, NMR methods and pH rate Profiles.

1. Glycosidases catalyze the hydrolysis of glycosidic bonds. Approximately 500 proteins have been identified that can catalyze this reaction and they have been classified into two categories based on their distinct mechanisms. In one class the enzymes catalyze the hydrolysis reaction with inversion of configuration and the second class they catalyze the reaction with retention of configuration. Xylanase, from *Bacillus circulans*, is in the second class and catalyzes the reaction shown in Eq. 1. Xylan is a major component of plant cell walls.

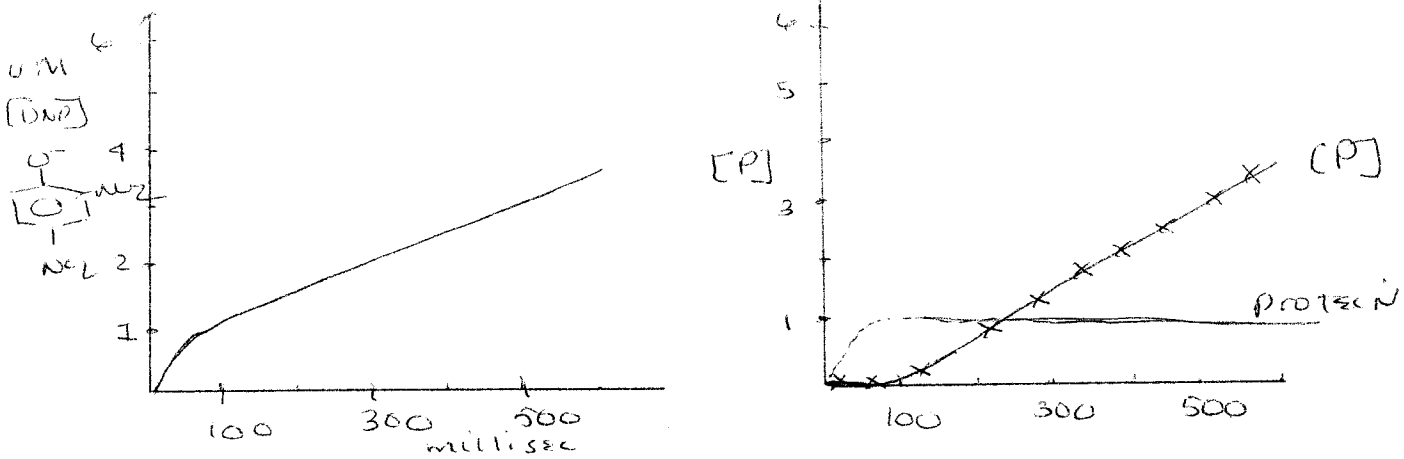


Eq. 1

The rate of the xylanase reaction can be followed spectrophotometrically and continuously by monitoring the changes in dinitrophenolate released at 412 nm. One can also monitor starting material (**1**, is uniformly radiolabeled with ^{14}C) disappearance and product formation (**2**) by HPLC and the use of a scintillation counter. The product elutes with a retention time of 15 min and the starting material with a retention time of 25 min. Stopped flow experiments monitoring dinitrophenolate release and rapid chemical quench experiments monitoring both starting material and product have been carried out. The results are shown in Figure 1.

Figure 1A Stopped Flow Kinetics 1B Rapid Chemical Quench

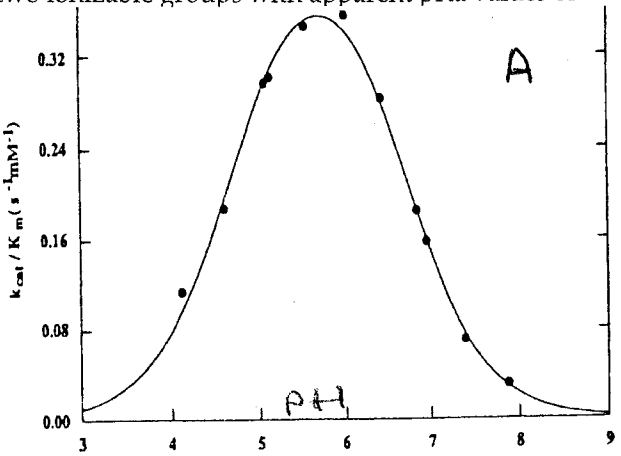
In one syringe was xylanase at 2 microM and in the second syringe was the 1.



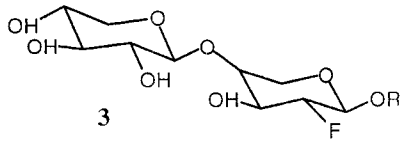
A study on the pH dependence of the xylanase reaction using the spectrophotometric assay is shown in Figure 2A. As noted in class with chymotrypsin, pKas of amino acid side chains can be perturbed. The investigators suggested from this

profile and additional experiments that two carboxylate side chains might play a role in catalysis.

Fig. 2. (A) Dependence of k_{cat}/K_m upon pH for hydolysis of ONPXb by xylanase at 25°C, fit to the kinetic expression for two ionizable groups with apparent pKa values of x and y (line).



Additional information about groups involved in catalysis and their function have been obtained using a mechanism based inhibitor. Incubation of 2',4'-dinitrophenyl 2-deoxy-2-fluoro ß-xylobioside (3, DNP2FXb) with



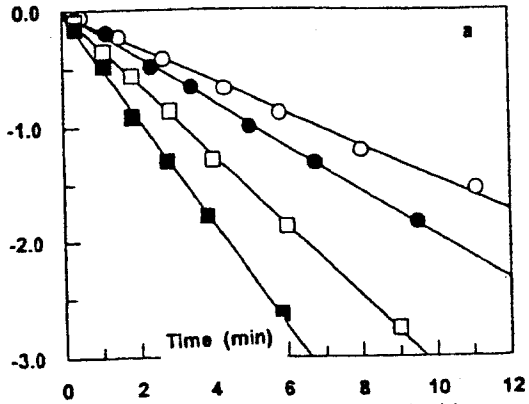
3

DNP2FXb

DNP = dinitro phenol

xylanase results in the enzyme inactivation as shown in Figure 3.
Electroscopy mass spectrometric analysis of the inactivated enzyme in comparison with the enzyme that has not been exposed to inhibitor reveals molecular masses of 20666 ± 2 Da and 20,348 ± 3 Da, respectively. Denatured inactivated-xylanase was degraded with pepsin (a non-specific protease) and analyzed by tandem mass spectroscopy (those interested in reading about this method can find review in PNAS 91, 11290-7(1994) and TIBS 20, 219-224 (1995)). A peptide containing 2FXb and residues 69 through 79 [Y69GWTRSPLIEY79] was identified by this method.

Figure 3 Inactivation of *B. subtilis* xylase by 3.

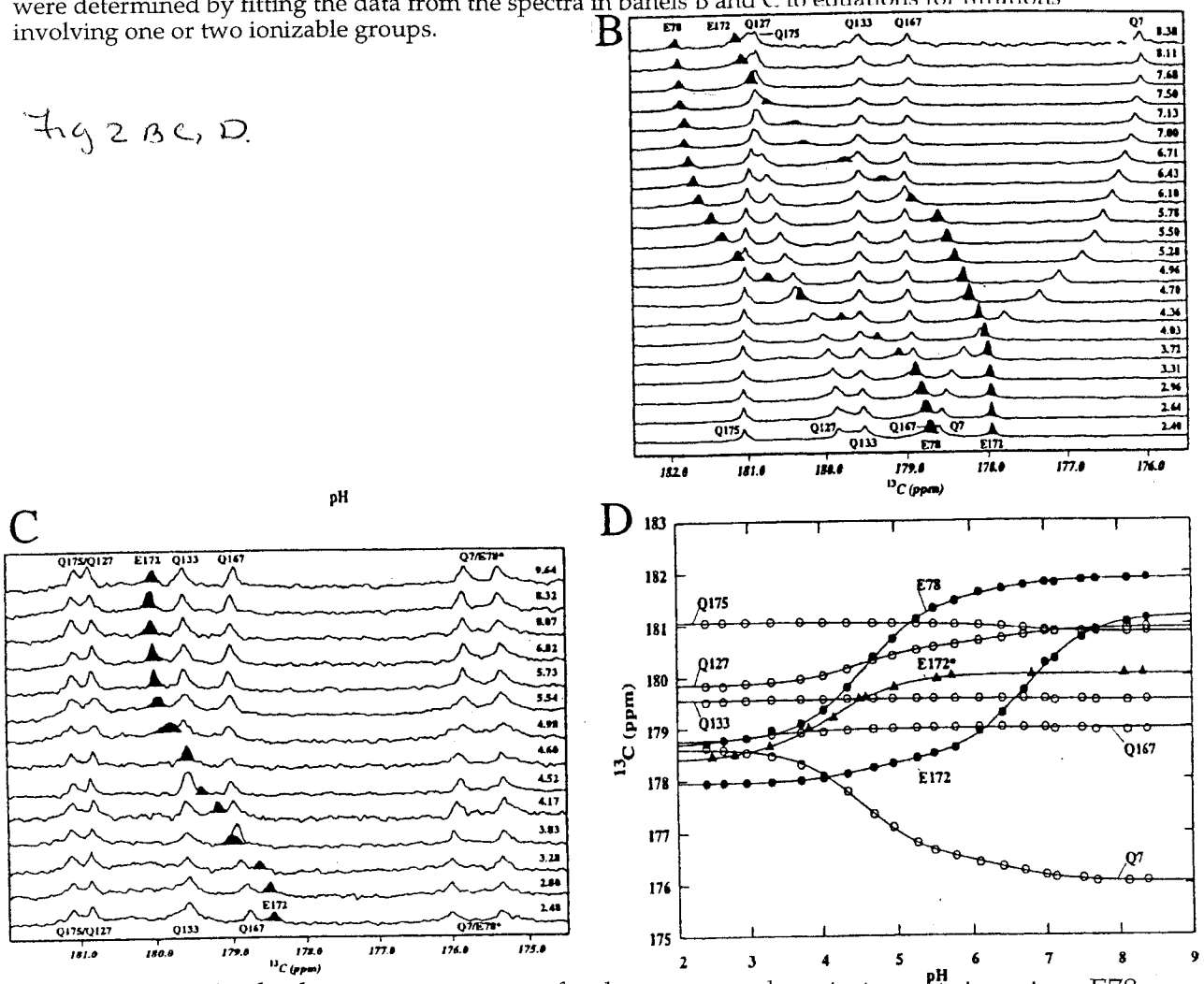


(a) Semilogarithmic plot of residual activity *vs* time at the indicated inactivator concentrations: (○) 0.42 mM; (●) 0.63 mM; (□) 1.05 mM; (■) 1.69 mM.

Figures 2B through 2D. (B) ^{13}C-NMR spectra of wild type xylanase recorded as a function of pH at 25°C. The peaks corresponding to E78 and E172 are highlighted in black to emphasize the titrations of these two residues. (C) ^{13}C-NMR spectra of wild type xylanase modified with DNP2FXb, as described

above. The assignments of the spectra of the modified protein are extrapolated from those in panel B, and E78* denotes the resonance from the experiment in which 3 is incubated with the enzyme. The peaks corresponding to E172 are shown in solid black to emphasize the titration of this residue. (D) The appparent pKa values descibing the pH dependence of the δ–^{13}C chemical shifts of the two Es and five glutamines (Q) in native xylanase (circles) and E172 in the inactivated-xylanase (triangles: labeled E172*) were determined by fitting the data from the spectra in panels B and C to equations for titrations involving one or two ionizable groups.

Fig 2 B, C, D.



Interestingly the gene sequence of xylanase reveals only two glutamates: E78 and E172. The role of these glutamates in catalysis has recently been studied by Withers and his co-workers using ^{13}C-NMR spectroscopy (Fig. 2B, C, D). Xylanase prepared uniquely with [δ-^{13}C] glutamate was examined as a function of pH in the absence of any sugar. The chemical shift of the δ-carboxylate is sensitive to the protonation state of the carboxylate. The results of this titration are shown in Fig. 2B. A similar experiment was carried out on the inactivated xylanase (xyalanse treated with 3) described above (Fig. 2C). The results of both of these studies are summarized in Fig 2D. The Q residues (175, 127, 133, 167, 7 serve as controls). The Es from the wt protein contain no *(data from Figure 2B), but the inactivated protein data contain a * (data from Figure 2C).

Questions:

(1) Pull up the structure of xylanase with xylobiose bound in its active site 1bcx-xylanase.pdb. The structure you have pulled up is of a mutant protein in which glutamate 172 (E172) is replaced by a cysteine (E172C). This mutation prevents any chemistry from occurring on the bound sugar. How far are the glutamate oxygens of the carboxylate from the sulphhydryl of the cysteine and how far are these side chains from the C-1 position of the sugar where the chemistry occurs? Draw an informative cartoon of what you see.

(2) Propose two mechanisms for glycosylases: one involving nucleophilic catalysis and one involving general acid/base catalysis.

(3) From the pre-steady state data in Figure 1 (both stopped flow and rapid chemical quench), what can you say about the kinetic mechanism? Does this data allow you to distinguish between the two mechanisms that you have proposed in question 2?

(4) Describe what you can learn from the pH rate profile in Figure 2A assuming that the authors interpretation of their data, that is , two carboxylates are involved in catalysis, is correct.

(5). What does the data in Figure 3 tell you about the interaction of xylanase with 3? What does the ESI mass spectrometric data and the peptide mapping tell you? Does this data allow you to favor one of the two mechanisms that you proposed in question 2? Why? Do the results described in Figure 3 help you to explain the observed stereochemistry of xylanase? How? From your chemical intuition (or look up acetal hydroysis in your organic text book if you cannot recall the mechanism), why did the authors use a fluorine at the C2 position of the bond being cleaved?

(6) A more subtle but important issue is raised by the ^{13}C-NMR studies described in Figures 2B, C and D. In light of your favored mechanism derived in questions 3 through 5, provide an explanation for the observed NMR titration experiments in the presence and in the absence of the inhibitor. How does this data give you insight into the results described in Figure 3?

(7) Can all of the data you have been given be rationalized in terms of one favored mechanism that you have proposed in question 2? If not go back and think about the information given.