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Characterization of substrate binding and catalytic mechanisms of an endoxylanase, amylosucrase, and porcine pancreatic alpha-amylase

Tao, Bernard Yi, Ph.D.
Iowa State University, 1988
Characterization of substrate binding and catalytic mechanisms of an endoxylanase, amylosucrase, and porcine pancreatic alpha-amylase

by

Bernard Yi Tao

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Major: Chemical Engineering

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For Major Department

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For the Graduate College

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ABBREVIATIONS USED

bcf  bond cleavage frequency
C    centigrade
C1   carbon 1 on the glucosyl residue
C3   carbon 3 on the glucosyl residue
C4   carbon 4 on the glucosyl residue
C6   carbon 6 on the glucosyl residue
cm   centimeter(s)
13C  carbon-13
d    day(s)
D2O  deuterium oxide
DEAE- diethylaminoethyl-
DMSO dimethyl sulfoxide
DP   degree of polymerization
FID  free induction decay
ΔG_a subsite affinity mapping acceleration factor
h    hour(s)
HPLC high performance liquid chromatography
Hz   hertz
3H   tritium
IU   international unit(s) of enzyme activity
kD   kilodalton(s)
KI   potassium iodide
K_i  inhibition constant
K_{int} intrinsic catalytic rate constant
kJ   kilojoule(s)
K_M  Michaelis binding constant
K_i  substrate inhibition constant
L    liter(s)
M    molar
MD   megadalton(s)
mg   milligram(s)
MHz  megahertz
min  minute(s)
mL  milliliter(s)
mm  millimeter(s)
mM  millimolar
mmol millimol(s)
mol  mole(s)
N  normality
nm  nanometer(s)
n.m.r. nuclear magnetic resonance
PPA  porcine pancreatic α-amylase
ppm parts per million
s  second(s)
[S]  substrate concentration
t.l.c. thin layer chromatography
v  rate of reaction
V_m  maximum enzymatic rate of reaction
W  watt(s)
X_1  xylose
X_2  xylolbiose
X_3  xylotriose
X_4  xylotetraose
X_5  xylpentaose
X_6  xylohexaose
X_7  xyloheptaose
X_8  xylooctaose
\lambda_{\text{max}}  maximum spectral absorbance frequency
\mu g  microgram(s)
\mu l  microliter(s)
\mu mol micromol(s)
\mu s  microsecond(s)
GENERAL INTRODUCTION

Due to their ubiquitous nature, carbohydrate enzymes offer an unusual opportunity to examine the relationships between enzymatic function and structure. Because of their importance in the environmental cycle of synthesis and degradation of naturally occurring polysaccharides, a plethora of bacteria, animals and plants have developed similar forms of many carbohydrate enzymes. While retaining essentially the same catalytic function with respect to their substrates, these enzyme systems often produce different products from the same substrates.

For example, amylases from a variety of origins exist, each capable of specifically cleaving the glycosidic bonds of amylopolysaccharides. While it is believed that the catalytic mechanism of these various amylases is conserved, the different binding properties of each enzyme results in a variety of products, such as different size, linear maltooligosaccharides and cyclic maltodextrins. The advantage of studying such systems of related enzymes is that direct comparisons of physical and chemical properties can highlight important structural and functional differences that can help to determine general relationships between structure and function. This in turn may provide clues for establishing more basic principles of function and structure for enzymes in general.

One of the challenges of biotechnology is to develop a sufficient understanding of the relationship between structure and function of enzymes in order to be able to predict the catalytic properties of synthetic polypeptides. While chemical modification and recombinant genetic techniques can be used to study functional-structural relationships, the majority of information has come from analysis and comparison of enzymes of recognized structure and function. The normal steps in this process involve establishing kinetic parameters, modeling catalytic behavior, obtaining structural information, and comparing results to determine similarities between enzymes. In the case of the enzymes xylanase and amylosucrase, which have relatively little available structural information, the action patterns and kinetic behaviors have been determined and compared to enzymes with similar functions. As more structural information becomes available on these enzymes, these observations of functional behavior can be corre-
lated to the physical structures. In the case of PPA, structural information was available, allowing a more sophisticated research approach to be used to determine the mechanistic action of this enzyme.

Carbohydrate Enzymes

The foundations of enzymology are inexorably intertwined with the study of carbohydrate enzymes. They were the first enzymes to be put to practical use (in the form of molds and microbes), when early man noticed that aging grains and fruits produced remarkable changes in flavor and taste, often accompanied by pleasing pharmacological effects. These early uncontrolled fermentations involved the degradation of starches, first to simple sugars, and subsequently to alcohol. In fact, the word 'enzyme' itself is related to carbohydrates, being derived from the Greek word, enzymos, meaning in yeast, referring to the catalytic action of yeast on sugars.

The nomenclature of enzymes also has its roots in carbohydrates. One of the earliest recorded isolations of an enzyme, by the addition of alcohol to malt extract in 1833, by Payen and Persoz\(^1\), was of a substance that converted starch to sugars. They called this substance 'diastase', which means separation, because it caused the separation of soluble dextrins from insoluble starch grains. While demonstrating the isolation of a carbohydrate enzyme was a remarkable feat, it did not receive widespread recognition. However, it did serve to coin the suffix 'ase', which is now almost universally used to denote enzymes.

The decade of the 1890s recorded the birth of the science of enzymology, again relying heavily upon carbohydrases. In 1894, the German chemist Emil Fischer\(^2\) proposed the famous lock-and-key model to explain the action of carbohydrate enzymes. In 1897 the Buchner brothers\(^3\) settled the fermentation-life issue by demonstrating that sugars were converted to alcohol in the absence of living cells. By showing that enzymes were entities independent of the living cells which produced them, the Buchners laid the foundation for the rapid growth in enzymology which was to follow in the next quarter century.
The early part of this century saw a flurry of research in the fledgling field of enzymology. The classical study of enzyme-substrate interactions by Michaelis and Menten in 1913, using invertase (another carbohydrase), laid the groundwork for the field of enzyme kinetics. The purification and crystallization of jack bean urease by Sumner and trypsin by Northrup and Kunitz put the final nail in the coffin of the 'living' ferment controversy and firmly established the proteinaceous nature of enzymes. However, nearly a quarter century would pass, due to the economic and political environment of the times, before the budding field of enzymology would gain significant use in industry and medicine.

In the late 1950's, enzymology once again saw a rapid growth period, riding on the shoulders of discoveries in antibiotics and immunization. For the next 20 years, remarkable discoveries into the nature of the enzymes abounded. The work of F. Sanger in protein chemistry and J. C. Kendrew and M. F. Perutz in X-ray crystallography demonstrated the three-dimensional structure of proteins and began to unravel the relationships between the structure and function of enzymes. Technology rapidly followed these discoveries, resulting in processing techniques to separate, isolate and immobilize enzymes on an industrial scale. This in turn has produced a $500 million industry using enzymes for detergents, food products, textiles and waste management applications, as well as for academic and medical research.

With the advent of recombinant genetic engineering and its promise for the creation of new and modified catalytic proteins, the field of enzymology is once more poised for an era of rapid growth. Nearly a century after Buchner and Fischer related remarkable discoveries about the basic nature of enzymes, the opportunity to develop a fundamental understanding of the structural-functional relationship of enzymes at the molecular level is the challenge. Understanding the basic principles which govern the remarkable catalytic specificity of enzymes will unlock the door to creating new, efficient protein catalysts with properties to dramatically change the condition of life in this world. It is towards this goal that this research has been performed.
Explanation of Dissertation Format

The experimental work in this dissertation is presented in three sections. Each represents a paper which has been or will be submitted to a scientific journal for publication. Section I is part of a larger paper published in *Carbohydrate Research*\textsuperscript{12}. Section II is currently in press in the same journal\textsuperscript{13}. Section III is being prepared for submission. The work in all three sections was performed entirely by myself, under the guidance of Dr. Peter Reilly and Dr. John Robyt. These papers (with the exception of the paper that includes Section I) were prepared by me, with assistance from Dr. Reilly and Dr. Robyt in editing and revising the manuscripts. Section I was originally written by me, re-written by Dr. Reilly for inclusion into a larger paper, which was re-edited by me for this dissertation.
SECTION I.

SUBSITE MAPPING OF A XYLOBIOSE- AND D-XYLOSE-PRODUCING
Aspergillus niger ENDO-(1→4)-β-XYLANASE
An endo-(1→4)-β-xylanase produced by *Aspergillus niger* was reacted with linear, $^3$H-reducing-end-labeled xyloooligosaccharides ranging in length from xylotriose to xylooctaose. No evidence of multiple attack or of condensation and transxylosylation reactions was found. Bond cleavage frequencies were highest near the reducing end of short substrates, with the locus of highest frequencies moving towards the middle of larger substrates. This endoxylanase has five major subsites, with the catalytic site located between the third and fourth subsites, counting from the non-reducing end of the bound substrate. The subsite to the non-reducing side of the catalytic site strongly repels its corresponding D-xylosyl residue, characteristic of endohydrolases that produce mainly oligosaccharides but do not catalyze bi-substrate reactions. The results demonstrate that this endoxylanase is similar in the structure of its active center to others previously subsite-mapped.
INTRODUCTION

Polysaccharide hydrolytic enzymes have recently received much attention, based on their role in the production of simple sugars from plant biomass, agricultural wastes and municipal wastes. These materials represent an enormous renewable resource for energy production, chemicals manufacture and fermentation feedstocks\(^1\). While much of the attention has focused on cellulose degradation, any efficient process which utilizes biomass must also effectively handle the hemicelluloses present, which may compose up to 40% of the biomass.

Hemicelluloses (xylans) are essentially polymers consisting of D-xylose (Fig. 1). Depending on the source of hemicellulose, branch linkages initiated by other carbohydrates may also be present. D-Xylose can be used as a feedstock for ethanol production\(^2\) or chemical production\(^3\). Hemicellulose hydrolysis to D-xylose can be done by either chemical or enzymatic means. Whereas chemical methods are usually more rapid, side reactions can occur and lead to a variety of undesirable byproducts, separation difficulties and waste disposal problems. The advantage of using enzymes is that they provide clean products, are non-polluting and biodegradable, and use mild process conditions, thereby reducing operating costs. However, the rate of reaction for enzymatic processes is usually much slower than by chemical means. A recent review by Woodward\(^4\) presents an overview of the functions, properties and applications of xylanases from a variety of sources.
Figure 1. Structures of D-xylose and xylan
LITERATURE REVIEW

Due to the existence of a variety of carbohydrate branches on the xylan backbone of various hemicelluloses, a number of different types of xylanases exist. A review by Reilly recognizes several categories of xylanases, based on their action pattern and substrate specificity. This research focuses on an A. niger endoxylanase that does not cleave L-arabinosyl-initiated branch points and is active on short xylooligosaccharides. The xylanase used in this study was first isolated by Frederick et al., purified for this project by J. M. Chow and kinetically characterized by Meagher. Xylanases with similar action patterns have been isolated from a number of organisms. However, the physical behavior and kinetic properties of these other enzymes has been found to be distinct from the xylanase investigated here.

There are many ways to characterize enzymes. In the specific case of depolymerase enzymes, such as xylanases or amylases, subsite mapping provides a convenient method for deriving energetic binding parameters that can be compared to other enzymes, yielding some insight into their binding and catalytic structure.

The technique of subsite mapping, pioneered by Robyt and French in their study of the hydrolysis of maltooligosaccharides with α-amylase, was extended to more quantitative applications mainly by the groups of Thoma and Hiromi, using experimental data from various amylases. With both exo- or endohydrolases, the technique requires the use of a homologous linear series of oligomeric substrates of lengths up to and beyond the number of subsites in the active site. From these the maximum rate ($V_m$) and Michaelis constant ($K_M$) of each substrate are determined, along with inhibition constants or their equivalents for smaller non-hydrolyzed materials. For exohydrolases this leads directly to calculation of the free energies of substrate binding for each subsite. However, with endohydrolases, the location of the catalytic site within the subsite array is not known a priori. In this case, a series of unsymmetrically-labeled oligomers must be used to determine the frequency of cleavage of each bond in each substrate. Together with substrate kinetic parameters, these bond cleavage frequencies are used to determine the location of the catalytic site and the subsite binding free energies. It must be recognized that either multiple attack, where more than one bond of the substrate is cleaved before the substrate is released by the enzyme, leading to a
rapid increase of small products, or two-substrate reactions such as condensation and transglycosylation, producing larger products than expected from simple hydrolysis, will distort binding energy determinations$^{25-28}$. Either conditions must be chosen where they do not occur or their effects must be accounted for when binding energies are calculated.

Several carbohydrate depolymerases have been characterized using this model$^{16,19,21,29-31}$. Of the xylanases, only the *Cryptococcus* and *A. niger* xylanases investigated by Biely *et al.*$^{32,33}$ have been subsite-mapped.
EXPERIMENTAL

Materials

Enzyme

The *A. niger* endoxylanase used in this work was isolated and characterized by Frederick *et al.*\(^6\) and purified for this project by J. M. Chow.

Carbohydrates

Unlabeled xylooligosaccharides (X3 through X8) were produced by Meagher\(^7\).

Xylooligosaccharides labeled with tritium at the reducing-end C-1 atom were produced by Research Products International by bubbling tritium gas through a pH 7 aqueous solution of 2.8% carbohydrate at 37°C over a catalyst of palladium oxide on barium sulfate\(^34\). Because this procedure yielded a mixture of labeled products, the samples were repurified by HPLC with either a Whatman Partisil 10 PXS 10/25 PAC cyano-amino column eluted with an 80% (v/v) aqueous acetonitrile solution or a Supelcosil LC-NH\(_2\) amino column eluted with 70% aqueous acetonitrile.

Methods

Determination of action pattern

To determine if multiple attack or transxylosylation and condensation reactions occurred during xylooligosaccharide hydrolysis, X6, X7, and X8 were subjected to attack with the endoxylanase in 0.01M sodium citrate buffer at pH 4.85 and 40°C. Product distributions of samples taken at 15% conversion or less were measured by HPLC with a Supelcosil LC-NH\(_2\) column operated at room temperature with 70% (v/v) aqueous acetonitrile eluent.
Measurement of bond cleavage frequencies

Hydrolyses of purified reducing-end-labeled xylooligosaccharides with endo-
xyylanase were carried out at 40°C in 0.01M or 0.05M sodium citrate buffer at pH 4.85.
Agitated 300-µL Pierce Reacti-Vials held 200 µL of solution, and 10-µL samples were
taken at specified intervals and added to 0.1 mL of 1N NaOH or 20 mL of 10% Na₂CO₃
solution.

Samples of 1 µL from the NaOH-quenched reaction mixture or 10 µL from the
Na₂CO₃-quenched material were spotted on E. Merck plastic-backed t.l.c. plates
precoated with 0.2-mm thick silica gel 60. After air-drying, the plates were developed at
room temperature with a single ascent of 70:20:10 (v/v/v) acetonitrile/water/1-propanol.
Dried plates were sprayed with tritium surface autoradiograph enhancer (En³Hance,
New England Nuclear) and contact-exposed to X-ray film (Eastman Kodak Type S) for
four days to locate radioactive spots corresponding to specific labeled xylooligosac-
charides. These were excised and added to 10 mL of toluene cocktail (4 g 2,5-
diphenyloxazole and 0.1 g 1,4-bis-2-(5-phenyloxazoyl)-benzene in 1 L toluene). Each
sample was counted for 4 min with a Packard TriCarb C2425 scintillation counter.
Counts for each xylooligosaccharide at each sampling time were normalized to the total
count at that time and yielded bcf’s of each bond in each xylooligosaccharide.

Calculation of subsite map

The number of subsites, location of the catalytic site, values of all binding ener-
gies, and kinetic parameters were calculated with the computer program of Allen and
Thoma. This program requires the input of Vₐ’s and Kₐ’s and their standard devia-
tions for each substrate, bcf’s with standard deviations for each bond of each substrate,
and the overall K_int with its standard deviation. Values of 1 ± 0.2M and 0.5 ± 0.1M were
assumed for the K_M’s of D-xylose and xylobiose, respectively, but much different values
for these parameters hardly changed the final results. The estimation routine places
weights on the experimental estimates of Vₐ’s, Kₐ’s, bcf’s, and K_int in proportion to the
reciprocals of the squares of their standard deviations. An acceleration factor (ΔGₐ)
can be made part of the optimization routine. This factor allows for the increase in the average intrinsic hydrolysis rate with increasing substrate chain length caused by conformational strain between the active site and the substrate.
RESULTS

Kinetics

Action pattern

When high concentrations (0.12M to 0.15M) of unlabeled X6 through X8 were hydrolyzed with the endoxylanase to low conversions, a symmetrical product distribution occurred (essentially equal amounts of X3 and X5 from X8 hydrolysis, for instance), indicating the absence of multiple attack. The absence of transxylosylation and condensation reactions was confirmed in three different ways: 1) Neither the hydrolyses of X6 through X8 described above nor hydrolyses of labeled X3 through X8 at much lower concentrations yielded evidence of production of xylooligosaccharides larger than the substrate. As hydrolytic rates of X6 and larger are essentially constant, measurable amounts of these substances should have accumulated had they been produced. 2) No lags at the beginning of hydrolytic reactions involving X3 through X8 at concentrations up to several times their $K_M$ values were observed. 3) Hydrolyses of X3 through X8 followed Michaelis-Menten kinetics, rather than having a sigmoidal dependence of rate on substrate concentration.

Kinetic parameters

Values of $V_m$ and $K_M$ at 40°C and pH 4.85 were determined for X3 through X8 and are shown on Table I.

Subsite Mapping

Bond cleavage frequencies

Values were obtained at low conversions with low concentrations (0.015mM - 0.15mM) of labeled X3 through X8. Two runs were conducted with X3 and three with X4, and results were averaged. One run was conducted for each of the other substrates. At shorter chain lengths, bonds closer to the reducing end were preferentially attacked. As chain length increased, highest incidence of attack moved towards the middle of the chain (Fig. 2).
Table I. Kinetic parameters from endoxylanase hydrolysis of xylooligosaccharides of different lengths at 40°C and pH 4.85

<table>
<thead>
<tr>
<th>Substrate No.</th>
<th>Experimental Values</th>
<th>Calculated Values</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( V_m ) (relative to xylan)</td>
<td>( K_M ) (mM)</td>
</tr>
<tr>
<td>X1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>X2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>X3</td>
<td>8</td>
<td>0.220 ± 0.028\textsuperscript{a}</td>
</tr>
<tr>
<td>X4</td>
<td>10</td>
<td>0.321 ± 0.024</td>
</tr>
<tr>
<td>X5</td>
<td>9</td>
<td>0.645 ± 0.046</td>
</tr>
<tr>
<td>X6</td>
<td>13</td>
<td>0.686 ± 0.044</td>
</tr>
<tr>
<td>X7</td>
<td>6</td>
<td>0.611 ± 0.047</td>
</tr>
<tr>
<td>X8</td>
<td>9</td>
<td>0.589 ± 0.048</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Standard deviation.
Figure 2. Bond cleavage frequencies for A. niger endoxylanase
Calculation of the subsite map

Calculated binding energies for eight subsites, four to each side of the catalytic site, are shown on Fig. 3 for the case where $\Delta G_a$ was set to zero. Only subsites 2 through 6, counting from the non-reducing end of the substrate, have major significance. Subsites 4 and 5, which bracket the catalytic site, strongly repel and moderately attract those residues, respectively. Subsites 2, 3, and 6 are strongly attracting. When $\Delta G_a$ was allowed to float, its value was only -0.2 kJ/mol, not significantly different from zero. As there were only small variations in subsite maps and calculated $V_m$, $K_M$, and $bcf$ values between accelerated and non-accelerated cases, only the latter will be discussed further.
Figure 3. Subsite affinity map for *A. niger* endoxylanase
DISCUSSION

Differences between experimentally obtained values of $K_M$ and those calculated after binding energies were determined were the largest of any of the dependent variables (Table I). In all cases calculated values were lower than experimental values, with the differences being greatest with the shortest substrates. Differences between calculated and experimental values of $V_m$'s and $b_c$f's (Tables I and II) were smaller. All but one calculated $V_m$ value was smaller than its corresponding experimental value; the calculated value that was higher was for $X_2$, for which no rate could be experimentally determined. No clear pattern in differences of $b_c$f values was apparent. The calculated value of $K_int$ was 1.82 mM, compared to an experimental value of 1.46±0.31 mM. Approximately 68% of the total residual error between experimental and calculated data were found in the $K_M$'s, with 23% associated with the estimate of $b_c$f's and 9% with that of $V_m$'s. Less than 1% of the residual error was associated with the $K_int$ estimate.

The observation that the experimental value of $K_M$ for $X_3$ was over a hundred-fold larger than its calculated value, as well as being much larger than experimental values of $K_M$ for larger xylooligosaccharides, led us to test the sensitivity of the calculated results to changes in this parameter and to data for $X_3$ in general. Changing the assumed experimental $K_M$ value for $X_3$ from 102mM to 5mM, barely above the value for $X_4$, had virtually no effect on calculated values of the binding energies, $b_c$f's, $K_M$'s, and $V_m$'s. Eliminating all the $X_3$ data led to somewhat greater, though hardly significant, changes in these four parameters. The residual error on the estimation decreased less than 25%, and the same skewed relationship between experimental and calculated kinetic parameters still appeared, suggesting that the major portion of the residual error was not associated with the $X_3$ data. The possibility that two-substrate reactions occurred at high concentrations of $X_3$, $X_4$, and $X_5$ cannot be rigorously excluded, as direct tests to observe them were not performed with these substrates. However, even if they occurred at high concentrations, which is unlikely given the uniformly negative results when this possibility was tested by presence of products larger than the substrate, presence of an initial lag phase, or lack of adherence to Michaelis-Menten kinetics, they did not occur at the concentrations at which $b_c$f values, which affect the subsite map more than any of the other experimental parameters, were measured.
The fact that estimates of $K_M$ and $V_m$ were nearly always smaller than experimental values indicates that the subsite model does not completely fit the experimental data, as a proper fit should have led to calculated values of $K_M$ and $V_m$ being both higher and lower than experimental values. This deviation suggests that the assumption that there is either no acceleration factor or a constant one is incorrect in this case. Instead it suggests that the intrinsic bond hydrolysis rate neither remains constant nor increases exponentially with increasing substrate chain length, but instead varies in a more individual fashion with substrates of different lengths.

Despite the disagreement between calculated and experimental $K_M$ and $V_m$ values, even with a non-zero $\Delta G_a$, the subsite map is capable of explaining why substrates of different lengths have such different $V_m$'s and $K_M$'s, and why individual bcf's in different substrates vary as they do. There is no significant endoxylanase activity on X2 because the only productive complex between enzyme and substrate is highly improbable, the result of the highly positive sum of the binding energies of the two subsites adjacent to the catalytic site. With X3, hydrolysis is possible because formation of enzyme-substrate complexes across subsites 3, 4, and 5 and across subsites 4, 5, and 6 both yield negative free energies. The difference in free energies of binding between the two productive enzyme-X3 complexes, the first being much more negative than the second, explains the large difference in the bcf's of the two glycosidic bonds of X3.

Values of $K_M$ decrease with increasing chain length because the free energies of binding of the complexes formed become progressively more negative as more subsites are involved in substrate binding, since there is only one subsite (the fourth) with a significantly positive binding energy, and two others (the first and eighth), with fairly small positive energies. Values of $V_m$ increase with increasing substrate chain length through X6 because the proportion of productive complexes to total complexes increases. The locus of highest bcf's moves toward the center of the substrate and becomes more diffuse with increasing substrate chain length because the overall free energies of binding of different productive complexes of enzyme and substrate become more similar. Only those complexes with the catalytic site acting on a bond near the end of long substrate chains have free energies of binding much less negative than those involving more subsites. The former are less probable, and therefore yield lower bcf's for bonds near the end of the substrate.
The structure of the subsite map found with this endoxylanase, with a subsite having a positive binding energy located adjacent to the catalytic site, is characteristic of endohydrolases that produce mainly oligosaccharides but do not catalyze bi-substrate reactions. If the two subsites adjacent to the catalytic site both had negative binding energies, disaccharides would be hydrolyzed to monosaccharides. The location of the subsite with positive binding energy to the non-reducing side of the catalytic site suggests that the D-xylosyl residue bound there is strongly distorted during the cleavage of its glycosidic bond, probably from chair to half-chair form\(^{15,35}\). Similar subsite patterns are found with lysozyme\(^{29}\) and *Bacillus amyloliquefaciens* \(\alpha\)-amylase\(^{15,18}\). Prodanov *et al.*\(^{30}\), however, found the opposite order of positive and negative binding energies about the catalytic site of porcine pancreatic \(\alpha\)-amylase, probably because of the extremely restrictive assumptions they used to determine their subsite map.

Several other groups have calculated a positive sum of binding energies for the two subsites on either side of the catalytic site, but have not determined how this total is allocated between the sites. This pattern is exhibited by Taka-amylase A from *Aspergillus oryzae*\(^{21}\) and endoxylanases from both *Cryptococcus albidus*\(^{32}\) and *A. niger*\(^{33}\) and presumably by an endodextranase from *Streptococcus mutans*\(^{31}\). It should be noted that the *A. niger* endoxylanase\(^{33}\) has different properties from the one studied in this project, in that it has strong transxylosylation activity\(^{36}\) and only four major subsites, two to each side of the catalytic site\(^{33}\). Three additional subsites that are more to the reducing end of the substrate being bound all have positive binding energies\(^{33}\).

This work has demonstrated that structural similarity exists between the catalytic centers of the endoxylanase characterized here and those previously described, based on their subsite affinity maps. The implication is that since the remarkable specificity of enzymes is highly dependent upon structure, the observed behavioral similarity between these enzymes is probably related to structural similarities. Therefore, it would be of great interest to obtain and compare the actual physical structure of these xylanases, to ascertain if any general binding or catalytic features can be observed which correspond to the functional model similarities. This will require amino acid sequencing, X-ray crystallography and possibly site-directed mutagenesis of these enzymes.
ACKNOWLEDGMENTS

This project was supported by National Science Foundation Grant PFR-8022895 and by the Engineering Research Institute of Iowa State University. The author thanks Professor John F. Robyt for the use of the scintillation counter and other laboratory facilities and Professor John A. Thoma for the subsite mapping computer program.
REFERENCES


SECTION II.

*Neisseria perflava* AMYLOSUCRASE:

CHARACTERIZATION OF ITS PRODUCT POLYSACCHARIDE

AND A STUDY OF ITS INHIBITION

BY SUCROSE DERIVATIVES
ABSTRACT

*Neisseria perflava* amylosucrase forms a polysaccharide from sucrose very similar to glycogen. Sucrose derivatives modified at position C3 (3-deoxysucrose and α-D-allopyranosyl-β-D-fructofuranoside), position C6 (6-deoxysucrose and 6-deoxy-6-fluorosucrose), and both positions C4 and C6 (4,6-dideoxysucrose) were tested as inhibitors of amylosucrase. These results were compared to similar studies with dextransucrase using the same inhibitors. The $K_M$ value of sucrose is $26.5 \pm 4.3$ mM. Derivatives modified at the C6-position were potent competitive inhibitors, with $K_i$ values of $6.2 \pm 0.3$ mM (6-deoxysucrose) and $0.50 \pm 0.06$ mM (6-deoxy-6-fluorosucrose). Sucroses modified at the C3-position were not significantly inhibitory over the concentration range tested. 4,6-Dideoxysucrose gave an unusual, non-competitive inhibition, in that increasing its concentration did not produce a commensurate increase in the level of inhibition, which instead appeared to approach a limit. None of these sucrose derivatives were substrates for amylosucrase, nor were they glycosyl donors to maltotriose.

These results show remarkable similarity to results obtained for dextransucrase, implying that the binding structures may be similar. Since the basic catalytic function of both enzymes is the formation of α-glycosidic bonds, albeit to different hydroxyls on the glucose ring, it would appear that the binding of the substrate may be the determining factor in the resulting polysaccharide structure.
INTRODUCTION

In the previous section, the hydrolytic action of a carbohydrate depolymerase was characterized by using an energetic subsite affinity model. The results indicate that similarities exist in catalytic properties of some xylanohydrolases, despite differences in origin and physical structure. However, hydrolytic enzymes are only part of the carbohydrate story. There are also many polymerase carbohydrate enzymes which play important roles in nature. Enzymes that move glucosyl residues between carbohydrates are known as glucosyltransferases. This research compares two such carbohydrate enzymes, amylosucrase and dextransucrase. Both synthesize glucans from sucrose, albeit with very different glycosidic linkages, resulting in polysaccharides with different physical and chemical properties.

Dextransucrase (EC 2.4.1.5, sucrose:1,6-α-D-glucano-α-D-glucosyltransferase) is an enzyme of industrial and medical interest. Derived from *Leuconostoc mesenteroides* and *Streptococcus mutans* species, its dextran product is used for a variety of analytical research applications such as molecular size exclusion chromatography; in addition it is a human blood plasma extender. Dextransucrase is also thought to participate in the formation of dental caries in humans.

The synthesis of a glycogen-like polysaccharide by *Neisseria perflava* amylosucrase (EC 2.4.1.4, sucrose:1,4-α-D-glucano-4-α-D-glucosyltransferase) is unique among enzymatic syntheses of amylopolysaccharides, as the glucan is formed directly from the substrate sucrose without the intervention of α-D-glucosyl-nucleotide diphosphate intermediates.
Amylosucrase was discovered by Hehre and Hamilton in 1946. Subsequent studies by Hehre, coworkers, and by others examined the behavior of this constitutive enzyme from \textit{N. perflava} and showed that it produced a glycogen-like polysaccharide directly from α-D-glucopyranosyl fluoride, as well as from sucrose. Their studies also showed that amylosucrase is strongly inhibited by sucrose concentrations greater than 200 mM and is activated by the presence of its native polysaccharide or a variety of other amylopolysaccharides. Amylosucrase has been postulated to have a role in the formation of dental caries. However, since these investigations, this enzyme has received very little additional attention.

The present work further characterizes the amylosucrase polysaccharide product, investigates the inhibitory behavior of various sucrose derivatives upon amylosucrase and compares the results with those obtained for dextran sucrase.
EXPERIMENTAL

Materials

Enzymes

*N. perflava* 19-34 was obtained from Professor E. J. Hehre and was grown in a 5-L fermentor, following the procedure of Okada and Hehre. After centrifugation, the cells were suspended in approximately 3 mL of 100mM sodium maleate buffer, pH 7.0, and rapidly disrupted by three passages through a French pressure cell previously cooled to 4°C. The remaining steps were all performed at 4°C. The suspension was centrifuged (120,000 x g, 40 min) and the supernatant was recovered by decantation. This solution was treated overnight with an equal volume of an 80% saturated ammonium sulfate solution at pH 6.4. Following centrifugation, the sediment was dissolved in 3 mL of 50mM sodium maleate buffer (pH 7.0) containing 0.025M 2-mercaptoethanol and 0.02% sodium azide. This solution was desalted by passage through a Sephadex G-10 column, using the same buffer as eluent. The enzyme fractions, which emerged at the void volume, were collected and pooled. This constituted the enzyme used in this study. The protein concentration of this solution was 11 mg/mL, determined by the method of Lowry et al., standardized with bovine serum albumin. The enzyme was kept at -20°C and retained full activity for over six months. Further purification was attempted by size exclusion chromatography [Bio-Gel P-150 (Bio-Rad Laboratories, Richmond, CA)], ion exchange chromatography (DEAE-cellulose) and adsorption onto cross-linked starch/glycogen, but did not yield stable, active enzyme. Therefore, further purification was not performed.

The specific activity of the enzyme was 0.50 U/mg of protein, where one unit of activity is defined as 1 μmol min⁻¹ of D-glucose incorporated into glucan at pH 7.0 and 35°C in the presence of approximately 200 mg/mL of non-radiolabeled native amylosucrase polysaccharide, using an initial concentration of 100mM [U-¹⁴C]sucrose. Details of the radiochemical assay are presented below.

This enzyme preparation possessed no amylolytic activity as measured by reducing value using the ferricyanide method and a 1% starch solution.
Pseudomonas amyloferans isoyamylase [EC 3.2.1.68, glycogen 6-glucanohydrolyase] was purchased from Sigma Chemical Co., St. Louis, MO.

Carbohydrates

[U-¹⁴C]Sucrose was obtained from Schwarz/Mann, Inc. (Spring Valley, NY). Allosucrose (α-D-allopyranosyl-β-D-fructofuranoside), 3-deoxyxysucrose, 4,6-dideoxyzucrose, 6-deoxyxysucrose, and 6-deoxy-6-fluorosucrose (Fig. 1) were all previously synthesized in this laboratory. Non-labeled and ¹⁴C-labeled polysaccharides from amylosucrase were made from sucrose (100mM) in 100mM sodium maleate buffer (pH 7.0) at 35°C, followed by ethanol precipitation. The recovered polysaccharide was dissolved in the same buffer to make a 200 mg/mL solution and dialyzed against the same buffer. Thin layer chromatography (t.l.c.) showed that no residual sucrose or D-fructose was present. No amylosucrase activity was detected in the purified polysaccharide preparation. T-series dextrans were obtained from Pharmacia (Uppsala, Sweden).

Methods

Liquid scintillation counting

¹⁴C-labeled carbohydrate samples were adsorbed onto pieces of paper or plastic-backed silica, placed face up in 10 mL toluene scintillation fluid in 20-mL scintillation vials, and the amount of radiolabel was measured.

Thin layer chromatography

Analysis for the presence of maltooligosaccharides was performed by t.l.c. Samples were spotted onto Whatman K5 silica gel plates (Whatman Chemical Separation, Inc., Clifton, NJ) and dried. Separation was effected by a single ascent of ethyl acetate:methanol:water (37:40:23, v/v/v), and visualized by spraying with methanol-sulfuric acid (4:1 v/v) followed by charring at 120°C for 10 min. For determination of higher oligosaccharides (DP 8-15), three ascents of nitromethane:water:1-propanol (2:3:5, v/v/v) were used.
Figure 1. Structures of modified sucrases tested as inhibitors for amylosucrase
Polysaccharide molecular weight determination

A sample of amylosucrase-produced polysaccharide in 100mM sodium maleate buffer, pH 7.0, was passed over a 10-mm x 500-mm Bio-Gel A-150m column that was previously equilibrated with the same buffer. Samples of 1 mL were collected and analyzed by the phenol-sulfuric acid method for total carbohydrate. Dextrans of 465 kD, 2 MD, and 150 MD were used as standards.

Polysaccharide permethylation analysis

Dr. M. E. Slodki of the Northern Regional Research Center of the U. S. Department of Agriculture (Peoria, IL) kindly performed this analysis.

Polysaccharide isoamylase digestion

Approximately 100 mg of $^{14}$C-labeled N. perflava polysaccharide, made from [U-$^{14}$C]sucrose and purified from residual sucrose and D-fructose by dialysis, were incubated with 100 units of isoamylase in 50mM sodium acetate buffer, pH 4.0, at 35°C for 6 h. Following digestion, the sample was placed onto a piece of 200-mm x 500-mm Whatman 3MM paper and developed with a descending flow of 1-propanol:water (7:3, v/v) for 24 h. Following drying, an autoradiogram of the paper was made. The radioactive compounds were excised and counted by liquid scintillation spectrometry. Six products were sufficiently resolved from the origin. The two fastest running were eluted from the paper with water, dried, and dissolved in 10 mL water. These solutions, when analyzed by t.l.c. and compared to maltooligosaccharide standards, corresponded to maltose and maltotriose. From this, the other four products were assumed to be maltotetraose through maltotriaose.

Polysaccharide iodine staining

A reaction mixture, consisting of 100 μL amylosucrase in a total volume of 1.5 mL 100mM sucrose in 100mM sodium maleate buffer, pH 7.0, was incubated at 35°C.
Samples of 100 μL were withdrawn at various times and added to 1 mL distilled water containing 100 μL of 0.2% I2 + 2% KI aqueous solution. The spectral absorbance was measured at various wavelengths from 400 to 600 nm.

**Measurement of enzyme activity**

Various concentrations of [U-14C]sucrose were incubated with 20 μL of enzyme solution in 300-μL reaction digests. Periodically, 25-μL aliquots were removed and deposited onto 1-cm² pieces of Whatman 3MM filter paper. The papers were washed five times in methanol to remove methanol-soluble label, followed by heterogenous liquid scintillation counting to quantitate the formation of methanol-insoluble labeled product.

**Reaction conditions**

All reactions were run at 35°C in 50-100mM sodium maleate, pH 7.0, containing 0.02% sodium azide. Except as noted, reaction mixtures also contained approximately 200 mg/mL of non-labeled amylosucrase-produced polysaccharide to eliminate glucan synthesis lag.

**Inhibitor kinetics reactions**

A series of reactions, using different concentrations of [U-14C]sucrose and sucrose derivatives, was performed to test the ability of the latter individually to inhibit glucan formation by amylosucrase. The concentration ranges used were 2.5-50mM [U-14C]sucrose, 10-50mM allosucrose, 0.1-50mM 3-deoxysucrose, 1-30mM 4,6-dideoxysucrose, 0.1-50mM 6-deoxysucrose, and 0.5-5mM 6-deoxy-6-fluorosucrose. 4,6-Dideoxysucrose was also tested as an activator in the absence of added native polysaccharide.

**Identification of substrates, acceptors, and glycosyl donors**

Each of the sucrose derivatives was tested to determine if it could act as a substrate in polysaccharide synthesis. Reaction mixtures (total volume 100-μL) consisting
of 50mM solutions of each derivative combined with 20 μL of amylosucrase were prepared without added native polysaccharide or other carbohydrate acceptors and were incubated for 6 d at 35°C. Polymer formation was tested by visual observation of the formation of opalescence and by t.l.c.

Each sucrose derivative was also tested as a glycosyl donor to maltotriose. A series of 300-μL mixtures, 50mM in sucrose derivative and 50mM in maltotriose and containing 20 μL of amylosucrase, was incubated at 35°C. Samples were withdrawn at 1, 2, 4, and 24 h and analyzed by t.l.c. for the presence of higher DP maltooligosaccharides.

Dextran was also tested as an acceptor of D-glucosyl residues from [U-\textsuperscript{14}C]sucrose. A mixture of 500 mL of a 1% solution of dextran T-10 (Pharmacia) and 50mM [U-\textsuperscript{14}C]sucrose was incubated with 20 μL of amylosucrase for 1 h. The product mixture was loaded onto a 10-mm x 500-mm Bio-Gel A-0.5m column and eluted with 100mM sodium maleate buffer, pH 7.0. Samples of 1 mL were collected and analyzed for total carbohydrate by the phenol-sulfuric acid method\textsuperscript{23}. In addition, 50-mL aliquots of each sample were spotted onto a 1 cm\textsuperscript{2} piece of 3MM Whatman paper and counted by liquid scintillation. Comparison of the elution profiles for the two methods were made to determine if significant amounts of label were incorporated into the dextran fraction.

**Transglycosylation**

Reaction mixtures of 50mM of each individual maltooligosaccharide, maltose through maltoheptaose, were incubated with amylosucrase for 48 h at 35°C. Isomaltose and isomaltotriose were also tested. Reaction samples were analyzed for transglycosylation products by t.l.c. and compared to maltooligosaccharide standards.

Transglycosylation activity was quantitated by using 50mM \textsuperscript{14}C-labeled maltotetraose under the same reaction conditions. Samples taken at various times were separated by t.l.c., using a plastic-backed t.l.c. plate (Silica Gel 80, MCB Reagents, Gibbstown, NJ). An autoradiogram of the plate was made, and the maltotetraose spots were excised and counted by liquid scintillation spectometry.
RESULTS

Characterization of Amylosucrase

Characterization of amylosucrase polysaccharide

The estimated molecular weight range of the amylopolysaccharide measured by size exclusion chromatography was approximately 1 MD to 20 MD. Methylation analysis of this polysaccharide indicated that about 90% of the linkages were (1→4), with the remaining 10% being (1→6). Addition of iodine-iodide solution produced a reddish-brown complex similar to iodine-stained glycogen. Adsorption measurements of the iodine-stained complex yielded a general spectral shift of $\lambda_{\text{max}}$ from 530 nm to 500 nm with increasing reaction time (Fig. 2). Quantitation of the radiolabeled products from isomylase debranching of radiolabeled polysaccharide by paper chromatography and liquid scintillation counting indicated that approximately 70 mol % of the D-glucosyl residues were contained in branch chains of seven or fewer residues (Fig. 3). Oligosaccharide branches up to D.P. 12 were detectable by t.l.c.; however, they could not be resolved by paper chromatography.

Amylosucrase kinetic parameters with sucrose substrate

As previously noted, amylosucrase is inhibited by concentrations of sucrose greater than 100 mM. Initial rate data fit the standard substrate inhibition model, assuming a second, non-productive sucrose binding site (Fig. 4):

$$ v = \frac{V_m [S]}{K_m + [S] + [S]^2/K_s} $$

Values calculated by non-linear regression using the SAS NONLIN program (SAS Institute Inc., Cary, NC) for $V_m$, $K_m$, and $K_s$ were $0.89 \pm 0.08 \mu$mol min$^{-1}$ per mg of protein, $26.5 \pm 4.6$ mM, and $201 \pm 35$ mM, respectively, where the second value presented is the standard deviation. These values are similar to those obtained by Okada and Hehre (K$M = 17$ mM, K$S = 305$ mM). Dextran T-10 was not an acceptor of D-glucosyl
Figure 2. Change of absorbance of iodine stain spectra of amylosucrase glycogen with reaction time
Figure 3. Mol % of radiolabel in isoamylase-digested polysaccharide branches (8 DP fraction was normalized by dividing the amount of radioactivity in the fraction by 10; the other fractions were normalized by dividing the amount of radioactivity in each fraction by its respective chain length)
Figure 4. Dependence of amylosucrase activity on sucrose concentration
residues from sucrose, because no significant amount of label was found in the dextran elution fraction. The observation by Okada and Hehre that the enzyme is activated by its native polysaccharide was also confirmed.

**Inhibition by sucrose derivatives**

Various sucrose derivatives were tested as inhibitors of glucan formation from sucrose. Fig. 5 shows Lineweaver-Burk plots of glucose incorporation into glucan by reaction of amylosucrase with [U-\(^{14}\)C]sucrose in the presence of these derivatives. \(K_i\) values were calculated by non-linear regression using the SAS NONLIN program. Table I summarizes the kinetic and inhibition parameters obtained from these analyses. Neither 3-deoxyxysucrose nor allosucrose exerted significant inhibition over the concentration ranges tested. Both 6-deoxysucrose and 6-deoxy-6-fluorosucrose were strong competitive inhibitors, with \(K_i\) values of 6.2 ± 0.3mM and 0.50 ± 0.06mM, respectively. 4,6-Dideoxysucrose was also an inhibitor, but showed an unusual behavior. The appearance of the reciprocal plot (Fig. 5) implies that 4,6-dideoxysucrose is a non-competitive inhibitor. However, increasing the inhibitor concentration did not produce a corresponding rise in the level of inhibition, which appeared to approach a maximum value (Fig. 6). Fitting the data for this reaction using a non-competitive model

\[
\frac{K_i V_m [S]}{V} = \frac{[S]}{(K_M + [S])(K_i + [I])}
\]

yielded a \(K_i\) value of 22.5 ± 4.0mM.

**Sucrose derivative and maltooligosaccharide reactivity**

None of the sucrose derivatives was a substrate for amylosucrase, as determined visually by the presence of opalescence in the reaction mixture and by t.l.c. Similarly, none was a glycosyl donor to maltotriose. However, a small amount of disproportionation of maltotriose to D-glucose and maltopentaose was observed. This disproportionation reaction was investigated further by incubating a series of maltooligosaccharides, from maltose to maltoheptose, with amylosucrase. Isomaltose and isomaltotriose were
Figure 5. Lineweaver-Burk plots of D-glucose incorporation into glucan by the reaction of amylosucrase with [U-14C]sucrose in the presence of the following inhibitors: (a) 6-deoxysucrose, (b) 6-deoxy-6-fluorosucrose, (c) 4,6-dideoxysucrose. (Concentrations on the plots are those of the inhibitors)
Figure 5 (continued)
Figure 5 (continued)
Table I. Kinetic and inhibition constants for modified sucrases with amylosucrase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kinetic Constant (mM)</th>
<th>Type of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose ($K_M$)</td>
<td>26.5 ± 4.6</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose ($K_s$)</td>
<td>201 ± 35</td>
<td>Substrate</td>
</tr>
<tr>
<td>6-deoxy-6-fluorosucrose ($K_i$)</td>
<td>0.50 ± 0.06</td>
<td>Competitive</td>
</tr>
<tr>
<td>6-deoxysucrose ($K_i$)</td>
<td>6.2 ± 0.3</td>
<td>Competitive</td>
</tr>
<tr>
<td>4,6-dideoxysucrose ($K_i$)</td>
<td>22.5 ± 4.0</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>3-deoxysucrose</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>$\alpha$-D-allopyranosyl-$\beta$-D-fructofuranoside</td>
<td>-</td>
<td>a</td>
</tr>
</tbody>
</table>

aDid not give significant inhibition over the concentration ranges tested.
Figure 6. Effect of inhibitor concentration on the slopes of the Lineweaver-Burk plots
Also used. T.l.c. analyses of the digests of maltotriose and maltotetraose indicated that maltosyl or maltotriosyl units, respectively, were exclusively transferred to form maltopentaose or maltoheptaose with the release of D-glucose. Faint spots corresponding to maltoheptaose appeared at long maltotriose digestion times. Incubation of maltopentaose, maltohexaose, and maltoheptaose with amylosucrase led to the initial formation of oligomers one D-glucosyl unit longer and shorter than the original oligosaccharide. These were followed by further disproportionation products of both lower and higher chain length. Maltose, isomaltose, and isomaltotriose did not undergo disproportionation reactions.

Amylosucrase and transglycosylase activities, using enzyme previously heated to 50°C for 10 min, were measured at 35°C and pH 7.0 to determine if these activities were related. The results were compared to the activity of the unheated enzyme. The heated enzyme retained 25% of the amylosucrase activity while losing all transglycosylase activity. The unheated enzyme had no measurable activity at pH 4.0 and 35°C.
Amylosucrase and \textit{S. mutans} dextranucrase have many points of similarity. Both are bacterial $\alpha$-D-glucosyltransferases that catalyze the formation of specific high molecular weight glucans from sucrose. Both are inhibited by high sucrose concentrations and are activated by the presence of native polysaccharide. The present work provides information showing several additional similarities, as well as some mechanistic differences between the two enzymes.

The present study confirms the structural similarity to glycogen of the amylopolysaccharide produced by amylosucrase. Its degree of branching (10\%) and iodine staining color (reddish-brown) are closest to those of various glycogens\textsuperscript{25-27}. It differs in having somewhat shorter branch chains than most glycogens and a $\lambda_{\text{max}}$ of 500 nm with iodine that is slightly higher than most glycogens but considerably lower than amylopectin\textsuperscript{25}. The iodine stain absorbance spectra of the amylosucrase glycogen shows a shift to shorter wavelengths with reaction time. This is due to increased branching, which implies that branching is not the initially predominant reaction during polymerization\textsuperscript{28}, but is similar to the branching reaction by dextranucrase and occurs by an acceptor mechanism by segments of the synthesized amylodextrin chains\textsuperscript{29,30}.

The presence of transglycosylation activity is apparently not directly related to amylosucrase activity, in that heating the enzyme mixture before reaction affects the two activities differently. Therefore, we believe that the transglycosylase activity observed is not an intrinsic function of the amylosucrase, but rather is caused by a separate transglycosylase present in the enzyme preparation. It is not known whether this transglycosylase activity affects the product polysaccharide structure.

In the case of 3-modified compounds, no inhibitory or synthetic activity catalyzed by amylosucrase was observed. This implies that the 3-position is critical to the binding of substrate to the enzyme, similar to \textit{S. mutans} glucansucrases\textsuperscript{31}. However, in the case of dextranucrase, 3-deoxysucrose is both a weak inhibitor and a glycosyl donor.

6-Deoxysucrose and 6-deoxy-6-fluorosucrose are strong inhibitors of both amylosucrase and dextranucrase\textsuperscript{19,32}, implying that substrate binding in both involves
the C-6-hydroxyl group. Because the main linkage in dextran is α-(1→6), it is not surprising that the 6-modified sucroses bind well to the active site of dextran sucrase but do not react. However, the difference in the $K_i$ values of 6-deoxy-sucrose and 6-deoxy-6-fluorosucrose for amylosucrase was not as great as with dextran sucrase. Replacement of the hydroxyl group by a hydrogen atom yields a sucrose derivative that is a competitive inhibitor with a $K_i$ of approximately 6mM. Substitution of a fluorine for the hydroxyl group, which more closely mimics the electronic nature of the oxygen atom in the hydroxyl group, increases the effectiveness of inhibition approximately tenfold, yielding a $K_i = 0.5$ mM and indicating that the hydroxyl group at the 6-position of sucrose acts as a hydrogen bond acceptor.

6-Deoxy-sucrose and 6-deoxy-6-fluorosucrose were not substrates or glycosyl donors to maltotriose for either enzyme. The inability of these derivatives to act as substrates for the enzyme suggests that the presence of the hydroxyl group at the 6-position of sucrose affects catalysis as well as being important to binding. A possible explanation is that the modifications alter the binding position of the sucrose derivative in the active site. This binding change leads to enhanced binding due to steric and electronic forces, but positions the glycosidic bond so that it cannot undergo reactions catalyzed by the enzyme.

The amylosucrase glucan product is composed predominantly of α-(1→4) glucosidic linkages; thus the 4-position is quite important for both binding and catalysis. A comparison of the inhibitory behavior of 6-deoxy-sucrose and 4,6-dideoxy-sucrose indicates that the substitution of hydrogen for the hydroxyl group at the 4-position reduces the inhibitory activity at high inhibitor concentrations and changes the type of inhibition from competitive to a mixed form resembling both competitive and non-competitive inhibition. However, increasing the inhibitor concentration does not produce a commensurate rise in inhibitory activity. The extent of inhibition levels off with increasing inhibitor concentration (Fig. 5), implying that a different inhibition mechanism may be occurring. A possible cause of this kind of behavior is that an alternative binding mode exists when the concentration becomes higher, which affects the catalytic process. It is interesting to note that 4,6-dideoxy-sucrose has a similar unusual inhibitory action on dextran sucrase.
Because of the similarity in behavior of 4,6-dideoxysucrose with both amylosucrase and dextran sucrase, and because of the modification at the 4- and 6-positions, where the glycosidic linkage is formed respectively by amylosucrase and dextran sucrase, it is tempting to speculate that the unusual inhibitory effects are related to binding at the active site. An alternative explanation is that the sucrose derivative binds preferentially at the sucrose inhibitory binding site to reduce the inhibitory effect. The addition of 4,6-dideoxysucrose in reaction mixtures of sucrose and amylosucrase with and without added polysaccharide does not eliminate the lag in activity observed in the absence of the exogenous polysaccharide. Therefore, the decreasing inhibitory effects at higher inhibitor concentrations are probably not caused by binding of the modified sucrose at the polysaccharide site.

In summary, the general behavior of *N. perflava* amylosucrase and both *L. mesenteroides* and *S. mutans* dextran sucrases towards sucrose and the sucrose derivatives used in these studies is quite similar, despite the different glycosidic linkages in the product polysaccharides. It is interesting to speculate on the similarity in mechanism, particularly with respect to branching, since the bacterial species that produce the two enzymes occupy the same environment and use the same substrate. In both types of enzymes, the 3-position is critical for binding. However, dextran sucrase is capable of donating glycosyl residues from sucrose derivatives modified at this position, whereas amylosucrase does not exhibit this ability. Inhibitions by sucrose derivatives modified at the 6-position indicate that this position is important for binding and catalysis in both enzymes. For amylosucrase, the absence of a hydroxyl group or substitution of a fluorine strongly enhances binding, indicating that both steric and hydrogen bonding forces may play an important role at this position. Simultaneous substitution of hydrogen for hydroxyl groups at the 4- and 6-positions of sucrose produced an unusual inhibitory effect. A possible interpretation may be that different modes of binding exist, affecting the observed inhibitory behavior.

As with Section I of this dissertation, the implication of this research is that the theme of conservation of catalytic mechanism is echoed, despite a greater difference in product formation. The same implications of relationships between structure and function are also noted. The results also indicate that there may be a means of relating structure to binding, independent of catalysis. This implies that for these polymerizing
carbohydrate enzymes, there may be a way to structurally alter binding without affecting the catalytic mechanism and effectively engineer polysaccharides with specific glycosidic linkages.
ACKNOWLEDGMENTS

The author wishes to thank Dr. Edward J. Hehre for providing the *N. perflava* culture, Dr. Morey E. Slodki for performing the permethylation analysis, Mr. Steve Eklund for the gift of 6-deoxy-6-fluorosucrose, and Mr. Aziz Tanriseven for the gift of 4,6-dideoxysucrose. I am also grateful to the Corn Refiners Association for a fellowship while conducting this research.
REFERENCES


SECTION III.

DETECTION OF A COVALENT INTERMEDIATE

IN THE MECHANISM OF ACTION OF

PORCINE PANCREATIC $\alpha$-AMYLASE

USING $^{13}$C NUCLEAR MAGNETIC RESONANCE
The catalytic mechanism of porcine pancreatic α-amylase was been examined by nuclear magnetic resonance (n.m.r.) at subzero temperatures using [1-13C]-labeled maltotetraose. Spectral summation and difference techniques revealed a broad resonance peak, whose chemical shift, relative signal intensity, and time-course appearance corresponded to a β-carboxyl-acetal ester covalent enzyme-glycosyl intermediate. This evidence supports the double-displacement covalent mechanism hypothesis for porcine pancreatic α-amylase-catalyzed hydrolysis of glycosidic linkages, based on the presumption that aspartic acid residues play a catalytic role in the active site of this enzyme.
INTRODUCTION

In the introduction to this dissertation, I stated that one of the challenges to biotechnology was to develop a better understanding of the relationship between function and structure of catalytic proteins. Sections I and II describe research which characterized two carbohydrate enzymes, an endo-β-xylosidase and amylosucrase, and compares their behavior to similar enzymes. This information provides a basis for future comparisons of enzymatic functional-structural features, as structural information for these enzymes becomes available. This section describes research investigating the catalytic mechanism of a much more widely studied enzyme, porcine pancreatic α-amylase (PPA). In the case of PPA, a great deal of structural information is currently available, providing the opportunity to begin to derive relationships between function and structure. The results of this research confirm the nature of the catalytic groups involved and provide evidence as to the mechanism of reaction.

Primary structural information from α-amylases of different origin have shown that while the degree of overall homology varies greatly, several short regions of amino acids are highly conserved. It is believed that some of these regions could be involved in the catalytic process, implying that the mechanism of catalysis is conserved among the α-amylases. This concept is not unusual in that all amylases catalyze the hydrolysis of α-(1→4) glycosidic linkages, regardless of the specific low molecular weight products that they form. The product specificities of the amylases are apparently obtained by the specific binding of the substrate, via both hydrophobic interactions and hydrogen bonding, resulting in the different action patterns observed with the different amylases.

Recent high resolution X-ray crystallographic data have allowed three-dimensional models to be generated that confirm the presence of these regions of homology within the catalytic sites of the α-amylases. Concrete enzymatic functional-structural principles have not yet been determined; however, these findings are a major step forward in understanding the puzzle of enzymatic catalysis. The research described here provides another piece of this puzzle by relating the mechanism of catalytic action to the structural features previously determined.
Amylases

Amylases are probably the most widely studied of the carbohydrate enzymes, due to their biological and industrial importance, as well as their ubiquitous distribution. As noted in the introduction to this dissertation, the nomenclature used to denote enzymes originated with malt amylase. Different types of amylases exist, with a variety of physical, chemical and catalytic properties. A review by Robyt¹ describes the classification, characterization and action patterns of different amylases on starch.

Of the many kinds of amylases that occur in nature, α-amylases are probably the most ubiquitous. The distinction between α- and β-amylases is that the former retains stereo-configuration of the product anomeric hydroxyl group while the latter forms a product with an inversion of configuration. α-Amylases are produced by a wide variety of species, including fungi, bacteria, plants, and animals. Within a particular organism, different forms of α-amylase can even be produced by specific organs, such as salivary and pancreatic α-amylases in mammals.

The catalytic properties of α-amylases have been extensively studied. α-Amylases are predominantly endohydrolases, cleaving α- (1→4) glycosidic bonds with retention of configuration in the products. A variety of oligosaccharides can be produced, depending the binding specificity of the particular enzyme. Many α-amylases cleave substrates in a multiple attack fashion² and do not cleave α- (1→6) glycosidic branch linkages³⁴. Several α-amylases have been previously subsite-mapped, showing binding sites from five⁵ to nine⁶ subsites in length. The amino acids involved in substrate binding have also been investigated⁷. Studies of the catalytic action of α-amylases⁷⁹ have revealed that carboxylate anions are involved in the catalytic process.

In general, α-amylases are calcium-metallo enzymes, requiring the presence of calcium ions to stabilize the tertiary structure of the protein¹⁰. For mammalian α-amylases, chloride ions are also required for optimum activity¹¹. Recent results on the three-dimensional structure of porcine pancreatic α-amylase imply that the structure of the calcium binding site is conserved in all α-amylases¹².
Many $\alpha$-amylases exist in slightly different isozymic forms, because of amino acid substitutions and modifications. Most $\alpha$-amylases are in the 50,000 molecular weight range, with the exception of a zinc-dimer of $B. amyloliquifaciens$ $\alpha$-amylase that has a molecular weight of 96,000. Most $\alpha$-amylases are acidic, due to a high level of aspartic and glutamic acids, and contain sulfhydryl groups. $\alpha$-Amylase amino acid composition and sequence information is available for $\alpha$-amylases of different origins. Comparison of these sequences has shown that while the degree of overall sequence homology varies greatly between proteins from different sources, small regions of apparently universal homology exist. Specifically, three short regions of porcine pancreatic $\alpha$-amylase (PPA), residues 95-101 (region 1), residues 193-201 (region 2), and residues 295-301 (region 3), are apparently conserved in all $\alpha$-amylases.

Most recently, X-ray crystallographic data have become available to explore the tertiary structure of $\alpha$-amylases. In 1980, Payan et al. presented a low-resolution model which indicated that two maltotriose binding sites exist in porcine pancreatic $\alpha$-amylase. Whereas the nature of this second site has not been determined, it is suspected to play a role in the multiple attack activity of PPA. Matsuura et al. have presented a crystal structure for Taka-amylase A ($Aspergillus oryzae$ $\alpha$-amylase). In this model, a glutamic acid residue is proposed to be involved in the catalytic site. Most recently, Buisson et al. have presented a high-resolution structural model for PPA. They proposed that two aspartic acid residues in conserved regions 2 and 3 (Asp 197 and Asp 300, respectively) are responsible for catalysis. An asparagine (Asn 100, region 1) and a histidine (His 201, region 2) are proposed to bind Ca, which is required to optimally position the catalytic groups. These models confirm the importance of carboxylic acid residues in the catalytic function of $\alpha$-amylases and the importance of the Ca chelations to maintain the integrity of the active site.

Of general interest is the discussion by Buisson et al. regarding the genetic code for $\alpha$-amylases and its implications about the possible common ancestry of PPA, pyruvate kinase, phosphogluconate aldolase, xylose isomerase and triose phosphate isomerase, based on similarities in the tertiary structure of these diverse enzymes.
Proposed Catalytic Mechanisms for $\alpha$-Amylases

Koshland\textsuperscript{22} originally proposed a nucleophilic catalytic displacement mechanism for enzymes that involve stereochemical reactions. In the case of enzymes which invert stereo-configuration, such as $\beta$-amylase, a single nucleophilic displacement was envisioned. In the case of enzymes which retain stereo-configuration, such as $\alpha$-amylase, a double displacement mechanism was invoked. The intermediate involved in this double displacement mechanism was a covalently bonded intermediate. This mechanism was further elaborated upon by Fischer and Stein\textsuperscript{13} to include a carboxylate anion as the nucleophilic catalytic group (Fig. 1).

Mayer and Lamer\textsuperscript{23} proposed an alternative mechanism employing a stabilized carbonium ion intermediate (Fig. 2). The stereospecificity of the hydration of the carbonium ion to form products was envisioned as an enzyme-mediated water interaction. The appealing point of the Mayer and Lamer scheme is that the catalytic action of both $\alpha$- and $\beta$-amylases could be explained by a single mechanism.

Detection of Transition Intermediates

Enzymatic catalysis is believed to proceed via a transition state intermediate. Detection of such intermediates has always been desirable from the viewpoint of determining and understanding catalytic mechanisms. However, the extremely short lifespans of such intermediates usually precludes their detection. A recent review\textsuperscript{24} describes the use of substrate analogs, which allow more stable transition state complexes. An alternative means of extending intermediate lifespans is by reducing the temperature of the reaction to cryogenic temperatures. Thus stabilized, these transition analogs allow various spectroscopic and crystallographic detection techniques to be employed\textsuperscript{25,26}. While some carbohydrate enzymes ($\beta$-galactosidase and lysozyme)\textsuperscript{26} have been investigated by using these techniques, much of this work has been performed with serine proteases (trypsin, chymotrypsin and papain)\textsuperscript{27-30}. More recently, Mackenzie \textit{et al.}\textsuperscript{29} used $^{13}$C n.m.r. in conjunction with cryological temperatures to directly demonstrate the presence of an actual covalent transition intermediate of trypsin, using a $^{13}$C-enriched substrate analog.
The main limitation of using cryogenic temperatures is the need for addition of a cryosolvent to reaction mixtures. Douzou\textsuperscript{25} and Fink and Cartwright\textsuperscript{26} discuss the use of a number of cryosolvents, including alcohols, polyols, and various organic solvents. In the studies performed here, dimethyl sulfoxide (DMSO) was used as a cryosolvent, because it does not fundamentally affect the catalytic mechanism of PPA\textsuperscript{31,32}. 
Figure 1. Proposed nucleophilic double displacement mechanism for α-amylase (E refers to the enzyme and B refers to an electrophilic catalytic group on the enzyme, proposed to be an imidazole. The circled structure is the proposed covalent intermediate.)
Figure 2. Proposed carbonium ion mechanism for α-amylase (E refers to the enzyme and B refers to an electrophilic catalytic group on the enzyme, proposed to be an imidizole. The circled structure is the proposed carbonium ion intermediate.)
EXPERIMENTAL

Materials

Enzymes

Porcine pancreatic α-amylase [EC 3.2.1.1] was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). *Pseudomonas stutzeri* maltotetraose-producing amylase [EC 3.2.1.60, exo-maltotetraohydrolase] was prepared by the method of Robyt and Ackerman\(^\text{33}\). *Pseudomonas amyloderamosa* isoamylase\(^\text{34}\) [EC 3.2.1.68, glycogen 6-glucano-hydrolase] was purchased from Sigma Chemicals, Inc. (St. Louis, MO). Amylosucrase [EC 2.4.1.4] was produced by the methods of Okada and Hehre\(^\text{35}\), as noted in Section II of this dissertation.

Carbohydrates

D-[1-\(^{13}\)C]-glucose (99 atom % enriched) was purchased from MSD Isotopes (Rahway, NJ).

\[ \alpha-D-[1-\(^{13}\)C]-glucopyranosyl fluoride \]

\[ \alpha-D-[1-\(^{13}\)C]-glucopyranosyl fluoride \] was prepared by using the methods described by Okada and Hehre\(^\text{36}\), except that D-[1-\(^{13}\)C]-glucose was used instead of D-glucose and the reaction temperature was \(-70^\circ\text{C}\) instead of \(-20^\circ\text{C}\).

Amylosucrase (0.46 IU) was added to \(\alpha-D-[1-\(^{13}\)C]-glucopyranosyl fluoride\) (0.16 mol) in 2 mL of buffer (50 mM sodium maleate, pH 7.0, 0.02% sodium azide) and incubated at 30°C overnight. The pH was maintained at 7.0 by addition of 0.25N sodium hydroxide. The resulting polysaccharide was precipitated with three volumes of ethanol, recovered by centrifugation, and dried in vacuo at 30°C. The yield was approximately 30 mg of polysaccharide.

This polysaccharide was dissolved in 3 mL of buffer by boiling for 1 h followed by ultrasonication. The nuclear magnetic resonance (n.m.r.) spectrum of this polysaccharide confirmed the presence of the \([1-\(^{13}\)C] label.\]
[1-13C]-Maltotetraose was produced in several steps. First, maltotetraose-producing amylase (approximately 0.1 IU) was added to the dissolved polysaccharide and incubated at room temperature for 12 h. Three volumes of ethanol were added to the digest to precipitate polysaccharide, followed by centrifugation. The supernatant containing the maltotetraose was decanted and reduced to approximately 1 mL by vacuum evaporation followed by isolation of the maltotetraose by paper chromatography.

The recovered polysaccharide was redissolved in 1 mL of buffer (pH 6.0), 22 IU of isoamylase were added, and the mixture was incubated overnight at room temperature. T.l.c. of the digest showed that several maltooligosaccharides were present. The pH was adjusted to 7.0 with NaOH, and maltotetraose-producing amylase (approximately 0.1 IU) was added to this mixture, which was incubated overnight at room temperature. Two volumes of ethanol were added to the digest to precipitate any remaining polysaccharide, followed by centrifugation. The supernatant was decanted and the maltotetraose was isolated from it using paper chromatography. The purified maltotetraose samples were combined and analyzed by a ferricyanide reducing method. The total yield of [1-13C]-maltotetraose was 4.93 mg.

Methods

Thin layer chromatography

Analysis for the presence of maltooligosaccharides was performed by t.l.c. Samples were spotted onto Whatman K5 silica gel plates (Whatman Chemical Separations, Inc., Clifton, NJ) and dried. Separation was effected by a single ascent of ethyl acetate:methanol:water (37:40:23, v/v/v), and visualized by spraying with methanol-sulfuric acid (4:1, v/v) followed by charring at 120°C for 10 min.

Paper chromatography

Preparative isolation of maltotetraose was performed by placing the sample on 22 x 56 cm Whatman 3MM filter paper and irrigating with one descent of 7:3 (v/v) n-propyl alcohol:water for 24 h. The maltotetraose was located by using silver nitrate. The maltotetraose was eluted from the paper with water and freeze-dried.
Determination of the effect of DMSO on PPA

The rates of hydrolysis of soluble starch and maltotetraose by PPA in the presence of various concentrations of dimethyl sulfoxide (DMSO) were measured by ferricyanide reducing analysis\textsuperscript{37}.

In the case of starch, reaction digests were composed of 590 μL of 1% soluble starch solution in 50 mM sodium glycerophosphate buffer, pH 7.0, containing 10 mM calcium chloride, and 10 μL of PPA (0.2 mg/mL in 50 mM sodium glycerophosphate buffer, pH 7.0, containing 10 mM calcium chloride). The remaining reaction mixture was composed of DMSO/water in appropriate proportions to make the reaction mixture 0, 5, 10, 20, and 40 % (vol.) in DMSO. Samples were taken at various time intervals and analyzed by the ferricyanide reducing sugar method\textsuperscript{37}. The reaction temperature was 30°C. The rate of hydrolysis was also measured at -15°C in the presence of 40 % (vol.) DMSO.

The rate of hydrolysis of maltotetraose was measured at 30°C at 0 % and 40 % (vol.) DMSO, and at -15°C in 40 % (vol.) DMSO.

N.m.r. conditions

PPA (10 mg) was dissolved in 0.6 mL of buffer (50 mM sodium glycerophosphate, pH 7.0, containing 10 mM calcium chloride, made with deuterated water) and made 40 % (vol.) in DMSO by the addition of 0.4 mL of DMSO. Deuterated buffer (1.2 mL) was added to the previously produced [l-\textsuperscript{13}]C-maltotetraose (4.93 mg). DMSO (0.8 mL) was added to produce a 40 % (vol.) DMSO solution. Both solutions were cooled to -20°C by using a dry ice-acetone bath prior.

Immediately prior to spectral acquisition, the two solutions were combined at -20°C and thoroughly mixed (approximately 1-2 min.). Total sample size was 3 mL in a 10-mm diameter n.m.r. sample tube, giving a final enzyme concentration of 70 μmol and a final maltotetraose concentration of 2.3 mM. Eighty spectra were recorded with a Bruker WM300 wide-bore spectrometer at 75 MHz for \textsuperscript{13}C nuclei. Spectral acquisition parameters were 8000 time domain points (zero-filled to 16,000 before Fourier transfor-
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mation), 10 μs pulse width (90 degree pulse = 32 μs), 0.22 s acquisition time, 10,000 acquisitions per spectrum, spectral width approximately 200 ppm and low power noise decoupling (0.4 W). Each spectrum took approximately 40 min to collect. Chemical shifts are reported relative to tetramethylsilane (0 ppm).

Spectral analyses were performed with the Lab One NMR1 program (New Methods Research, Inc., Syracuse, NY) on a MicroVax computer (Digital Equipment Corp., Maynard, MA). First, the individual free-induction decay (FID) spectra were Fourier-transformed, applying an exponential line broadening of 5 Hz. The three peaks obtained were assignable to the C1 in the α-(1→4) glycosidic linkages between glucosyl residues (100 ppm), and the α- and β-anomeric carbon residues (96 and 92 ppm, respectively). These separate peak areas were individually integrated and the total signal strength was obtained from the sum of the three areas.

To detect the intermediate, the FID spectra were added together sequentially in groups of ten. To remove residual resonances caused by the enzyme and to observe the formation of products, a control spectrum was obtained by multiplying the initial FID spectrum by a factor of ten. This control spectrum was subtracted from each sum of ten subsequent spectra. The remaining FID spectrum was Fourier-transformed by using the parameters previously described.
RESULTS

Reaction Conditions

The effects of temperature and DMSO concentration on PPA

Hydrolytic rates of PPA on starch in the presence of DMSO are presented in Table I. The addition of DMSO up to 10 % (vol.) did not significantly reduce the rate of hydrolysis. At 20 % and 40 % (vol.) DMSO the rate was reduced by approximately 40 % and 80 %, respectively. At -15°C and 40 % (vol.) DMSO, the hydrolytic rate of PPA on starch was reduced by approximately two orders of magnitude.

The hydrolytic rate of PPA on maltotetraose at 30°C in the absence of DMSO was roughly 100-fold lower than on starch. This is in accordance with results previously obtained by Robyt and French. In 40 % (vol.) DMSO, the rate decreased roughly 10-fold. The rate at -15°C in 40 % (vol.) DMSO was not detectable.

N.M.R. Results

N.m.r. spectral analysis

A typical transformed spectrum of the reaction mixture is presented in Fig. 3. Based on their chemical shifts, the three peaks are assignable as the C1 resonances of glucosyl residues in the α-(1→4) glycosidic linkages (100 ppm), the β-anomer (96 ppm), and the α-anomer (92 ppm). Fig. 4 presents the changes in the peak areas (% of total signal) vs. spectrum number. As expected, the loss of signal in the glycosidic linkage peak and corresponding increase in the signal of the anemic peaks indicate the progress of the hydrolytic reaction. Plotting the total signal strength vs. spectrum number (Fig. 5) shows an initial decrease (approximately 5%) in signal strength. After approximately 60 spectra were acquired, the total signal strength began to increase. Fig. 6 shows the results of summation of the spectra in groups of ten after subtraction of the initial substrate and enzyme spectra.
Table I. Hydrolytic rates of PPA on starch and maltotetraose in the presence of DMSO

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Temperature °C</th>
<th>% (Vol.) DMSO</th>
<th>Rate^ (\mu\text{mol}) min-mg enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>30</td>
<td>0</td>
<td>106.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>5</td>
<td>105.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>10</td>
<td>115.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>20</td>
<td>63.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>40</td>
<td>17.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>-15</td>
<td>40</td>
<td>0.6</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>30</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>40</td>
<td>0.06</td>
</tr>
<tr>
<td>&quot;</td>
<td>-15</td>
<td>40</td>
<td>not detectable</td>
</tr>
</tbody>
</table>

^Calculated as equivalent \(\mu\text{mol}\)s of maltose released based on ferricyanide reducing sugar analysis.
Figure 3. N.m.r. spectrum of maltotetraose in 40% DMSO
Figure 4. Changes in peak signal strength (peak area) with spectrum number
Figure 5. Total signal strength vs. spectrum number (Signal strength values presented are the sum of the areas under the glycosidic bond and anomeric $^{13}$C peaks.)
Figure 6. Direct observation of a β-carboxyl-acetal enzyme intermediate by $^{13}$C n.m.r. (Spectra are sums of ten sequential individual spectra, after subtracting initial substrate and enzyme background spectra. Spectra a-h correspond to spectra 1-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, respectively. Arrows indicate the resonance peak corresponding to the intermediate.)
DISCUSSION

The ability to trap intermediates in enzyme-catalyzed reactions at cryogenic temperatures has been demonstrated for a number of enzymes\textsuperscript{26}. In most instances, the detection of intermediates was dependent upon the use of modified substrates or inhibitors. In this study, a specifically-labeled natural substrate was employed to minimize any possibility of changes in the mode of action of the enzyme and to uniquely identify the intermediate species.

Reaction Parameters

Effects of cryosolvent and temperature on PPA activity

As Fink and Cartwright\textsuperscript{26} noted, the choice of cryosolvent is largely empirical. The selection of DMSO for this study was based on the fact that it does not completely inactivate PPA at the concentrations used here. While the rate of reaction is greatly diminished under cryological conditions, the basic mechanism of reaction is believed to remain unchanged. However, the large increase in viscosity of DMSO with decreased temperature does affect the tumbling speed of large molecules, such as PPA, resulting in severe broadening of spectral peaks. Thus, while the presence of a peak corresponding to the covalent intermediate is detectable, the high noise level of the spectra did not allow direct quantitation of the amount of intermediate by measuring the peak area.

The results on the effect of DMSO on PPA activity obtained here are in accordance with those found by Lineback and Sayeed\textsuperscript{31} and Griffin and Fogarty\textsuperscript{32}. The cause of the decreased activity at high DMSO concentrations is not known. However, it is believed that the presence of DMSO does not affect the basic conformation or catalytic activity of PPA. The enzyme is thought to exist within a hydrated shell, allowing normal catalytic behavior\textsuperscript{26}. Fink and Cartwright\textsuperscript{26} suggested that the decreased enzymatic rate in cryosolvents such as DMSO is caused by reduced mass transport due to increased viscosity and interference by the DMSO in substrate binding.
[1-13C]-Maltotetraose as substrate

The detection of enzyme intermediates relies on the ability to reduce the rate of reaction to a level that allows a sufficiently long intermediate lifetime. The choice of maltotetraose as a substrate was based on its size and reduced reaction rate compared with that of starch. As the smallest natural substrate with an appreciable rate of hydrolysis, maltotetraose added relatively little mass to the enzyme-substrate intermediate, minimizing n.m.r. peak broadening. Additionally, the hydrolytic rate of PPA on maltotetraose was less than 1% that of starch. Finally, as a natural substrate of PPA, the use of maltotetraose precluded any possibility of artifacts due to non-carbohydrate residues.

The use of [1-13C]-labeled substrate allowed the detection of the intermediate by n.m.r. and eliminated the possibility of detecting any resonances of non-labeled species.

Analysis of N.M.R. Spectra

Identification of the intermediate

As previously mentioned, the major difficulty in using an enzyme such as PPA is its large size and the effect of that size on the detection of covalent intermediates by n.m.r. The effect of using such a large enzyme is to greatly broaden the width of the n.m.r. peak, making detection and quantification more difficult. Previous work using the same techniques have used much smaller enzymes, such as trypsin, chymotrypsin and lysozyme, which yield sharper peaks. For example, the line width of the trypsin intermediate detected by Mackenzie et al.29 was approximately 100 Hz, whereas the line width of the peak detected here is about 200 Hz.

As with the case of these other enzymes, peaks corresponding to an intermediate were not directly observable from the individual spectra. However, summation of spectra does allow detection of peaks assignable to intermediates by difference techniques. While this methodology works for the detection of peaks, accurate quantifica-
tion of peak intensity and area is difficult, particularly with very broad line widths. In the present study, this limitation is apparent. Whereas the presence of the intermediate is clear from the broad peak detected at approximately 91 ppm, the peak width and noise precluded accurate integration so that accurate quantification of signal strength was not possible.

X-ray crystallographic studies of PPA indicated that the amino acid residues involved in catalysis are aspartic acids\textsuperscript{12}. The reaction mechanism for PPA proposed by Fischer and Stein\textsuperscript{13} employs a carboxylate anion as the nucleophile in a double-displacement catalytic scheme. The reaction intermediate of this mechanism is a $\beta$-linked enzyme carboxyl-acetal ester involving the C1 carbon of the glucosyl residue in the catalytic site.

The structural similarity of this carboxyl-acetal ester to that of the C1 $\beta$-acetate of glucose is the basis for the assignment of the n.m.r. resonance of the intermediate detected. The n.m.r. resonance of carbon 1 of $\beta$-D-acetyl glucose has a chemical shift of 91.3 ppm\textsuperscript{40}, approximately the same as the signal detected here. Since the only signal detectable in this mixture is due to the [1-$^{13}$C] label, and no other products exist for this enzyme, this resonance was assumed to be a $\beta$-carboxyl-acetal ester intermediate.

Additional evidence supporting this conclusion was obtained by monitoring the total signal strength vs. spectrum number. The initial loss of approximately 5\% of the total signal strength corresponds precisely to the molar loss of substrate which would be observed if the enzyme were assumed to be saturated by substrate (based on two binding sites per enzyme molecule). This loss of signal strength is also matched by both the appearance of the broad spectral peak corresponding to the carboxyl-acetal ester intermediate and its apparent decrease in intensity near the end of the spectral acquisition period. Therefore, it is presumed that the detected resonance reflects the presence of a covalent intermediate involving the enzyme and labeled substrate.

Based on the sequence homology and structural information available for different $\alpha$-amylases, it would appear that carboxylic acid residues are present in the
catalytic regions of all of these \( \alpha \)-amylases. This implies that the fundamental mechanism of all these enzymes is essentially identical, despite differences in physical properties and product formation.

The observation of a n.m.r. peak corresponding to an enzyme-glucosyl intermediate precludes the carbonium ion mechanism proposed by Mayer and Larner\(^{23} \), since such a highly reactive species would undoubtedly not be stable for the length of time needed to acquire a single spectrum (approximately 40 min). While the existence of this intermediate cannot be completely discarded based on the evidence obtained here, it is unlikely that it plays a major role in the catalytic mechanism of other amylases. As previously noted, the available structural information on amylases indicates that the amino acid sequences involved in catalysis are conserved, implying that the mechanism of action is also conserved. Therefore, while the formation of different product configurations for \( \alpha \)- and \( \beta \)-amylases undoubtedly occurs via different catalytic processes, the fundamental mechanism of action is probably the same for both enzymes.
ACKNOWLEDGMENTS

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APPENDIX TO SECTION III.

NUCLEAR MAGNETIC RESONANCE SPECTRA OF $^{13}$C-LABELED GLYCOGEN AND MALTOOLIGOSACCHARIDES
INTRODUCTION

Carbon-13 nuclear magnetic resonance ($^{13}$C n.m.r.) is a very useful tool for identifying the structure of biopolymers and studying the catalytic mechanisms of enzymes. Information on both structural linkages and tertiary conformation can be obtained from n.m.r. spectra. The low natural abundance of the $^{13}$C isotope, however, makes high-resolution spectra difficult to obtain. Very long scan times and high material concentrations are usually required. For biopolymers such as polysaccharides, low solubility can further limit the degree of spectral resolution.

To overcome these constraints, $^{13}$C-enriched monosaccharides have been synthesized and very precise spectra are available. However, no one has made $^{13}$C-labeled oligo- and polysaccharides, limiting investigation of these materials by n.m.r. spectroscopy. The availability of [1-$^{13}$C]-glycogen, produced as previously described in Section III of this dissertation, allowed us to obtain high resolution n.m.r. spectra for this polysaccharide, as well as [1-$^{13}$C]-labeled maltotetraose, maltotriose, and maltose.
EXPERIMENTAL

Materials

[1-$^{13}$C]-Labeled glycogen and maltotetraose were produced as previously described in Section III of this dissertation. Labeled maltose and maltotriose were produced by hydrolysis of the labeled maltotetraose by porcine pancreatic $\alpha$-amylase and were isolated by paper chromatography.

Methods

$^{13}$C n.m.r. spectra were obtained using a Nicolet NT-300 Fourier-transform spectrometer (75 MHz) in the proton-decoupled mode (256 scans per spectrum, 8K time domain data points, 0.4 s acquisition time, 0.6 s delay between acquisitions, 8 $\mu$s pulse ($12 \mu$s = 90°), zero-filled before Fourier transformation, using 1 Hz exponential line broadening). Peak assignments were relative to an external tetramethylsilane standard (0 ppm).
RESULTS AND DISCUSSION

$^{13}$C n.m.r. spectra from [1-$^{13}$C]-labeled glycogen, maltotetraose, maltotriose, and maltose are presented in Fig. 1.

The chemical shifts obtained for the polysaccharide and maltooligosaccharides are in agreement with those identified from non-labeled materials for the C1 carbon. However, due to the enhanced level of $^{13}$C in the saccharides, we were able to observe distinct chemical shifts for the different $\alpha$-(1→4) linkages in the labeled maltooligosaccharides.

The spectrum of the polysaccharide shows a single, broad resonance peak at 100 ppm corresponding to the $\alpha$-(1→4) glycosidic linkages. The spectrum of maltose shows a fused doublet, apparently due to different conformations of the disaccharide in solution. Maltotriose gives three peaks, a doublet corresponding to the glycosidic linkage at the reducing end and a single additional peak at 100.97 ppm, presumably due to the glycosidic linkage at the non-reducing end. With maltotetraose, three fused peaks were observed. While these peaks are resolvable by n.m.r., their proximity to one another indicates considerable rotational freedom around the glycosidic bonds of this oligosaccharide, corresponding to an increased number of available configurations. This implies that as the DP of the oligosaccharide increases, more configurations become available, and the ability to resolve specific configurations of individual glycosidic linkages under these conditions is reduced. Therefore, configurational resonance peaks of higher DP oligosaccharides corresponding to individual glycosidic bonds would probably not be expected to be resolvable using the present method.

These results demonstrate that n.m.r. spectroscopy of [1-$^{13}$C]-labeled maltooligosaccharides is a very sensitive probe into their structure and conformation. For di- and trisaccharides, distinct resonance peaks can be resolved, apparently corresponding to specific conformations of the internal glycosidic bonds. For maltotetraose, resonance peaks corresponding to the individual glycosidic linkages are also resolvable. However, the present instrumental limitations (magnetic field strength) and methodology did not allow sufficient resolution of the specific conformational forms for the
tetramer. However, with the availability of instruments of greater magnetic field strength and perhaps by reducing solution temperatures, resonance peaks corresponding to specific conformations of these higher oligomers may be obtainable.
Figure 1. N.m.r. spectra of [1-\textsuperscript{13}C]-labeled amylopectin, maltotetraose, maltotriose, and maltose.
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GENERAL CONCLUSIONS

As stated in the introduction of this dissertation, the objective of this research was to characterize several carbohydrate enzymes, thereby providing basic information for developing relationships between the function and structure of these biocatalysts. The underlying theme has been the concept that while the catalytic and binding mechanisms of enzymes are mutually dependent upon each other, the structural features related to each these functions are distinct and capable of being independently altered. While the goal of delineating the relationships between structure and function are yet to be determined, these research results have provided useful, important groundwork towards accomplishing this goal.

In the case of the endo-β-lanase research, subsite binding affinity mapping was used to characterized an *A. niger* endo-β-lanase and provided a basis for explaining the product distribution observed. Comparison of the subsite affinity maps of endo-β-xylanases of different origins indicated that while the catalytic function is conserved by the different enzymes, they possess different substrate binding features, resulting in markedly different product size distributions. From a pragmatic viewpoint, these results implied that the potential exists for modifying the binding structure to make different length products. This conclusion was recently demonstrated by the chemical modification of PPA to alter its product specificity14.

The amylosucrase work carried this theme a bit further, implying that binding structure may also be responsible for the creation of structurally different products. In this work, the similarity between the binding features of amylosucrase and dextran-sucrase was highlighted by inhibition studies using modified substrates. Since both enzymes use the same substrate and perform the same basic function of glucosyl transfer, it is conceivable that the fundamental mechanism of catalysis is also the same. Therefore, the product specificities of these enzymes must be a result of the specific structural binding arrangements of each enzyme. Again, from a pragmatic viewpoint, this implies that the potential exists for making different, new products from an enzyme by engineering its binding structure.
Finally, the PPA work provided evidence for defining the mechanism of action of a carbohydrate hydrolase. Combined with structural information from other researchers, the results of this work further emphasize the structural independence of binding and physical properties from mechanism. Less than 10% primary sequence homology exists between various α-amylases with different physical properties, such as pH optima and thermal stability. Even though this low degree of homology exists, it apparently is sufficient to allow the catalytic mechanisms of these various enzymes to be fundamentally identical. Therefore, the remaining 90% of the structure must be independently responsible for the observed differences in product formation and physical properties. Again, from a practical viewpoint, this means that it may be possible to effectively engineer the physical behavior of the protein independently from the mechanism.

As demonstrated by this work, the carbohydrate enzymes, both hydrolytic and synthetic, provide an excellent system for studying the basic relationships between structure and function of enzymatic binding and catalysis. The diversity among carbohydrate enzymes with similar catalytic functions provides opportunities to specifically compare the relationships between binding function and structure. These comparisons will eventually lead to determining the more general principles of structure and function of biocatalysts.

As of today, we still do not have a sufficient understanding of the general structural principles governing the binding and catalytic properties of enzymes. However, the research described here and by other scientists are rapidly providing the fundamental groundwork from which these principles will eventually be derived. Protein modification tools, such as site-directed mutagenesis, chemical modification, and recombinant genetics, provide exquisitely sensitive methods to pinpoint the role of specific amino acid residues in enzymes. These tools, in combination with the type of physical and kinetic information obtained in this research, will provide the means to further explore and eventually allow us to directly engineer new biocatalysts.
GENERAL LITERATURE CITED


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