Enzymatic Mechanism of N-acetylglucosaminidase Revealed by Structural Studies on Enzyme Substrate-Inhibitor Complexes

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N-acetylglucosaminidase from the soil bacterium *Serratia marcescens* has been studied by X-ray crystallography. Here, we propose a reaction mechanism for the hydrolysis of chitobiose to N-acetylglucosamine by the enzyme. The analysis of protein crystals complexed with chitobiose, glucose, and the inhibitor 2-acetamido-2-deoxy-D-gluconhydroximo-1,5-lactone (Lognac) is presented. One protein carboxyl side chain donates a proton to the glycosidic linkage in general acid catalysis. The intermediate is stabilised by the acetamido group of the substrate acting as nucleophile. There is no requirement for a second functional protein group. The substrate is held by an arginine in a binding pocket and distorted towards the reaction transition state. The mechanism and the active site architecture are novel for glycosyl hydrolases.

Keywords: *N-acetylglucosaminidase, chitobiase, chitobiose, structure, reaction mechanism, Serratia marcescens, hydrolysis, inhibitor, general acid catalysis.*

Introduction. While cellulose turnover is understood in some detail, our knowledge of natural chitin processing is relatively limited (1). Structural studies on chitinolytic enzymes are required to learn about the overall shape of these proteins and to gain insight into the active site geometry and reaction mechanism. Protein-substrate and protein-inhibitor interactions as studied by X-ray crystallography reveal the reaction mechanism at a structural level.

Bacterial chitinolytic systems are of special interest due to their potential application as biological control agents. *Serratia marcescens* is one of the most active chitinolytic bacteria (2) which produces chitinases and N-acetylglucosaminida-

ses with high activity over a wide pH range (3). Chitinases break the polysaccharide down to the disaccharide chitobiose, which is hydrolysed by N-acetylglucosaminidase to the monosaccharide N-acetylglucosamine.

Primary structure classifies prokaryotic and eukaryotic chitinases into family 18 and 19 of glycosyl hydrolases (4). 3-D structures are available for several chitinases (5, 6, 7). We have reported preliminary data on the first family 20 enzyme (8, 9), the N-acetylglucosaminidase from *Serratia marcescens*. All known structures have an 8-stranded βα-barrel in common which is the catalytic domain. Another feature is hydrolysis of the β-1,4 glycosidic linkages with retention of the anomeric configuration. The active site is at the carboxy terminal end of the barrel — in agreement with other enzymes that have a βα-barrel motif..

Materials and Methods. The 4.5 kb genomic DNA fragment from *Serratia marcescens* cloned into pEMBL18 (10) has been sequenced (11). The construct has been used to obtain excess of protein for crystallographic studies.

Crystallisation conditions, structure determination and preliminary refinement statistics of the native enzyme have been presented previously (8). The refinement converged to an R_{factor} of 13.9 % (R_{free} 19.6 %), the R_{factor} using all data is 14.6 %. The current model with good geometry contains 6801 protein atoms, 880 water molecules and 2 sulphates. Details of this analysis are presented elsewhere (9). 3-D comparisons were carried out with the program DALI (12).

To study substrate and inhibitor binding crystals of the native enzyme were soaked in solutions of the desired compounds by adjusting the crystal mother liquor to the desired substrate concentration. Protein crystals were kept in this buffer for about 2 to 10 minutes as longer soaks reduced the diffraction limit. Datasets to a resolution of 2 Å or better were collected on single crystals using a MAR Research imaging plate scanner at the EMBL beamlines X31 (2-acetamido-2-deoxy-D-gluconhydroximo-1,5-lactone Lognac) and BW7B (chitobiose, glucose). All complex structures were individually refined to similar $R_{factors}$ as the native structure.

Results. The chitobiase structure is shown in Figure 1. Overall dimensions of the protein are roughly 90 x 80 x 60 Å. One side of the molecule is an essentially flat surface. Three disulphide bridges stabilise the structure. There are no cofactors, metals or other ligands.

The monomeric protein is divisible into four domains. Both the N-terminal domain I, 154 amino acids, and the C-terminal domain IV, 67 amino acids, have two β-sheets opposing each other to form compact modules. The fold of the domains relates to known structures. Domain I is almost identical to the cellulose binding domain from *Cellulomonas fimi* (13), although there is no detectable se-

quence homology. The cellulose binding domain lacks the N-terminal helix which in chitobiase establishes a cleavage site for the leader peptide (8). Although no function has been assigned to this domain, an involvement in substrate binding appears likely. The two β-sheets in the C-terminal domain IV show a topology similar to the immunoglobulin β-sandwich. A good structural superposition is obtained with the human growth hormone receptor (14), but only six out of seven strands are present. Many chitinases and cellulases show either an immunoglobulin β-type sandwich or the related FNIII motif.

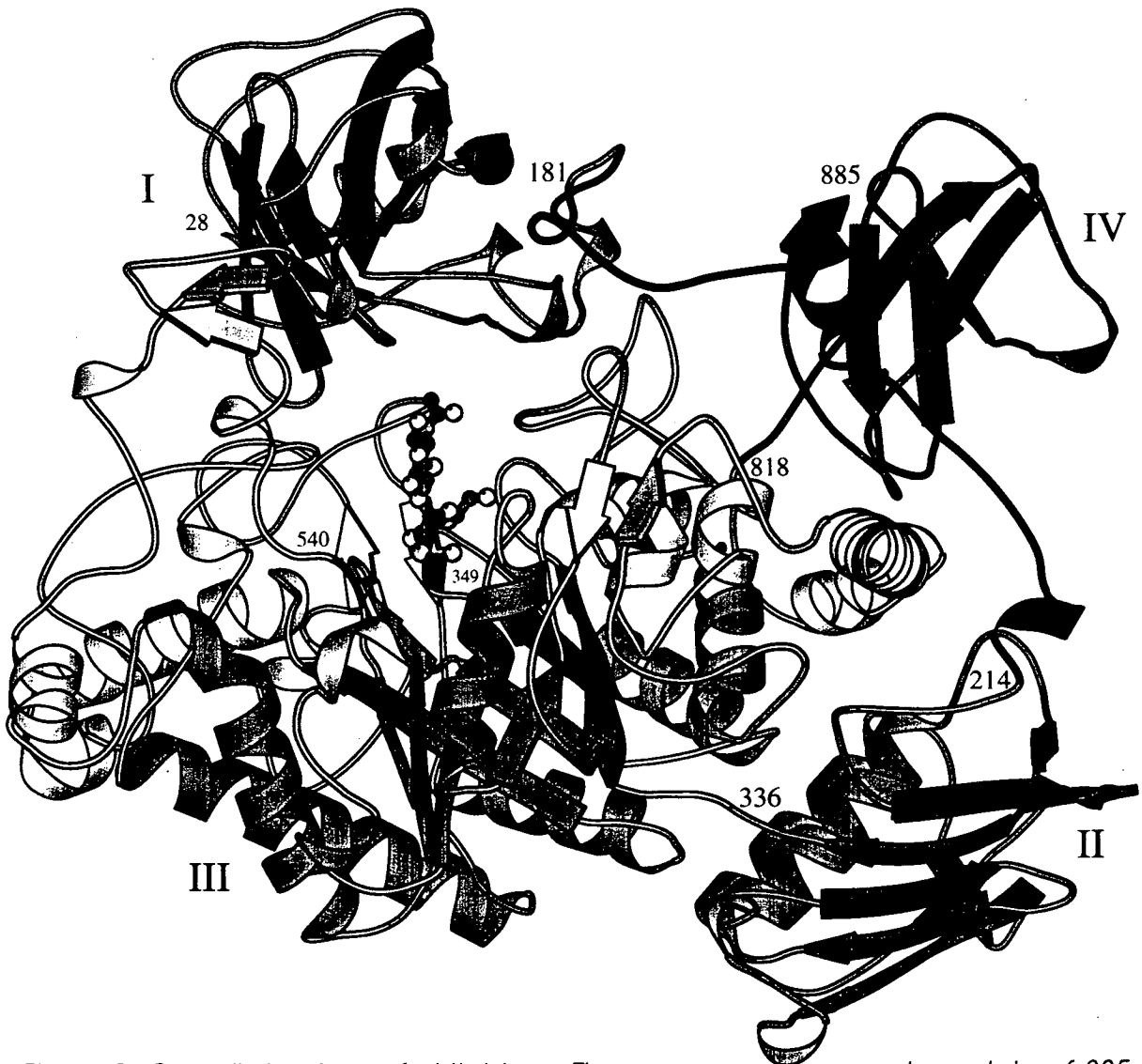


Figure 1: *Overall structure of chitobiase. The enzyme, a monomeric protein of 885 amino acids, is shown in complex with the natural substrate chitobiose. The binding pocket is open to the viewer. Arabic numerasl indicate amino acid positions, roman numerals indicate domains. The first amino acid is numbered 28 as a leader peptide is cleaved off the mature protein.*

Domain II, 122 amino acids, has an $\alpha+\beta$ topology with similarities to the *Serratia* metalloprotease serralysin (15). The exposed sheet and the parallel organisation of the buried helices represents a novel feature of protein organisation.

Domain III is the catalytic domain and the central feature of the structure. A 483 amino acid long segment folds into the classical $\beta\alpha$-barrel motif. Eight β-strands form a barrel surrounded by seven α-helices, one helix is absent ($\alpha 7$ in the common notation). Long loops extend from the C-terminal end of the barrel and interact with domain I. The active site identified by substrate binding lies on this end of the barrel.

Figure 2 shows schematic views of the active site of the complexes of N-acetylglucosaminidase with the substrate chitobiose, the substrate analogue glucose and the synthetic inhibitor LOGNAC (2-acetamido-2-deoxy-D-gluconhydroximo-1,5-lactone, 16). The schemes show differences in binding. The residues highlighted in the scheme are identified as being critical in substrate binding and generally conserved among N-acetylglucosaminidases. Differences are explained in respect to the chitobiose complex.

Figure 3 presents the binding of chitobiose in detail. Individual figures show single amino acids in side-by-side stereo to explain their individual contribution in substrate binding and in the proposed reaction mechanism. Electron density maps are shown, the stick representations of the molecule come from the refined enzyme substrate complex structure.

Chitobiose, the natural substrate of the enzyme, is not digested to N-acetylglucosamine in the substrate enzy me complex. The chitobiose site is fully occupied and highly ordered. The conditions of the crystallisation buffer may present unfavourable conditions for the reaction to occur. The low pH of 5.6 may alter the protonation state of the active site. More likely, the high salt concentration, i.e. the limited availability of water, may inhibit hydrolysis. Restricted flexibility in the crystal lattice may be another factor.

Figure 2: Enzyme-substrate and -inhibitor complexes.

Chitobiose. *The substrate 'docks' to Arg349. The catalytic residue Glu540 binds to the glycosidic linkage. Various direct hydrogen bridges are formed with the non-reducing sugar ring which is in the sofa conformation, while the reducing sugar residue is in the chair conformation.*

LOGNAC. *Binding is similar to the non-reducing sugar ring of chitobiose. This sugar residue is likewise distorted and takes the unusual sofa conformation. The catalytic Glu540 binds to nitrogen rather than to the glycosidic oxygen. The proximal OH group of the oxime superimposes with the reducing sugar of chitobiose.*

Glucose. *Binding is analogous to LOGNAC, but without distortion of the sugar ring which is in the chair conformation. In comparison to the two other structures, hydrophobic interactions and one hydrogen bond between enzyme and the N-acetyl group are missing. OH1 hydrogen bonds to Tyr669.*

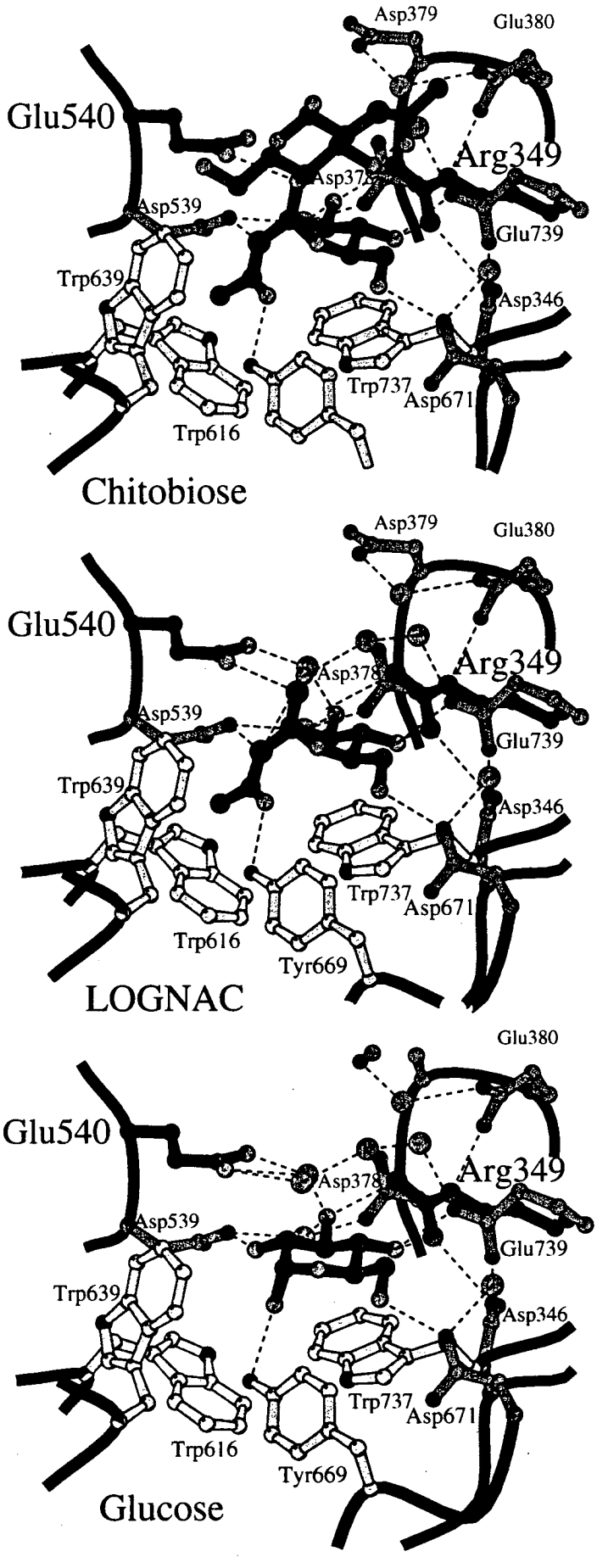


Figure 3: the enzyme substrate complex.

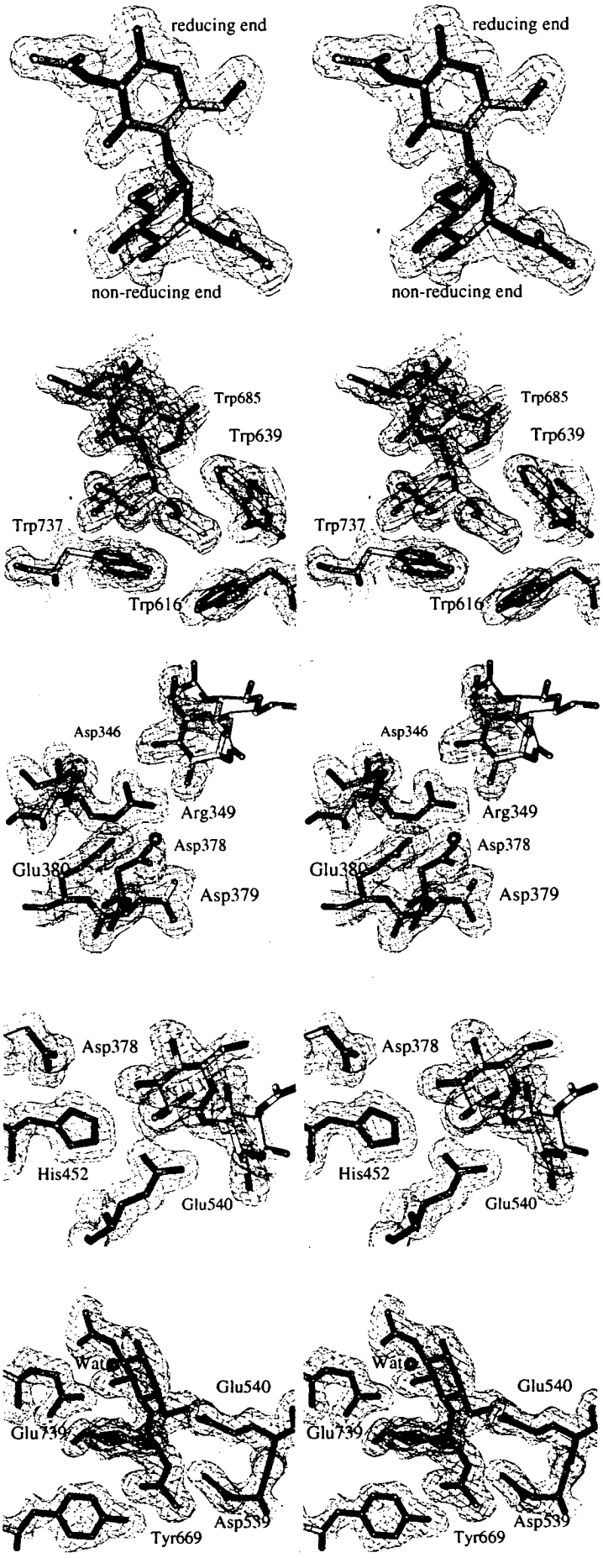
The sugar residues of chitobiose are in the chair (reducing end) and in the sofa conformation (non-reducing end). The H-bond O5—OH3 is disrupted.

The sugar residues are stacked against Trp685 and Trp737. Trp616 and Trp639 stabilise the essentially planar N-acetyl group of the non-reducing sugar residue.

Arg349 binds to OH3 and OH4 of the non-reducing sugar ring to anchor the saccharide. Hydrogen bonds to Asp346, Asp378, Asp379, Glu380 and a water stabilise Arg349.

The catalytic residue Glu540 forms a hydrogen bond to the glycosidic linkage. The second carboxygen binds to His452 which in return binds to Asp378.

A water hydrogen bonded between OH5 and Glu739 is a candidate for the hydrolysis of the glycosidic bond. The intermediate is stabilised by the N-acetyl group, which is hydrogen bonded to Asp539 and Tyr669 and packed against Trp639 and Trp616.



Discussion. The reaction mechanism of lysozyme (17) often serves as an example for hydrolysis of glycosides. Two carboxyl groups act in general acid-base catalysis. The first group is protonated and uncharged and acts as proton donor to the glycosidic oxygen. The second group, charged, stabilises the intermediate.

In general, two different mechanisms are known as classified by the stereospecific formation of product: a reaction with retention and a reaction with inversion of the anomeric configuration. A characterisation is possible by the distance between the two functional carboxyl groups which is roughly 9 Å to 9.6 Å for inverting and 4.8 Å to 5.4 Å for retaining enzymes. In inverting enzymes the two carboxyl-oxygen are on opposite sides of the bond to place a water in between which is necessary for hydrolysis. No stable intermediate is formed, while retaining reaction modes are characterised by a carbonium ion that has to be stabilised as a water has to diffuse in place of the product.

The proposed reaction mechanism in chitobiase occurs with retention of the anomeric configuration. The protonated carboxyl-group which binds to the glycosidic linkage is identified from the substrate-enzyme complex. This amino acid protonates the glycosidic oxygen, the bond is split and the reducing sugar ring leaves the complex as product.

The general scheme of retaining reaction modes is altered by the way the remaining sugar moiety is stabilised without a second carboxyl-group. The oxygen of the acetyl-group of the non-reducing sugar residue lies 3.0 Å from the anomeric C1 and takes up the role of the second carboxyl. It acts as nucleophile in catalysis. An H-bond with Tyr669 is formed in the complex, and the partial negative charge can stabilise the carbonium ion required for the transition state. A covalent intermediate may well be formed. A water can diffuse in to complete hydrolysis, the second sugar is released. Although there is no direct biochemical evidence, the geometry of the active site strongly supports such a mechanism. This also agrees well with biochemical evidence for the homologous hexosaminidases demonstrating a retaining mechanism and showing involvement of the acetyl group in catalysis (18). Indirect evidence is given here by the complexes with the inhibitor and with glucose. The results underline the importance of the N-acetyl group. It appears that interactions around this group are critical in obtaining the reactive complex with the typical distortion of the sugar moieties.

This result should be related to the known chitinases (5). These enzymes have a groove through which the polysaccharide threads. The unusual geometry of the hevamine active site suggests a similar mechanism with involvement of the acetyl group (7, 19). The complex shown here demonstrates such a finding *in situ* on bound substrate for N-acetylglucosaminidase and is of great importance in understanding chitinase reaction mechanisms.

Conclusion. The active site of N-acetylglucosaminidase was identified using a substrate-enzyme complex. An arginine, at the bottom of a binding pocket, forms two hydrogen bonds to the OH3 and OH4 groups of the non-reducing end of chitobiase. The substrate is distorted towards the transition state by the arrangement of hydrophobic surfaces and hydrogen bonds. The reducing sugar is in the normal chair conformation, while the non-reducing sugar takes the sofa conformation. A comparison with a complex with glucose shows a similar binding mode, the monosaccharide takes the position of the non-reducing ring, however, this sugar is not distorted. This is largely due to the missing interactions which in the substrate complex are formed between the N-acetyl-group of the non-reducing sugar and protein side chains. The unusual conformation allows the N-acetyl group to come close to the anomeric C1 and to play a key role in catalysis. It takes over the role of a nucleophile stabilising the reaction intermediate. The reaction is catalysed by only one protein carboxyl group, which, protonated, binds to the glycosidic oxygen acting in general acid catalysis. This is the first demonstration of such an enzymatic mechanism by structural means.

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