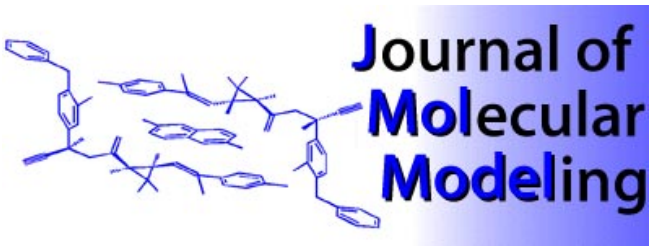
J. Mol. Model. **1997**, *3*, 249 – 260



Molecular Dynamics Simulations on the Complexes of Glucoamylase II (471) from *Aspergillus awamori* var. X100 with 1-Deoxynojirimycin and Lentiginosine

Francesca Cardona[1], Andrea Goti[1], Alberto Brandi*[,1], Maria Scarselli[2], Neri Niccolai[2], and Stefano Mangani*[,3]

[1]Dipartimento di Chimica Organica "U. Schiff", Centro di Studio C. N. R. sulla Chimica e la Struttura dei Composti Eterociclici e loro Applicazioni, Università di Firenze, via G. Capponi 9, I-50121 Firenze, Italy; Fax: +39 55 2476964 (brandi@chimorg.unifi.it)

[2]Dipartimento di Biologia Molecolare, Università di Siena, Italy

[3]Dipartimento di Chimica, Università di Siena, Pian dei Mantellini 44, I-53100 Siena, Italy; Fax: +39 577 280405 (stefano@neriserv.chim.unisi.it)

Received: 28 February 1997 / Accepted: 7 July 1997 / Published: 17 July 1997

Abstract

Molecular dynamics (MD) simulations on the complexes of glucoamylase II (471) from *Aspergillus awamori* var. X100 with two powerful inhibitors, 1-deoxynojirimycin and (+)-lentiginosine, have been performed, in order to build a model for these complexes in solution and to clarify the structure-activity relationship.
MD calculations were carried out for 105 ps, over a 15 Å sphere centered on the inhibitors. A 8 Å residue-based cut-off was used, and the calculations were performed with explicit inclusion of solvent molecules. The MD structure of the complex 1-deoxynojirimycin-glucoamylase shows only minor deviations from the available X-ray structure. The MD structure of the complex of (+)-lentiginosine-glucoamylase, obtained by docking the inhibitor into the active site, suggests us a suitable orientation for the molecule into the enzyme cavity, which can rationalize the high inhibition activity found for (+)-lentiginosine towards amyloglucosidase from *A. niger*.

Keywords: Glycosidase inhibitors, protein-substrate adduct, enzyme cavity, azasugar, polyhydroxylated indolizidines

Introduction

Glycosidases are enzymes which hydrolize the glycosidic bonds in oligo- and polysaccharide chains. Some glycosidases are key enzymes in the biosynthesis and processing of glycoproteins in man and mammals,[1] which accounts for the huge interest recently devoted to the search for inhibitors of this class of enzymes in general, as potential antibacterial, antiviral and antitumoral agents.[1,2]

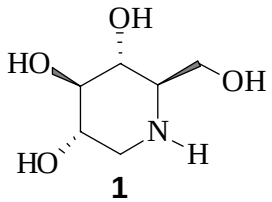
By far, the broadest and most studied class of glycosidases inhibitors is represented by monocyclic azasugars (e.g.

* *To whom correspondence should be addressed*

1-deoxynojirimycin (**1**)) and related natural and unnatural polyhydroxylated indolizidines (e.g. castanospermine (**2**), lentiginosine (**3**)), and pyrrolizidines.[1-3] These compounds are mimics of glycosidases natural substrates.

Recently, we have synthesized (+)-lentiginosine (**3**)[4] and its analogue **9,**[4c,5] which have shown a high and selective biological activity towards Glucoamylase (α-D-1,4-glucan glucohydrolase EC 3.2.1.3), a glycosidase which catalyzes the release of β-D-glucose from the non-reducing ends of starch and other related oligo- and polysaccharides.[6] This enzyme is widely used in industry for the production of fructose sweeteners, ethanol by fermentation, etc.,[7] by conversion of starch to glucose. Glucoamylase cleaves the α-1,4-glucosidic bond preferentially and, at a slower rate, the α-1,6-glucosidic bond,[8] thus allowing the complete hydrolysis of starch.[9] The enzyme also shows a preference for the hydrolysis of maltooligosaccharides with at least 6 residues.[8-10]

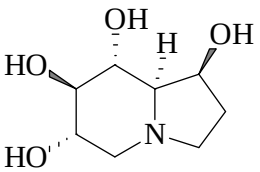
Several species of *Aspergillus* produce a single parent protein (called glucoamylase I) of 615 or 616 amino acid residues.[11] Glucoamylase I has three functional domains: [12] an *N*-terminal catalytic domain of approximately 440 residues, an *O*-glycosylated linker domain of approximately 70 residues and a *C*-terminal starch binding domain of approximately 100 residues. The catalytic domain consists of 13 α-helices,[13] 12 of which are arranged in a polypeptide fold that is a variation on the α/β-barrel.[13a] This fold has been called α/α-barrel[13a] or twisted α-barrel.[14] The



1

1-deoxynojirimycin

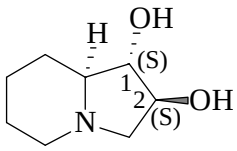
IC_{50}=29 μM [a]
K_i=2 μM [b]



2

castanospermine

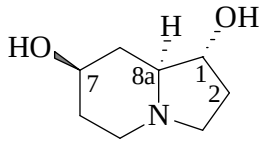
IC_{50}=4.3 μM [c]



3

(+)-lentiginosine

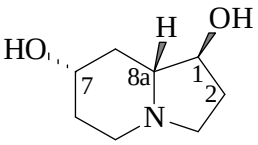
IC_{50}=2.7 μM [c]
K_i=2 μM [c]



4

(1*R*,7*R*,8a*S*)-
1,7-dihydroxyindolizidine

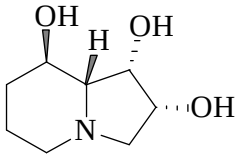
INACTIVE [a]



5

(1*S*,7*S*,8a*R*)-
1,7-dihydroxyindolizidine

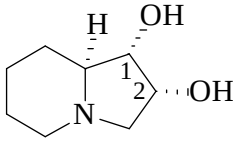
INACTIVE [a]



6

swainsonine

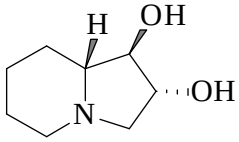
INACTIVE [d]



7

2-epilentiginosine

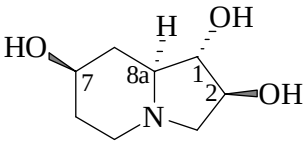
INACTIVE [e]



8

(–)-lentiginosine

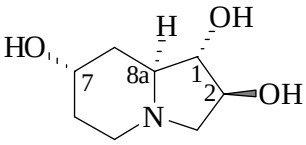
IC_{50}=108 μM [c]



9

(1*S*,2*S*,7*R*,8a*S*)-
1,2,7-trihydroxyindolizidine

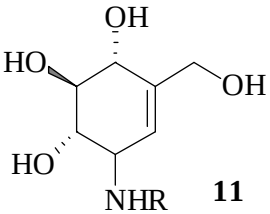
IC_{50}=9.8 μM [a]
K_i=4.4 μM [a]



10

(1*S*,2*S*,7*S*,8a*S*)-
1,2,7-trihydroxyindolizidine

IC_{50}=290 μM [a]
K_i=69 μM [a]



11

acarbose
(R=trisaccharide unit)

IC_{50}=0.06 μM [f]
K_i=10^{-6} μM [b]

Figure 1. *Some examples of* Aspergillus niger *amylogluco-sidases inhibitors. [a] Ref. 5; [b] ref. 20; [c] ref. 4b; [d] ref. 21; [e] ref. 22; [f] ref. 23.*

O-glycosylated domain prevents the protein from thermal denaturation[15] and it may play a role in the movement of the enzyme through the extracellular environment.[16]

Typically the parent protein (glucoamylase I) undergoes limited proteolysis, resulting in the appearance of glucoamylases (often called glucoamylase II) of smaller molecular weight. The shortened forms of glucoamylase I lack the entire starch binding domain and, in some instances, all or part of the *O*-glycosylated domain.[17] Hence, glucoamylase II has catalytic properties identical to those of glucoamylase I, but has lost the ability to digest raw starch.[18] The X-ray structure of the glucoamylase II (471) from *Aspergillus awamori* var. X100 (which is a fragment corresponding to residues 1 to 471 of glucoamylase from *Aspergillus niger*) has been determined,[13a,b] as well as the 1-deoxynojirimycin (**1**)-ligated[13c] and the acarbose (**11**)-ligated complex.[13d]

Like many other glycosidases, glucoamylase is inhibited by sugar analogues having a structure which resembles that of the enzyme's natural substrate, amylose in the present case. Inhibitors of amyloglucosidase have a basic nitrogen[19] generally replacing the cyclic oxygen. They all behave as competitive inhibitors. Some of such compounds tested towards amyloglucosidase from *Aspergillus niger* are listed in Figure 1 with their IC_{50} and/or Ki values. In the case of 1-deoxynojirimycin (**1**) and castanospermine (**2**), the analogy with amylose is apparent, since both molecules have the same absolute configuration of glucose at all the stereogenic carbon atoms.

(+)-Lentiginosine (**3**), recently synthesized in our laboratories,[4] belongs to this class of compounds and, in spite of being the first glycosidase inhibitor bearing only two hydroxy groups, has been shown to be among the most powerful inhibitors towards amyloglucosidase found so far. Moreover, the OH groups of (+)-lentiginosine are located on the 5-membered ring of the indolizidine nucleus rather than on the piperidine portion. For (+)-lentiginosine and related compounds, such as **9** and **10**, the analogy with the natural substrate of the enzyme is not immediate.

On the basis of the simple observation of the structure of molecules such as (+)-lentiginosine and similar compounds (see Figure 1) correlated by biological activity, we proposed a model[5] which compares amylose (the natural substrate), (+)-lentiginosine and 1-deoxynojirimycin as shown in Figure 2.

Indeed, according to this model, not only the basic nitrogen is an absolute requirement for activity, but also the *trans*-dihydroxypyrrolidine unit, and moreover, the *S*,*S* absolute configuration of the two carbon atoms bearing these hydroxy groups seems to be essential. Compounds lacking of hydroxy groups on C(2) such as **4** and **5**[24] are in fact inactive.[5] Furthermore, molecules having a *cis*-dihydroxypyrrolidine unit such as swainsonine (**6**) and 2-epilentiginosine (**7**) are inactive towards amyloglucosidase.[21,22] Finally, (–)-*R*,*R*-lentiginosine (**8**) is 35 times less active then the enantiomeric lentiginosine (**3**).[4b] Our model was also in agreement with the different activities towards amyloglucosidase measured for the two trihydroxyindolizidines **9** and **10**.[5] A validation of this hypothesis on a more solid structural basis was necessary, and a computational study seemed to be the best mean to provide an answer to the problem.

Molecular mechanics and molecular dynamics (MD) techniques[25] are being applied to an increasing variety of drug design problems.[26] Molecular dynamics calculations can provide structural models even for those systems for which the X-ray characterization is not available. These calculations allow also the study of the mobility of residues in the protein and are well suited for the structural analysis of protein-substrate adducts which cannot be crystallized.

We report in this paper computational studies performed on complexes of glucoamylase with inhibitors, namely deoxynojirimycin (**1**) and lentiginosine (**3**), aimed to a better understanding of the structure-activity relationship. This study might allow the design of new more powerful glycosidases inhibitors having the necessary features to fit the enzyme cavity.

To accomplish this goal, we have performed MD calculations on the complex 1-deoxynojirimycin-glucoamylase, starting from the X-ray structure, as well as on the lentiginosine-glucoamylase adduct, obtained by docking the *trans*-fused indolizidine molecule **3** in the cavity according

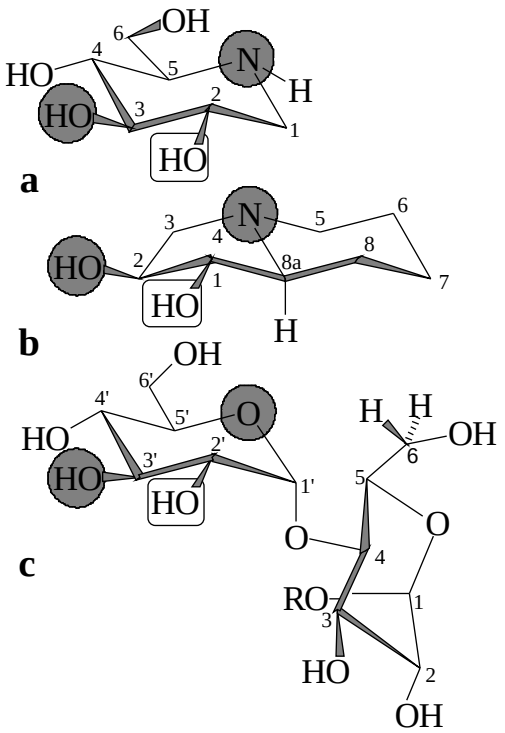


Figure 2. *Fitting between the non-reducing end of amylose (**c**), (+)-lentiginosine (**b**) and 1-deoxynojirimycin (**a**). The superimposed groups are similarly evidenced*

to our initial hypothesis (Figure 2). We were encouraged by the knowledge that glucoamylase II does not undergo global conformational changes upon binding with inhibitors molecules.[13c,d] The dynamic simulation of the complex deoxynojirimycin-glucoamylase allowed us to test the validity of our force field parameters, to refine the structure of this complex in solution and to have an insight about its dynamic behavior. In addition, we could confidently make a dynamic study on the complex lentiginosine-glucoamylase, having a suitable landmark.

Methods

The labels used for the residues are the same as reported by the crystallographers.[13c]

Computational procedure

The simulations were carried out using the SYBYL program (Molecular Modeling Software Package, Version 6.2, Tripos Associates Inc.) running on a Indy Silicon Graphics R4400 workstation.

Enzyme-inhibitor models

Atomic coordinates of the complex of glucoamylase II (471) from *Aspergillus awamori* var. X100 with deoxynojirimycin (2.4 Å resolution) were retrieved from the Protein Data Bank file (entry 1DOG).[13c] Two molecules of deoxynojirimycin are found by the crystallographers into the active cavity. The glycosidic residues and the sugar chains were not included in the model, since they are placed on the surface of glucoamylase and therefore far from the interesting atom set (see below). The hydrogen atoms were added using the BUILD/EDIT module of Sybyl. The endocyclic nitrogen (N5) of 1-deoxynojirimycin in both binding sites was protonated on the basis of the pKa (6.6 in water)[27] of the molecule, which implies that approximately 80% of the inhibitor should be positively charged at physiological pH (pH= 6.0). The local environment of the inhibitor reported in the X-ray structure is unable to solve this point. The whole system was neutralized by the addition of 27 sodium ions far from the active site, avoiding to break salt bridges among residues. A sphere of interesting atoms within a radius of 15 Å was defined, centered on the O6 atom of the deoxynojirimycin molecule in the primary binding site. This subset was surrounded by a 8 Å shell of water molecules with the SOLVENT/SILVERWARE algorithm, which resulted in the addition of 675 water molecules, besides the 605 crystallographic water molecules. The resulting system consisted of about 10800 atoms. The simulations were carried out over approximately 2400 atoms; the remaining atoms have been held as static local sets.

The complex glucoamylase-lentiginosine was constructed placing the indolizidine skeleton according to the previously proposed model,[5] (Figure 2), for both the binding sites. The subset of interesting atoms was the same as defined for deoxynojirimycin. The fitting of the molecules in the cavity was briefly optimized using the COMPUTE/DOCK command of Sybyl. The bridgehead nitrogen atoms were also protonated.

Charges and Force Field Parameters

The standard Kollman "all atoms" force field parameters[28] were used for all the residues, while for the deoxynojirimycin and lentiginosine molecules, which were assigned the charge +1 electrons, charges calculated by MOPAC package (F. J. Seiler Research Laboratory, U. S. Air Force, Colorado Springs, CO) were employed. The PM3 Hamiltonian was used for the SCF calculation. The sodium ions were assigned the charge +1 electrons, van der Waals radius R = 1.87 Å, and well depth ε = 0.0028 Kcal/mol.

Molecular Dynamics Calculations

The same procedure has been used for the complexes glucoamylase-deoxynojirimycin and glucoamylase-lentiginosine. MD simulations were carried out over a 15 Å sphere centered on the inhibitors, using a residue-based cutoff value of 8 Å for the nonbonded interactions (the number of pair interactions during the MD simulations was about 1.3×10^6). The time step of the dynamics was 1.5 fs, and bond lengths were constrained to their equilibrium values using the SHAKE[29] algorithm. A value of dielectric constant of 1 was chosen. The pair list was updated every 10 fs during the heating phase and every 25 fs during the rest of the time. The values of the energies and the coordinates were saved every 200 steps. All the water molecules present in the system and the sodium ions were equilibrated by minimizing

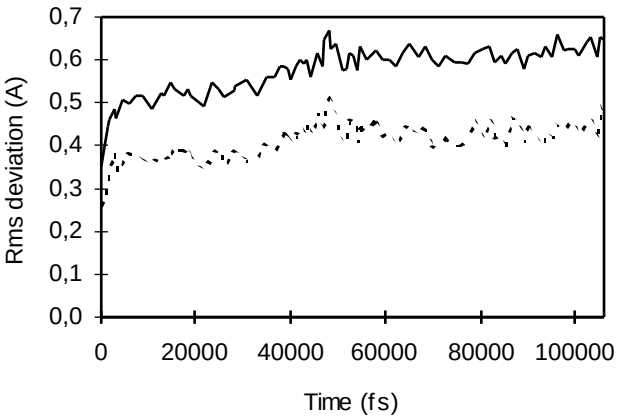


Figure 3. *Root mean square deviation between the instantaneous MD and crystal structures of the complex glucoamylase-deoxynojirimycin for the backbone atoms (dashed line) and for all the atoms (solid line).*

the rms energy gradient within 0.1 Kcal·mol^{-1}·Å^{-1} (20 iterations of simplex method followed by conjugated gradient method proceeded until the convergence condition was met). This minimization was followed by 3 ps of molecular dynamics performed over the water molecules within the sphere of interesting atoms previously defined. To avoid slow temperature shift of the system, it was coupled to a thermal bath[30] at T = 300 K with a relaxation time of 0.2 ps. The whole sphere of interesting atoms was then minimized with the same method and convergence criterion used for the water molecules. Then, molecular dynamics simulations were performed, gradually warming the system using the following procedure: 0-100 K, 1.5 ps; 100-200 K, 1.5 ps; 200-298 K, 1.5 ps. The system was coupled with a thermal bath at 300 K for the remaining 100 ps with a coupling constant of 0.2 ps.

Data Analysis

The coordinates of the first 50 ps were discarded, using the last 55 ps for structural analysis. Averaged structures were calculated using the AVERAGE_MOL command of Sybyl. The averaged structures were fully energy minimized. These structures were taken as models of the enzyme-inhibitor complexes in solution. The rms deviations of the average structure with respect to the X-ray structure or the starting structure (for the complex with lentiginosine) were calculated as $(\Sigma_{i=1}^{n} |\Delta r_i|^2/n)^{1/2}$ where Δr_i is the displacement of an atom in the average MD structure with respect to the X-ray structure, and the sum is performed over all atoms in a residue (deviation per residue), over the backbone atoms or over all atoms (deviation of the entire structure).

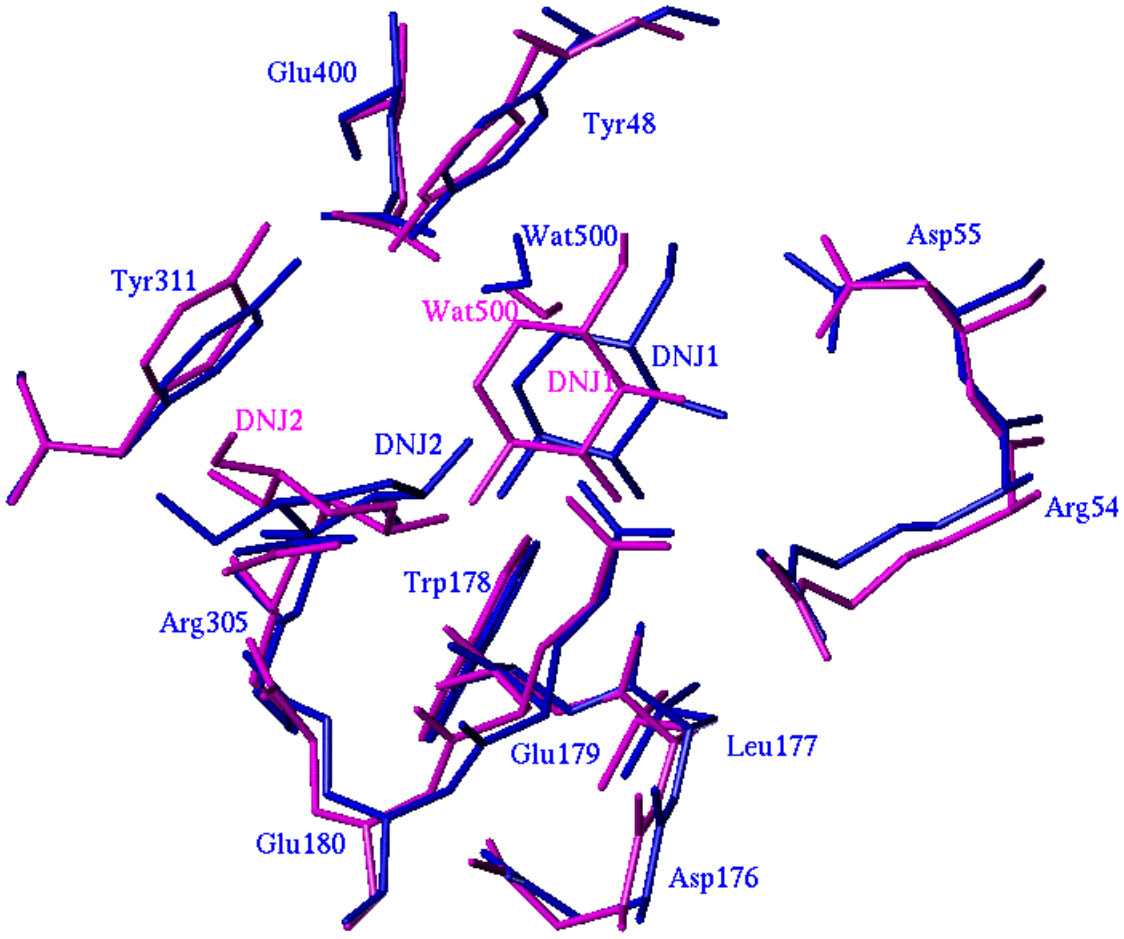


Figure 4. *Comparison between the active site of the X-ray structure (blue) and the MD average structure (magenta), for the complex glucoamylase-deoxynojirimycin. DNJ1 and DNJ2 are inhibitors in the primary and secondary binding sites, respectively.*

Table 1. *Selected relevant distances (Å) in the X-ray, minimized, and MD average structures for deoxynojirimycin at its primary binding site.*

	X-ray structure	minimized	MD averaged
N5-water 500	2.82	2.70	2.76
N5-OH Tyr 48	3.54	2.77	2.95
OH Tyr 48-Oε2 Glu 400	2.49	2.65	2.66
water 500-Oε2 Glu 400	2.18	2.11	2.56
C1-water 500	3.28	3.21	3.36
O6-water 500	2.97	3.69	3.08
O6-Oδ2 Asp 55	2.57	2.70	4.33
O6-Oδ1 Asp 55	3.81	3.38	4.41
C6-Oδ2 Asp 55	3.41	3.52	5.00
O4-Oδ1 Asp 55	2.73	2.69	3.55
O4-Nε Arg 54	3.32	3.98	4.86
O4-NH2 Arg 54	2.91	3.42	3.64
O3-C=O Leu 177	2.65	2.77	2.83
O3-Nε Arg 54	3.27	3.00	4.11
O3-NH2 Arg 54	3.55	2.80	3.62
O3-Cζ3 Trp 417	3.41	3.67	4.79
C3-C=O Leu 177	3.46	3.70	3.99
O2-NH1 Arg 305	3.23	4.11	3.02
O2-C=O Leu 177	3.83	3.41	4.31
O2-C=O Trp 178	3.74	3.54	2.95
O2-Cα Trp 178	3.43	3.40	3.42
O2-O3 (DNJ2) [a]	2.57	3.09	3.59
O2-O2 (DNJ2) [a]	3.85	4.79	3.05

[a] DNJ2 is the 1-deoxynojirimycin molecule at its secondary binding site.

Table 2. *Selected relevant distances (Å) in the X-ray, minimized, and MD average structures for deoxynojirimycin at its secondary binding site.*

	X-ray structure	minimized	MD averaged
O2-Oε1 Glu 179	3.20	2.59	2.79
O2-C1 (DNJ1) [a]	2.92	3.70	3.63
C2-Oε1 Glu 179	3.20	3.36	3.74
O2-C=O Trp 178	4.41	4.59	2.74
O3-C=O Trp 178	2.61	2.78	3.28
C3-C=O Trp 178	3.44	3.59	3.90
O3-NH2 Arg 305	4.19	4.89	3.21
O4-Oε2 Glu 180	2.43	2.64	4.58
O4-Cδ Glu 180	3.39	3.57	4.57
O4-NH1 Arg 305	3.49	3.00	5.05
O4-NH2 Arg 305	3.01	3.03	4.37
C4-Oε2 Glu 180	2.95	3.18	3.82

[a] DNJ1 is the 1-deoxynojirimycin molecule at its primary binding site.

Results and discussion

Complex glucoamylase-deoxynojirimycin

The root mean square deviation between the instantaneous MD and crystal structures of the complex glucoamylase-deoxynojirimycin (DNJ) for the backbone atoms and for all the heavy atoms is reported in Figure 3.

During the final 55 ps, used for the generation of the MD average structure, the system is stable. The rms deviation of the average MD structure with respect to the starting (X-ray) one is 0.57 Å, averaged over all the atoms, and 0.37 Å, averaged over the backbone atoms, while the rms deviation of the minimized structure with respect to the starting one is 0.35 Å, averaged over all the atoms, and 0.26 Å, averaged over the backbone atoms.

In the X-ray crystal structure the active site is located in the packing void of the α/α-barrel,[13c] and two molecules of 1-deoxynojirimycin are found in close proximity to each other in the active cavity. The deeper one (DNJ1) has an unambiguous placement (a strong electron density is present for the whole molecule). A weak electron density present in the upper part of the active cavity has been interpreted as a second inhibitor molecule (DNJ2).[13c] The authors themselves do not exclude alternative orientations of 1-deoxynojirimycin in the secondary binding site. A comparison between the active site of the X-ray structure and the MD average structure is reported in Figure 4.

Nonbonded contacts between glucoamylase and 1-deoxynojirimycin at its primary binding site and at its secondary binding site are listed in Table 1 and 2, respectively, for all the X-ray, minimized and MD average structures.

The strongest hydrogen bonds in the X-ray structure for deoxynojirimycin at its primary binding site are between O6, O3 and O4 of deoxynojirimycin and Oδ2 Asp 55, C=O Leu 177, Oδ1 Asp 55 and NH2 Arg 54 (Table 1). Moreover, deoxynojirimycin at its primary binding site is strongly hydrogen bonded to deoxynojirimycin at its secondary binding site. A water molecule (water 500) has an unambiguous orientation which has been determined in the X-ray structure, [13c] and the water oxygen is directly oriented toward the

C1 atom (the "anomeric" carbon)[31] of the inhibitor (Table 1). After minimization of the enzyme, the position and orientation of this water molecule remains unaltered (Table 1). The protonation of the endocyclic nitrogen (N5) of 1-deoxynojirimycin (see experimental section) brings in close contact N5 and OH Tyr 48 (the latter one as a proton acceptor), which is in turn bonded to Oε2 of Glu 400, acting, of course, as a proton donor. Thus, the minimization of the system forms a square-hydrogen bonding network (involving Oε2 of Glu 400, water 500, N5 of deoxynojirimycin and OH of Tyr 48), which keeps the amino moiety anchored in its position (Figure 5).

As regards to the other interactions involving the hydroxy groups of deoxynojirimycin at its principal site, is noteworthy that O2 looses its hydrogen bond to NH1 of Arg 305 while approaching to C=O of Leu 177 and C=O of Trp 178; at the same time hydroxy group on C3 reinforces its interactions with NH2 of Arg 54 and with Nε of Arg 54, while keeping a close contact to C=O Leu 177 (Table 1). This small shift, which involves deoxynojirimycin at its principal site, causes a weakening of the interaction with deoxynojirimycin at its secondary site (Table 1). Interactions involving deoxynojirimycin in the secondary binding site and its nearest residues remain essentially the same after minimization with respect to the X-ray structure (see Table 2).

Along all the MD simulation, the extensive H-bond network involving N5 of deoxynojirimycin, besides Glu 400, Tyr 48 and water 500 is strong enough to be maintained, despite the absence of any constraint imposed on the water molecule. Water 500 remains in close contact to N5 of deoxynojirimycin and to Glu 400 (Figure 6a, Table 1) during all the simulation, maintaining the same orientation. Thus, the role of water 500 as the nucleophile of a general base-catalyzed mechanism in which Glu 400 is the catalytic base has been confirmed, as well as the essential role for inhibition played by the amino moiety, anchored by the square-hydrogen bonding network which involves also OH group of Tyr 48 (see also Figure 6b).

While remaining anchored to water 500, deoxynojirimycin experiences a slight shift during the simulation which weakens its interactions with Asp 55 and Arg 54. The hydrogen bond which remains strong and stable during all the simulation is the one between O3 and C=O Leu 177 (2.83 Å is the distance in the MD average structure, see Table 1 and Figure 6c); O2 also experiences an approach to C=O Trp 178 during the dynamic run (Table 1, Figure 6c).

In conclusion, 1-deoxynojirimycin in its primary binding site experiences small fluctuations into the enzyme cavity, the fit of this molecule remaining essentially the one detected in the X-ray structure. On the contrary, the fit of deoxynojiri-

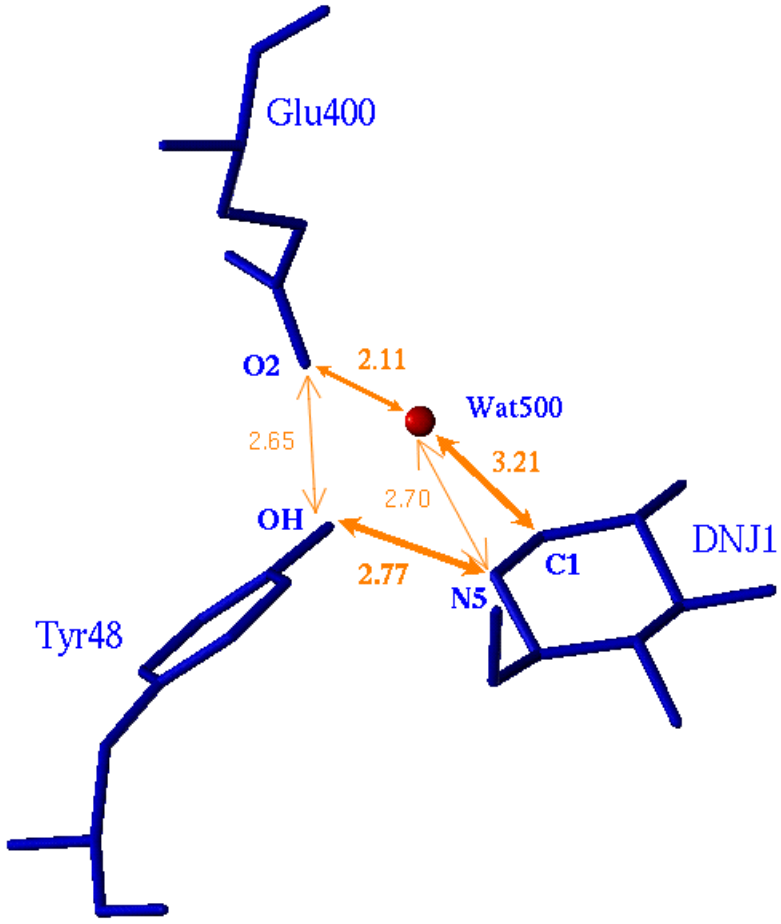


Figure 5. *The square-hydrogen bonding network which is formed after minimization of the complex glucoamylase-deoxynojirimycin. A nonbonded van der Waals contact is present between C1 of the inhibitor and water 500. DNJ1 is the 1-deoxynojirimycin at its primary binding site.*

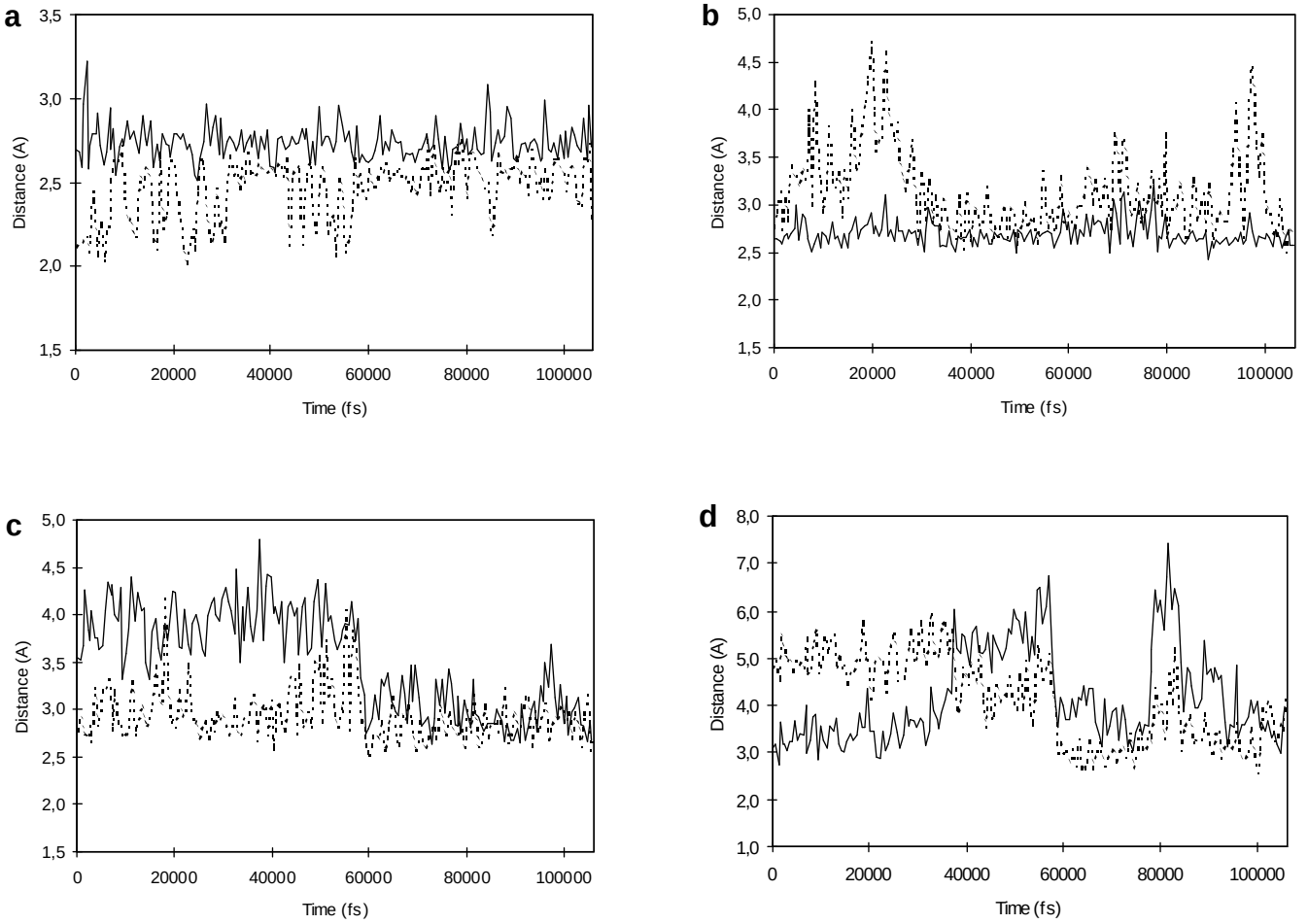


Figure 6. *Fluctuations of selected distances during the MD simulation for the complex glucoamylase-deoxynojirimycin. (a) N5-water 500 (solid line) and water 500-Oε2 Glu 400 (dashed line), (b) OH Tyr 48-Oε2 Glu 400 (solid line) and N5-OH Tyr 48 (dashed line), (c) O2-C=O Trp 178 (solid line) and O3-C=O Leu 177 (dashed line) and (d) O2-O3 DNJ2 (solid line) and O2-O2 DNJ2 (dashed line). DNJ2 is the 1-deoxynojirimycin molecule at its secondary binding site.*

mycin in its secondary binding site is different from that of the X-ray structure, particularly at hydroxy groups on C4 and C6, albeit strong interactions with residues and with inhibitor in the primary site still remain (Table 2). This result was not unexpected, since the authors themselves[13c] had asserted that what reported in the X-ray structure might not reflect the true binding of substrate at the second subsite. Deoxynojirimycin in the secondary binding site still interacts with the inhibitor molecule at its primary site, but there is a mutable interaction which involves, in turn, hydroxy groups on C2 and C3 of the second molecule of deoxynojirimycin to the hydroxy group on C2 of the inhibitor in the primary site (Figure 6d).

Complex glucoamylase-(+)lentiginosine

The root mean square deviation between the instantaneous MD and the starting structures of the complex glucoamylase-(+)lentiginosine (LENT) for the backbone atoms and for all the heavy atoms is reported in Figure 7.

Also in this case, similarly to the complex glucoamylase-deoxynojirimycin, the system is equilibrated during the final 55 ps, which were used for the generation of the MD average structure. The rms deviation of the average MD structure with respect to the starting one is 0.64 Å, averaged over all the atoms, and 0.40 Å, averaged over the backbone atoms, while the rms deviation of the minimized structure with respect to the starting one is 0.35 Å, averaged over all the atoms, and 0.24 Å, averaged over the backbone atoms.

Two molecules of (+)-lentiginosine were initially docked into the enzyme cavity according to our working hypothesis,[5] as shown in Figure 2, trying to superimpose, as far as possible, N4 of lentiginosine to N5 of deoxynojirimycin, and O1 and O2 of lentiginosine to O2 and O3 of deoxynojirimycin. N4 resulted at a distance of 2.87 Å from water 500. However, placed in this position, lentiginosine suffers bad van

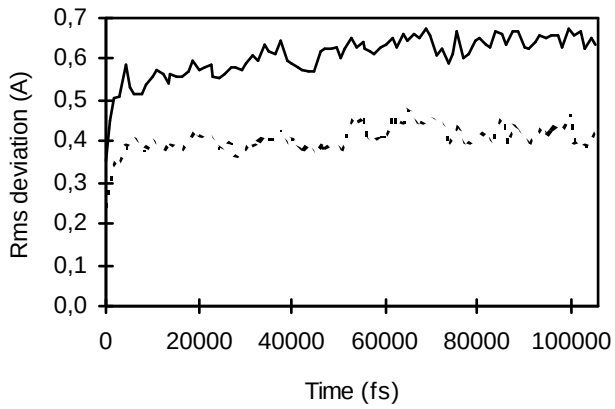


Figure 7. *Root mean square deviation between the instantaneous MD and starting structures of the complex glucoamylase-(+)lentiginosine for the backbone atoms (dashed line) and for all the atoms (solid line).*

der Waals contacts with Tyr 48. Moreover, no significant hydrogen bonds can be formed between O2 of lentiginosine and C=O Leu 177 (4.84 Å) or between O2 and Nε of Arg 54 (4.81 Å). As a matter of fact, hydroxy group on C2 cannot form hydrogen bonds with any of the near residues, while O1 of lentiginosine is strongly hydrogen bonded to NH1 of Arg 305 (2.91 Å). This fact is due to the rigidity of the bicyclic *trans*-fused molecule inhibitor. Indeed, in the initial position of lentiginosine, it is not possible to avoid bad v. d. W. contacts while maintaining hydrogen bonds for both the hydroxy groups on lentiginosine. In other words, there is no room for lentiginosine in this position. After minimization of the system, the v. d. W. contacts can be optimized to reasonable values, but, while the distance O1-NH1 Arg 305 decreases a little bit (2.84 Å), no significant hydrogen bond can be attained which can involve hydroxy group on C2. N4 of lentiginosine remains in close contact to water 500, which is in tum strongly bonded to Oε2 of Glu 400 (Table 3).

During the dynamic run, the molecule of lentiginosine in the secondary binding site goes away from the cavity towards

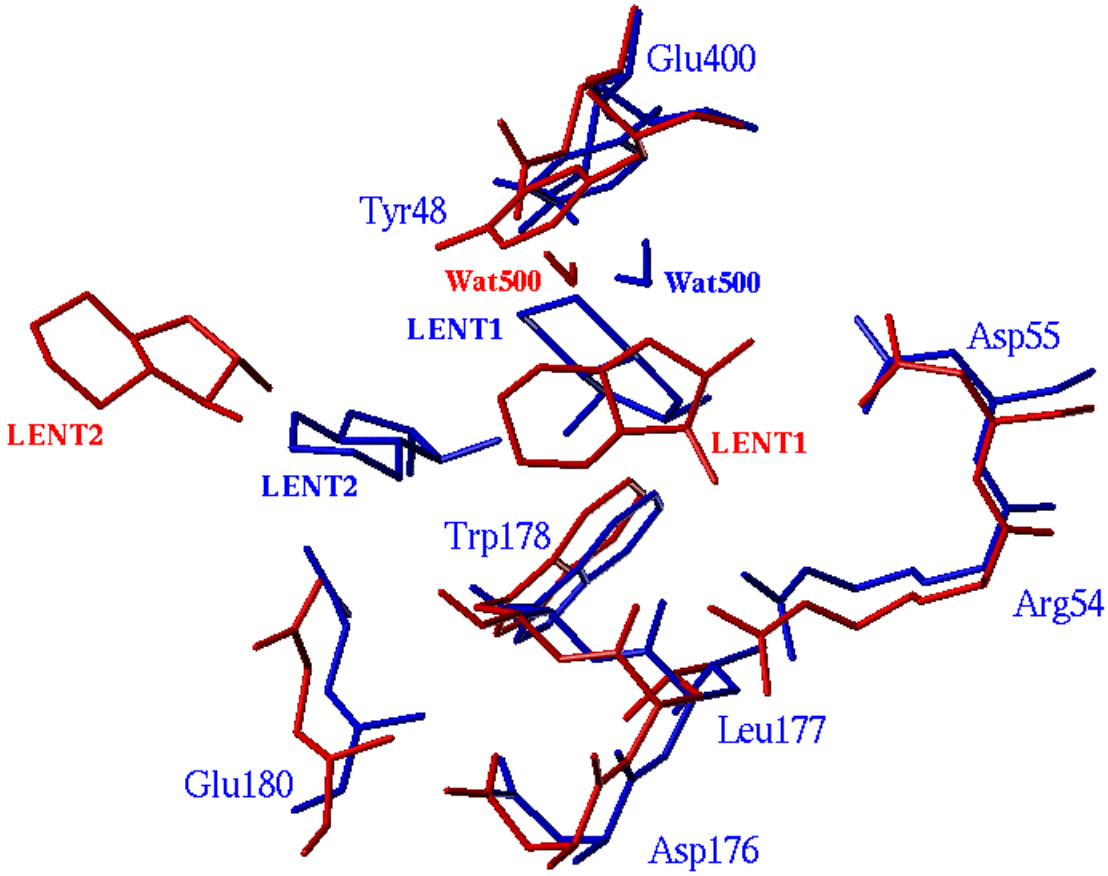


Figure 8. *Comparison between the active site of the starting structure (blue) and the MD average structure (red) for the complex glucoamylase-lentiginosine. LENT1 and LENT2 are inhibitors in the primary and secondary binding sites, respectively.*

Table 3. *Selected relevant distances (Å) in the minimized and MD average structures for lentiginosine in the active site.*

	Minimized structure	MD averaged
O2-Oδ2 Asp 55	5.92	3.22
O2-Oδ1 Asp 55	5.48	2.93
O1-Nε Arg 54	7.29	3.57
O1-NH2 Arg 54	6.92	2.88
N4-water 500	2.70	2.64
water 500-Oε2 Glu 400	2.14	2.16
Oε2 Glu 400-OH Tyr 48	3.28	4.29
N4-OH Tyr 48	5.53	4.84

the solvent (Figure 8) losing all its nonbonded interactions with nearest residues. Thus, for lentiginosine, a molecule in a secondary binding site (LENT2) is not relevant.

Lentiginosine in the primary binding site (LENT1) experiences a large rotation, which occurs approximately after 30-40 ps. This wide displacement leads O1 and O2 of lentiginosine in close contact with Arg 54 and Asp 55, respectively (Figure 9a). Thus, in the MD average structure, two strong hydrogen bonds can be formed between the only two hydroxy groups on lentiginosine and the sidechains of Arg 54 and Asp 55. Indeed, small values are found for the distances O2-Oδ1 Asp 55 and O1-NH2 Arg 54 in the MD average structure (Table 3), in contrast to the minimized structure. In this context, it is remarkable that mutation of Arg 54 to Lys or Thr and of Asp 55 to Asn or Tyr led to a complete loss of enzymatic activity.[32]

Despite the extensive movement experienced by the molecule in the dynamic run, the bridgehead nitrogen atom remains anchored to the nucleophile water 500, due to the strong hydrogen bond interaction (Figure 9b). Even in this case, despite the lack of any constraint imposed on the water molecule, the field created by the protonation of the inhibitor is strong enough to create a hydrogen bonding network, which increases the nucleophilic power of water 500. Water 500 is strongly bonded to Oε2 of Glu 400 (2.16 Å is the distance in the MD average structure) and remains in the same orientation during all the dynamic run (Figure 9b), being close to N4 and to C5 of inhibitor molecule (Table 3). This fact is a further corroboration of the crucial requirement of the basic amino moiety in inhibitor molecules. In this case, the lack of a second proton on nitrogen atom prevents the formation of a square-hydrogen bonding network involving Tyr 48.

In Figure 10 a superimposition between the MD average structures (a view of the active site) of the complex glucoamylase-deoxynojirimycin and the complex lentiginosine-ligated is reported. Fitting of O1 and O2 of (+)-lentiginosine with O4 and O6 of deoxynojirimycin appears to be a most sounding model than the initially chosen. The present study suggests that formation of strong H-bonds with Arg 54 and Asp 55 is essential for inhibition activity towards glucoamylase.

Conclusion

In conclusion, during the dynamic run, lentiginosine finds its optimal placement within the enzyme cavity by forming two strong hydrogen bonds with Arg 54 and Asp 55, without losing the bond with water 500 and accommodating the six-membered ring without severe van der Waals nonbonded interactions.

Our findings, therefore, rationalize the high inhibition activity of (+)-lentiginosine towards amyloglucosidase, as a

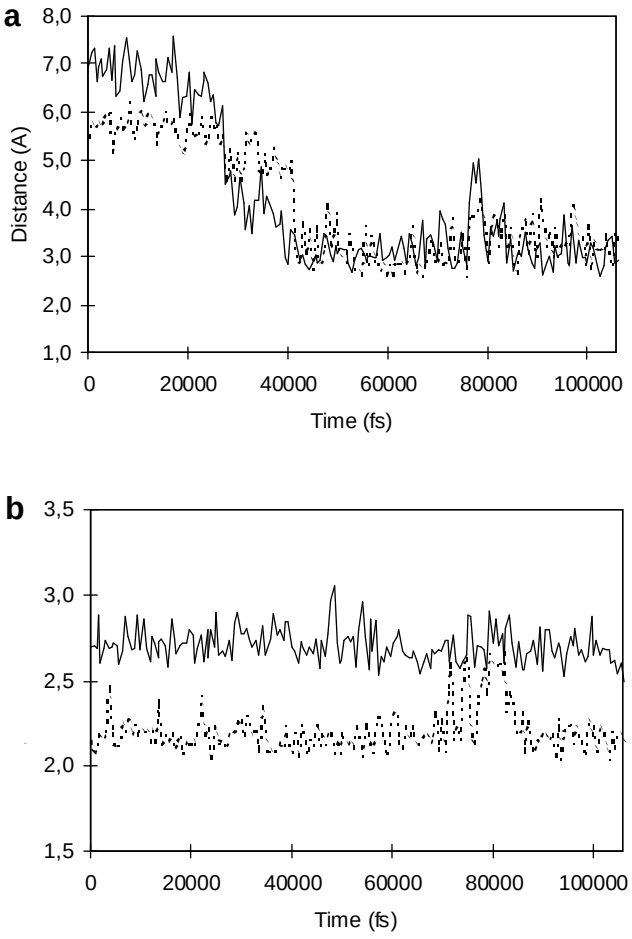


Figure 9. *Fluctuations of selected distances during the MD simulation for the complex glucoamylase-lentiginosine. (a) O1-NH2 Arg 54 (solid line) and O2-Oδ1 Asp 55 (dashed line) and (b) N4-water 500 (solid line) and water 500-Oε2 Glu 400 (dashed line).*

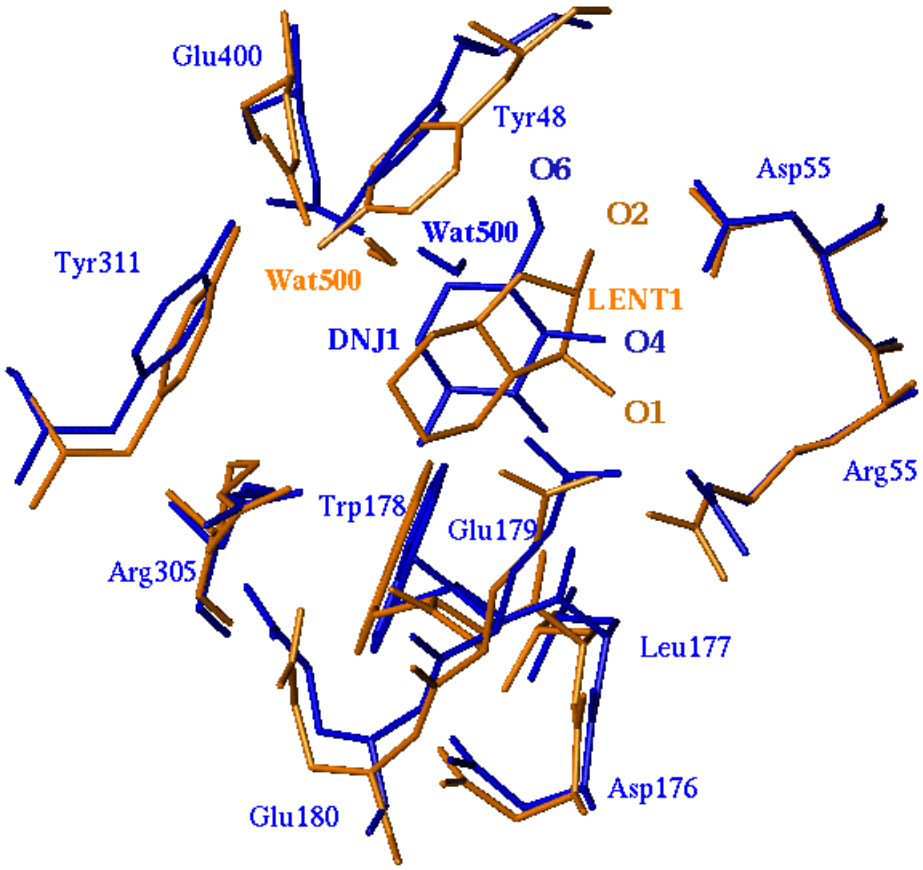


Figure 10. *Comparison between the complex glucoamylase-deoxynojirimycin MD average structure (blue) and the complex glucoamylase-lentiginosine MD average structure (orange). Only a view of the active site is reported. DNJ1 and LENT1 are the inhibitors in their primary binding site.*

result of the selective interaction of lentiginosine's hydroxy groups towards the enzyme's key residues for bioactivity. For this reason, (+)-lentiginosine might represent the prototype of a new class of low hydroxylated inhibitors. The present study provides a deeper knowledge of the mechanism of the interaction of the glycosidase inhibitors with the enzyme, and allows the design of new, hopefully more potent, inhibitors related to (+)-lentiginosine.

Acknowledgments: We deeply thank Dr. Paolo Carloni (Università di Firenze) for precious suggestions and illuminating discussions. We thank also the Ministry of University and Scientific and Technological Research (M. U. R. S. T. 60%/40%), Italy and the C. N. R. (National Research Council), Italy, for financial support.

References

1. Reviews: (a) Elbein, A. D. *Annu. Rev. Biochem.* **1987**, *56*, 497-534. (b) Vogel, P. *Chimica Oggi* **1992**, *10*, 9-15. (c) Legler, G. *Adv. Carbohydr. Chem. Biochem.* **1990**, *48*, 319-384.
2. Antibiotics: Ishida, N.; Kumagai, K.; Niida, T.; Tsuruoka, T.; Yumoto, H. *J. Antibiot.*, Ser A **1967**, *20*, 66-71. Anti-HIV: Sunkara, P. S.; Kang, M. S.; Bowlin, T. L.; Liu, P. S.; Tyms, A. S.; Sjoerdsma, A. *Ann. N. Y. Acad. Sci.* **1990**, *616*, 90-96. Anticancer: Dennis, J. W.; Koch, K.; Beckner, D. *J. Nat. Cancer Inst.* **1989**, *81*, 1028-1033.
3. (a) Elbein, A. D.; Molyneux, R. J. *Alkaloids: Chem. Biol. Perspect.* **1987**, *5*, 1-56. (b) Howard, A. S.; Michael, J. P. *Alkaloids* **1986**, *28*, 183-308. (c) Picasso, S.; Chen, Y.; Vogel, P. *Carbohydr. Lett.* **1994**, *1*, 1-8.
4. (a) Cordero, F. M.; Cicchi, S.; Goti, A.; Brandi, A. *Tetrahedron Lett.* **1994**, *35*, 949-952. (b) Brandi, A.; Cicchi, S.; Cordero, F. M.; Frignoli, R.; Goti, A.; Picasso, S.; Vogel, P. *J. Org. Chem.* **1995**, *60*, 6806-6812. (c) Goti, A.; Cardona, F.; Brandi, A. *Synlett* **1996**, 761-763.
5. Goti, A.; Cardona, F.; Brandi, A.; Picasso, S.; Vogel, P. *Tetrahedron: Asimmetry* **1996**, *7*, 1659-1674.

6. (a) Weill, C. E.; Burch, R. J.; Van Dyk, J. W. *Cereal Chem.* **1954**, *31*, 150-158. (b) Manjunath, P.; Shenoy, B. C.; Raghavendra Rao, M. R. *J. Appl. Biochem.* **1983**, *5*, 235-260.
7. Saha, B. C.; Zeikus, J. G. *Starch* **1989**, *41*, 57-64.
8. Abdullah, M.; Fleming, I. D.; Taylor, P. M.; Whelan, W. J. *Biochem. J.* **1963**, *89*, 35-36.
9. Takahashi, T.; Kato, K.; Ikegami, Y.; Irie, M. *J. Biochem.* **1985**, *98*, 663-671.
10. Meagher, M. M.; Nikolov, Z. L.; Reilly, P. J. *Biotechnol. Bioeng.* **1989**, *34*, 681-688.
11. (a) Boel, E.; Hjort, I.; Svensson, B.; Norris, K. E.; Fiil, N. P. *EMBO J.* **1984**, *3*, 1097-1102. (b) Nunberg, J. H.; Meade, J. H.; Cole, G.; Lawyer, E. C.; McCabe, P.; Schweickart, V.; Tal, R.; Wittman, V. P.; Flatgaard, J. E.; Innis, M. A. *Mol. Cell. Biol.* **1984**, *4*, 2306-2315.
12. (a) Svensson, B.; Larsen, K.; Svendsen, I.; Boel, E. *Carlsberg Res. Commun.* **1983**, *48*, 529-544. (b) Svensson, B.; Jesperson, H.; Sierks, M.; MacGregor, E. A. *Biochem. J.* **1989**, *264*, 309-311.
13. (a) Aleshin, A.; Golubev, A.; Firsov, L.; Honzatko, R. B. *J. Biol. Chem.* **1992**, *267*, 19291-19298. (b) Aleshin, A.; Hoffman, C.; Firsov, L.; Honzatko, R. B. *J. Mol. Biol.* **1994**, *238*, 575-591. (c) Harris, E. M. S.; Aleshin, A. E.; Firsov, L. M.; Honzatko, R. B. *Biochemistry* **1993**, *32*, 1618-1626. (d) Aleshin, A.; Firsov, L.; Honzatko, R. B. *J. Biol. Chem.* **1994**, *269*, 15631-15639.
14. Juy, M.; Amit, A. G.; Alzari, P. M.; Poljak, R. J.; Claeyssens, M.; Béguin, P.; Aubert, J. -P. *Nature* **1992**, *357*, 89-91.
15. Takegawa, K.; Inami, M.; Yamamoto, K.; Kumagai, H.; Tochikura, T.; Mikami, B.; Morita, Y. *Biochim. Biophys. Acta* **1988**, *955*, 187-193.
16. Yamashita, I. *Agric. Biol. Chem.* **1989**, *53*, 483-489.
17. (a) Svensson, B.; Larsen, K.; Gunnarsson, A. *Eur. J. Biochem.* **1986**, *154*, 497-502. (b) Hayashida, S.; Nakahara, K.; Kuroda, K.; Miyata, T.; Iwanaga, S. *Agric. Biol. Chem.* **1989**, *53*, 135-141. (c) Hayashida, S.; Nakahara, K.; Kanlayakrit, W.; Hara, T.; Teramoto, Y. *Agric. Biol. Chem.* **1989**, *53*, 143-149.
18. (a) Svensson, B.; Pedersen, T. G.; Svendsen, I.; Sakai, T.; Ottesen, M. *Carlsberg Res. Commun.* **1982**, *47*, 55-69. (b) Dalmia, B. K; Nikolov, Z. L. *Enzyme Microb. Technol.* **1991**, *13*, 982-990. (c)Williamson, G.; Belshaw, N. J.; Williamson, M. P. *Biochem. J.* **1992**, *282*, 423-428. (d) Belshaw, N. J.; Williamson, G. *FEBS Lett.* **1990**, *269*, 350-353. (e) Belshaw, N. J.; Williamson, G. *Biochim. Biophys. Acta* **1991**, *1078*, 117-120.
19. (a) Bock, K.; Sigurskjold, B. W. *Stud. Nat. Prod. Chem.* **1990**, *7*, 29-86. (b) Wong, C. -H.; Provencher, L.; Porco, J. A., Jr.; Jung, S. -H.; Wang, Y. -F.; Chen, L.; Wang, R.; Steensma, D. H. *J. Org. Chem.* **1995**, *60*, 1492-1501.
20. Svensson, B.; Sierks, M. R. *Carbohydr. Res.* **1992,** *227*, 29-44.
21. Tadano, K.; Morita, M.; Hotta, Y.; Ogawa, S.; Winchester, B.; Cenci di Bello, I. *J. Org. Chem.* **1988**, *53*, 5209-5215.
22. Pastuszak, I.; Molineux, R. J.; James, L. F.; Elbein, A. D. *Biochemistry* **1990**, *29*, 1886-1891.
23. Truscheit, E.; Frommer, W.; Junge, B.; Müller, L.; Schmidt, D. D.; Wingender, W. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 744-761.
24. Cicchi, S.; Goti, A.; Brandi, A. *J. Org. Chem.* **1995**, *60*, 4743-4748.
25. (a) Mc Cammon, J. A.; Harvey, S. C. *Dynamics of Proteins and Nucleic Acids*; Cambridge University Press: Cambridge **1987**. (b) Brooks, C. L., III; Karplus, M.; Pettitt, B. M. *Proteins: A perspective of Dynamics, Structure, Thermodynamics*; J. Wiley and Sons: New York, **1988**.
26. (a) Wasserman, Z. R.; Hodge, N. *Proteins* **1996**, *24*, 227-237. (b) Banci, L.; Schröder, S.; Kollman, P. A. *Proteins* **1992**, *13*, 288-305. (c) Banci, L.; Carloni, P.; Gori, G. *Biochemistry* **1994**, *33*, 12356-12366.
27. (a) Inouye, S.; Tsuruoka, T.; Niida, T. *J. Antibiot., Ser A* **1966**, *19*, 288-292. (b) Inouye, S.; Tsuruoka, T.; Ito, T.; Niida, T. *Tetrahedron* **1968**, *24*, 2125-2144.
28. Weiner, S. J.; Kollman, P. A.; Case, D. A.; Singh, U. C.; Ghio, C.; Alagona, G.; Profeta, S.; Jr. *J. Am. Chem. Soc.* **1984**, *106*, 765-784.
29. van Gunsteren, W. F.; Berendsen, H. J. C. *Mol. Phys.* **1979**, *34*, 1311-1327.
30. Berendsen, H. J. C.; Postma, J. P. M.; van Gunsteren, W. F.; DiNola, A.; Haak, J. R. *J. Chem. Phys.* **1984**, *81*, 3684-3690.
31. (a) Banait, N. S.; Jenks, W. P. *J. Am. Chem. Soc.* **1991**, *113*, 7951-7958. (b) Banait, N. S.; Jenks, W. P. *J. Am. Chem. Soc.* **1991**, *113*, 7958-7963. (c) Firsov, L. *Biokhimiya* **1978**, *43*, 2222-2232. (d) Matsui, H.; Blanchard, J. S.; Brewer, C. F.; Hehre, E. J. *J. Biol. Chem.* **1989**, *264*, 8714-8716.
32. Itoh, T.; Sakata, Y.; Akada, R.; Nimi, O.; Yamashita, I. *Agric. Biol. Chem.* **1989**, *52*, 3159-3167.