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GLYCOSYLATION OF PROTEINS: A COMPUTER BASED METHOD FOR THE RAPID EXPLORATION OF COMFORMATIONAL SPACE OF N-GLYCANS

A BOHNE, C.-W. VON DER LIETH
DKFZ (German Cancer Research Centre) , Molecular Modelling, INF 280, 69120 Heidelberg, Germany, {a.bohne,w.vonderlieth}@dkfz.de

Inspection of protein databases suggests that as many as 70% of proteins have potential N-glycosylation sites. Unfortunately glycoproteins often refuse to crystallize and NMR techniques do not allow an unambiguous determination of the complete conformation of the sugar part. Therefore, time-consuming complex simulation methods are often used to explore the conformational space of N-glycans. The generation of a comprehensive data base describing the conformational space of larger fragments of N-glycans taking into account the effects of branching is presented. High-temperature molecular dynamics simulations of essential N-glycan fragments are performed until conformational equilibrium has been reached. Free energy landscapes are calculated for each glycosidic linkage. All possible conformations for each N-glycan fragment are automatically assigned, ranked according to their relative population and stored in a database. These values are recalled for the generation of a complete set of all possible conformations for a given N-glycan topology. The constructed conformations are ranked according to their energy content. Since this approach allows to explore the complete conformational space of a given N-glycan within a few minutes of CPU-time on a standard PC, it is well suited to be used as a Web-Based application.

1 Introduction

Glycosylation is one of the most abundant forms of covalent protein and lipid modification. [1] There are two main types of protein glycosylation: N-glycosylation, in which the oligosaccharide is attached to an asparagine residue, and O-glycosylation, in which the oligosaccharide is attached to a serine or threonine residue. Glycoproteins usually exist as complex mixtures of glycosylated variants (glycoforms). Glycosylation occurs in the endoplasmic reticulum (ER) and Golgi compartments of the cell and involves a complex series of reactions catalyzed by membrane-bound glycosyltransferases and glycosidases. Many of these enzymes are exquisitely sensitive to other events taking place within the cell in which the glycoprotein is expressed. The populations of sugars attached to an individual protein will therefore depend on the cell type in which the glycoprotein is expressed and on the physiological status of the cell, and may be developmentally and disease regulated [2,3].

Inspection of protein databases suggests that as many as 70% of proteins have potential N-glycosylation sites (sequence motif ASN-X-[SER/THR] where X is not PRO) [4] The biological function of glycosylation is still not completely understood [1,5]. However, it is clear that glycoproteins are fundamental to many biological processes including fertilisation, immune defence, viral replications,

parasitic infection, cell growth, cell-cell adhesion and inflammation. Glycoproteins and glycolipids are major components of the outer surface of mammalian cells. They represent key structures for the interaction of cells with toxins, viruses, bacteria, antibodies and micro-organisms. The spatial structures of glycans provide the driving force for many intermolecular interactions and thus predetermine their function. Their flexibility and dynamics frequently play a key role in biological activity and must be taken into account.

Glycoproteins often refuse to crystallise [6,7]. The conformational flexibility of the glycan antennae at the surface of the protein obviously hampers crystal growth. In cases where glycoproteins do crystallise, the electron density is effected by high thermal motion of the glycan moiety: the detectable electron density is so low that no defined spatial arrangement can be assigned. For example, despite the existence of a well-resolved X-ray crystal structure of the enzyme ribonuclease B, the poor definition of the electron density associated with the oligosaccharide has prohibited any determination of the N-glycan conformation [1,8]. The current version of the Protein Database [9] contains about 500 glycosylated proteins. Usually only the co-ordinates of the rigid core region of N-glycans are available [7,10]. The question, if complex oligosaccharides do exhibit certain secondary or tertiary structural motifs, still remains to be answered due to the lack of crystallographic data on these molecules.

NMR techniques have been widely used in the conformational analysis of N-glycans [11-14]. However, an unambiguous determination of the complete conformational space is hampered by two effects; First, NMR-derived geometric constraints represent an average value based on conformations for flexible oligosaccharides; Second, only a few (one to three) contacts across a given glycosidic linkage can often be detected. To overcome this drawback, a combination of theoretical and experimental methods is applied to elucidate the dynamic behaviour of flexible N-glycans. This includes complex and time-consuming force field based modeling protocols like molecular dynamics simulations (MD) or Monte Carlo (MC) approaches to explore the conformational space accessible to various glycosidic linkages. Calculations resulting in systematic conformational energy maps are feasible to perform for di- and trisaccharides. A specialised collection of conformational profiles of disaccharides does exists and can be used to construct conformations of complex N-acetyllactosaminic type N-Glycans [15]. Two to seven possible conformations for disaccharides have been described for each glycosidic linkage. An exhaustive generation of all possible conformation of a middle-sized N-glycan possessing eleven residues would easily result in the evaluation of several thousand possible conformations. However, this number would be reduced as it is well known that the extent of branching influences the flexibility of N-Glycans in such a way that the majority of highly-branched glycosidic linkages populate only a restricted conformational area which is generally smaller than that observed in N-linked glycans with smaller numbers of antennae [16].

The aim of this study is a) to derive a comprehensive data base describing the conformational space of larger fragments of N-glycans taking into account the effects of branching, b) to automatically generate a complete set of all possible conformations for a given N-glycan and c) to rank the constructed conformations according to their strain energy. This should allow us to explore the complete conformational space of N-glycans rapidly and add this tool as additional option to our Web-based real-time online application services (see http://www.dkfz.de/spec/)

2 Materials and Methods

The current version of the Complex Carbohydrate Structural Database (known as CarbBank), [17] which is probably the most comprehensive data base of complex carbohydrates, contains about 2000 structurally different N-glycan entries. An analysis revealed that about 225 different tri-, tetra and pentasaccharide fragments are needed to construct all N-glycan structures contained in CarbBank.

The generation of a data base which can be used to enable a rapid exploration of the conformational space accessible to N-glycans can be accomplished in six steps.

Step 1: Extract all N-glycans from CarbBank to create a comprehensive set of all known substructures (CarbBank contains about 2000 N-glycans)
Step 2: Divide all N-glycans into meaningful Tri-, Tetra- and Pentasaccharide substructures. The result of this division is a set of 225 basic fragments which can describe all other N-glycans.
Step 3: Convert the 225 topology data sets of the fragments into 3D structures using the program Sweet II [18].
Step 4: Perform high temperature molecular dynamics simulations until conformational equilibrium has been reached
Step 5: Automatic assignment of conformations for each glycosidic linkage. Rank all possible conformations for each fragment according to their degrees of population.
Step 6: Store Φ,Ψ and ω and their relative populations for each fragment.

2.1 Topology of N-glycans

Complex Carbohydrates differ from the two other classes of biological macromolecules (proteins and DNA) in two important characteristics: their residues (monosaccharide units) can be linked by many different linkage types and they can form highly branched molecules. N-linked glycans, which can be classified structurally into four main types typically consist of 11 to 25 residues exhibiting multiantennary structures (see Figure 1a). Normally, the number of antennae varies between two and five. To take into account the degree of branching we

```
a-D-Manp-(1-2)-a-D-Manp-(1-6)+
```

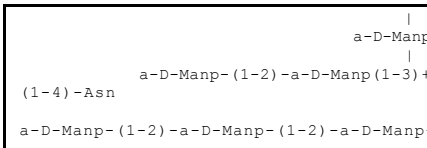

Figure 1a. The Primary structure of the carbohydrate chain of soybean agglutinin (SBA): a typical oligomannose N-glycan with four antennae. Nomenclature of carbohydrates as used in CarbBank is displayed. (Manp = mannose in its pyranose form , GlcpNAc= Glucosamine). Instead of the commonly used α and β, a and b are used to define the stereochemistry of the anomeric C atom.

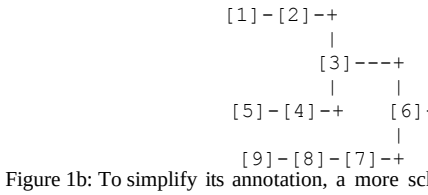

Figure 1b: To simplify its annotation, a more schematic representation of SBA is used throughout the rest of this paper: the residues of SBA are indicated by numbers.

a) Branching points (Tetrasaccharides)

```
a-D-Manp-(1-6)+                                  [2]
              |                                    |
          a-D-Manp-(1-6)-b-D-Manp                [3]—[6]
              |                                    |
a-D-Manp-(1-3)+                                  [4]
```

```
a-D-Manp-(1-6)+                                  [3]
              |                                    |
          b-D-Manp-(1-4)-a-D-GlcpNAc             [6]—[10]
              |                                    |
a-D-Manp-(1-3)+                                  [7]
```

b) Linear fragments (Trisaccharides)

```
a-D-Manp-(1-2)-a-D-Manp-(1-6)-a-D-Manp       [1,2,3]
a-D-Manp-(1-2)-a-D-Manp-(1-3)-a-D-Manp       [5,4,3]
a-D-Manp-(1-2)-a-D-Manp-(1-3)-b-D-Manp       [8,7,6]
a-D-Manp-(1-2)-a-D-Manp-(1-2)-a-D-Manp       [9,8,7]
b-D-Manp-(1-4)-b-D-GlcNAc-(1-4)-b-D-GlcpNAc  [6,10,11]
b-D-GlcNAc-(1-4)-b-D-GlcpNAc-(1-4)-Asn       [10,11,12]
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Figure 1c) Saccharide fragments(CarbBank nomenclature left side, simplified annotation right side) into which the carbohydrate chain of SBA is divided. a) two tetrasaccharides b) five different trisaccharides.

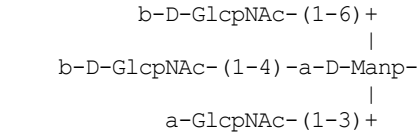

Figure 1d) Example of a complex branching point (pentasaccharides, not contained in carbohydrate chain of SBA) as can be found in complex N-glycans.

decided to define three topological different types of fragments, a) linear chains (trisaccharides), b) simple branching points (tetrasaccharides) and complex branching

points (pentasaccharides) (see Figure 1b). The fragments are assigned in such a way that one residue is always present in two connected fragments.

2.2. The use of Molecular Dynamics Simulation to create free energy landscapes

Computational approaches such as systematic conformational searches, Monte Carlo calculations and molecular dynamics (MD) simulations have been used to explore the conformational space accessible to biological macromolecules [13]. Most of the published methods are based on force-field calculations and produce energy maps which only reflect enthalpy effects and ignore entropic contributions. However, evaluation of both terms including the effects of the aqueous surroundings is necessary to produce reliable free energy landscapes. It is well known, in principle, that with a sufficiently long MD simulation it should be possible to produce a free energy map of a molecular ensemble. However, only recently have technical resources (computation speed, disk storage space) become available which can allow simulations to be continued until equilibrium between all populated conformations is reached. Now that such lengthy simulations are feasible, convenient criteria are required for judging when conformational equilibrium among several conformations has been achieved. Therefore, we have used an empirical algorithm developed by Martin Frank [19] which automatically calculates a parameter called *E*Q(*t*) at time point *t* from the available MD trajectory data. The value of *EQ*(*t*) can be used to define if and when any given MD simulation has approached conformational equilibrium (within a desired degree of accuracy).

It has been shown that high temperature MD-simulations (HTMD) of saccharides produce very similar energy landscapes for glycosidic linkages as long-time simulations at room temperature. However, HTMD simulations require significantly shorter time to reach conformational equilibrium than the standard simulations [19]. Such HTMD simulations allowed us to finalise the conformational analysis for all 225 N-glycan fragments on reasonable time scale, resulting thus in important reduction of required computational resources.

The applied simulation protocol was as follows: The SWEET-II environment [18] was used to generate the initial 3D structures of the fragments. The MM3-TINKER force field was used for energy calculation. The influence of the aqueous solvent environment was also considered, based on the GB/SA water model. Additional tethering force was applied during the HTMD simulations in order to avoid puckering of the pyranose ring atoms. The simulation parameters were set to: temperature 1000 K, integration step 10^{-15} s; the lengths of simulations were in the range of several nano-seconds (10^{-9}s) . Two molecular co-ordinates were stored per pico second (10^{-12} s). These structures were subsequently used to perform the population analysis. The TINKER software package [20] was used to perform MD simulations.

2.3 Characterising the conformational space of N-glycan fragments

Glycosidic bonds form the most flexible part of complex carbohydrates (see Figure 2). For the conformational analysis of individual glycosidic linkages most often Ramachandran-type profiles have been used to describe the effects of rotation around the glycosidic bond **Φ**,**Ψ** co-ordinates. **Φ**,**Ψ** torsion angles are calculated for each glycosidic linkage and all stored snapshots.

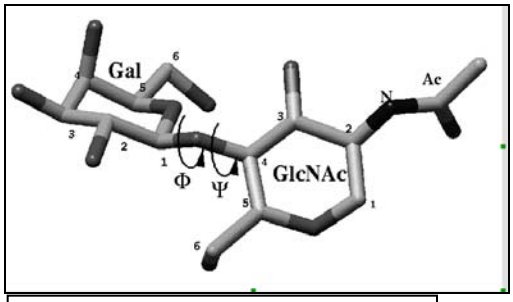

Figure 2: 3D structure of α–D-Galactose (Gal) 1-4 linked to Glucosamine (GlcNAc). Hydrogen atoms are omitted The determination of conformational preferences of oligosaccharides is best approached by describing their preferred conformations of the glycosidic linkage Φ,Ψ torsion angles

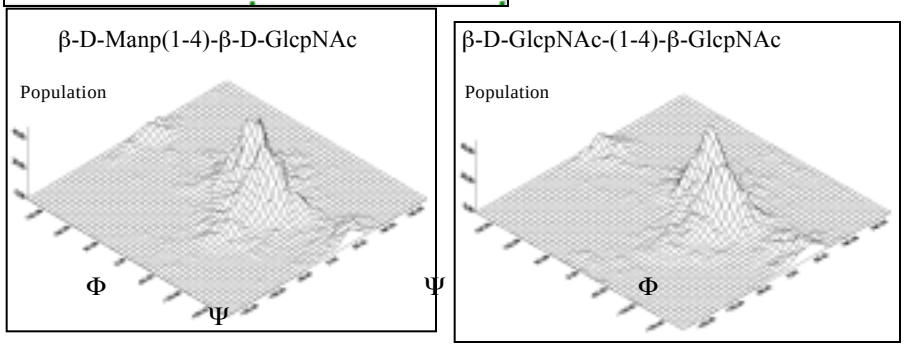

Figure 3: Population maps for both glycosidic linkages of the trisaccharide of β-D-Manp(1-4)-β-D-GlcpNAc-(1-4)-β–GlcpNAc generated from a high temperature molecular dynamics simulations.

In a first step the **Φ**,**Ψ** torsion angles are analysed separately for each glycosidic linkage. Since we continued HTMD simulations until equilibrium between all populated conformations was reached, we were allowed to assign conformations based on the populations of Φ,Ψ values (Figure 3). This procedure was accomplished in two steps. First, conformations were assigned separately for each individual glycosidic linkage. Therefore, areas showing a maximum in population density had to be identified. Normally, several maxima exist for each glycosidic linkage. Areas belonging to one conformation are encircled around each population maximum. The circled areas are not allowed to overlap in order to maximise the number of assigned values. Figure 4 illustrates how areas defining one conformation were assigned. Each selected conformation is then characterised by

the Φ,Ψ values of the centre of the encircled area and its relative population for each glycosidic linkage

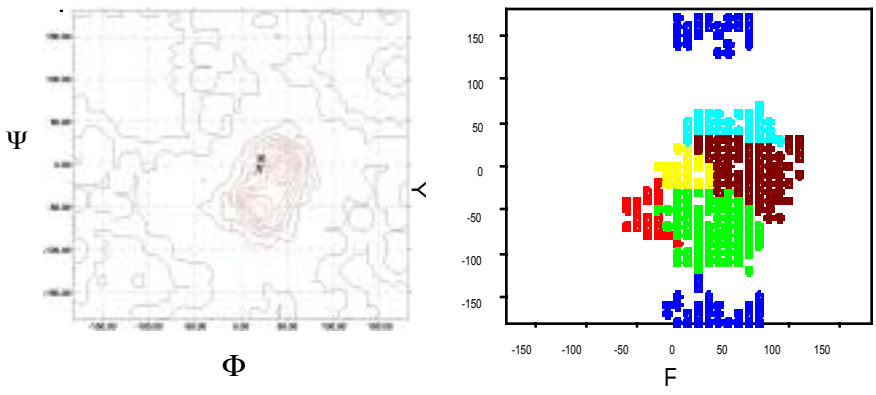

Figure 4: Assignment of areas defining the conformations of the β-D-GlcNAc-(1-4)-β-D-GlcNAc linkage. The analysis is based on the population of the Φ,Ψ space (see Figure 3) derived from the high temperature MD simulation of the trisaccharide β-D-Man-(1-4)-β-D-GlcNAc-(1-4)-β-GlcNAc. The maximum population together with its relative population is stored in the data base for each conformation. The assignment procedure works as follows: starting from maximal values, concentric rings are encircled in such a way that areas are not allowed to overlap and that the number of assigned values becomes maximal.

In a subsequent step the relative population for all existing combinations of conformations taking into account all glycosidic linkages of one fragment (e.g. a pentasaccharide has four glycosidic linkages) is calculated based on assignments for the individual glycosidic linkages. Figure 3 depicts the population of the space for both glycosidic torsion angles of the trisaccharide fragment β-D-Man-(1-4)-β-D-GlcNAc-(1-4)-β-GlcNAc. The automatic assignment procedure found six conformations for the β-D-Man-(1-4)-β-D-GlcNAc glycosidic linkage(Φ/Ψ: 24^{o} /-39^{o}; 38^{o}/4^{o}, 48^{o}/-171^{o}, -42^{o}/-50^{o}, 72^{o}/-155^{o}, 18^{o}/177^{o}) and six for β-D-GlcNAc-(1-4)-β-GlcNAc (Φ/Ψ: 56^{o}/8^{o}; 11^{o}/-39^{o}, 19^{o} /-5^{o}, 33^{o}/173^{o},-35^{o}/-44^{o} and -56^{o}/50^{o}). The maximum number of possible conformation in this case is 6x6 = 36. The population of all twenty-four conformations is calculated, ranked in descending order and stored. Table 1 shows the combination of values ordered by their relative population as stored in the data base. These data are recalled to exhaustively calculate all possible conformations of larger N-glycan conformations.

Table 1: Relative population and Φ,Ψ value for both glycosidic linkages for the 7 conformations of β-D-Man-(1-4)-β-D-GlcNAc-(1-4)-β-GlcNAc [6-10-11] showing more than 3% population are given.

No	Percent.	**Φ [10–11]**	**Ψ [10–11]**	**Φ[6–10]**	**Ψ [6–10]**
1	18.43	56	8	24	-39
2	17.76	11	-39	24	-39
3	17.25	56	8	38	4
4	15.80	11	-39	38	4
5	3.53	33	173	38	4
6	3.36	33	173	24	-39
7	3.14	19	-5	24	-39

2.4. Exploring the conformational space of N-glycans

To explore the conformational space of N-glycans an ensemble of possible conformations are generated for a given N-glycan. This task is accomplished in five steps.

Step 1: The structure, which has to be input using the condensed format for oligosaccharides, is divided into its basic structural fragments as described above.

Step 2: An initial 3D structure is generated using SWEET-II. The corresponding Φ,Ψ,ω values and their ranking are recalled form the data base for each fragment.

Step 3: Starting with the largest fragment contained, all possible conformations are constructed setting the glycosidic angles to the recalled Φ,Ψ,ω values one by one according to their ranking. Since the fragments were generated in such a way that always one residue overlaps with the previous fragment, the Φ,Ψ,ω values and the ranking of the already assigned fragments is maintained.

Step 4: The geometry of all generated conformations is relaxed applying a small number of molecular mechanics steps. The resulting force field energy is used to rank the computed structures. A hit list of the low energy structures is displayed.

Step 5: Since the number of generated conformations can be in the order of several thousand, it is not meaningful to perform a complete optimisation for all structures. Therefore, the user is given the possibility to define the number of best hits to be optimised applying a complete optimisation using the MM3 force field.

2.5. Implementation

MD simulations were performed on various high performance computing facilities (IBM-SP2 and HP V class machines) using the MM3 force field implemented in the TINKER software. Analysis of the various MD-trajectories and the generation of the N-glycan structures to explore their conformational space were accomplished using our own software running on a standard PC under Linux. Currently the software is installed on a Pentium 250 MHz PC. Generation of about 1000 conformations and evaluation of the energy content for each conformation takes less than two minutes of CPU time for an N-glycan consisting of eleven residues. Most of the required time is used to relax the large number of generated conformations.

3. Results and Discussion

Low energy conformations as a list of its glycosidic torsion angles Φ,Ψ,ω for some typical N-Glycan can be looked up at http://www.dkfz.de/spec/glydict/ and ordered according to their force field energy content. Here we discuss sugar chain attached to SBA. Overall more than 1000 conformations were generated and ranked according to their energy content. An examination of the glycosidic torsion angles reveals that several energetically similar conformations exist where only the orientation of one linkage has been changed. From X-ray and NMR measurements it is well known that only one conformation dominates the linkages of the core residues close to the protein attachment site. This finding is reflected in our approach for the preferred orientation of the [6-10], [10-11] and [11-12] linkage where only one conformation is present. The predicted torsion angles for the [6-10] (Φ/Ψ =19/-25) and the [10-11] (Φ/Ψ =56/8) linkage have values which are close to experimental data. A statistical analysis of the available X-ray data for glycoproteins as found in the PDB-files showed the following distribution: β-D-GlcNAc-(1-4)-GlcNAc [6-10] (Φ = 47±8; Ψ = -4 ±16). β-D-Man-(1-4) –β-D-GlcNAc [10-11] (Φ = 32±11 ; Ψ = -13 ±20).

A comparison of the preferred orientations of glycosidic linkages found in N-glycan structures as revealed by different experimental and theoretical methods is shown in Table 2. It is well known that complex carbohydrates are rather flexible molecules and therefore often refuse to crystallise. Moreover, in cases when crystals are available, often only for the atoms of the rigid core region have sufficient electron density to be measured making an unambiguous assignment of the conformation of the complete carbohydrate chain impossible. Therefore, only a limited number of X-ray structures and glycosidic linkages exist which can be used for comparison

Table 2: Comparison of generated torsion angles for various glycosidic linkages as found N-glycans with experimental data. Torsion angles are given in degrees.

Method	**systematic search**	**MD und NMR**	**MD and NMR**	**X-Ray**	**Knowledge base**
source	Imberty [7]	Woods [16]	Homans [21-23]	Petrescu[10]	
force field	MM2 Carb	Amber-Glycam	Amber-Homan		MM3 + GB/SA
β-GalNAc (1-4) β-GlcNAc	40/10 35/15 30/-55	36±15/ -33±23	0/-33 54/-2 27(25)/-17/(24) 34(27)/-14(22)	47±8/-4±16	56/8 11/-39 33/173
β-D-Man (1-4) β-GlcNAc	45/10 35/15 30/-55	46±13/-10±16	15/-27 15/-5 13(26)/-16(20) 25(30)/-7(18)	32±11/-13±20	32±11/-13±20
α-D-Man (1-3) α–D-Man	-35/55 -50/-20 30/30	-45/30 -45±10/0±50	-20/30 -20/50 -40(15)/12(27) -38(17)/ 2(23)	-48±11/6±22	-26/47 -39/-25 -55/64 -53/38
α-D-Manp (1-6) α-D-Manp	-50/-170/? -45/100/? -50/-170/? -45/90/?	- 35±15/180±10/? 40+-/10/145+-15/	-2/81/179 8/59/-52 -8(27)/95/31)/?	-55±9/182±5/ -174±10 -54±11/180±15/-55±15 -53±14/109±13//-37±22	-52/-179[1]/ -45 -44/-157/ -43
α-D-Man (1-2) α–D-Man	-30/60 -50/-20 30/30	-39±9/56±14 -42±9/55±14 -39±9/52±22 -46±8/22±14	-40/-20 -40/0	-58±8/-55±10 -49±8/16±15	35/36 -33/-3 -45/-11 -54/-37 -6/46 -38/-27

with theoretically generated structures. Complex simulation protocols based on force-field calculations like molecular dynamics simulations (MD) in combination with MR-derived constraints are intensively used to explore the conformational space accessible for various glycosidic linkages. All approaches support the fact that complex carbohydrates are rather flexible molecules so that several conformations for each glycosidic linkage can be populated. Nevertheless, the number of conformations found and their exact localisation varies considerably between different methods. An exact correspondence of Φ,Ψ values calculated with different force fields and varying computational approaches cannot be expected. As pointed out by Rasmussen and Fabricius, a minimum in a two-dimensional conformational map may represent an entire family of points in multidimensional space. Therefore,

a "difference in Φ,Ψ of 10^o, 10^o is really no difference at all" [24]. Comparing the Φ,Ψ values generated with our approach with those found in crystals we find at least one conformation which probably populates the same conformation as found in crystal structures. The intention of our approach is not compete with more complex and time consuming simulation techniques which include experimental constraints and explicit solvent molecules. Nevertheless, the ensemble of generated N-glycan conformations represents a realistic description of the conformational space accessible to such molecules.

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The presented approach is able to generate a comprehensive set of all possible conformations for a given N-glycan and to rank them according to their energy content. It is very efficient an is therefore well suited to be used as web-based application. We have established an internet interface (http://www.dkfz.de/spec/glydict/), which enables the user to input an N-glycan of interest and to receive an ensemble of possible conformations within a few minutes. The results are distributed via E-mail when the procedure has finished. For each conformation co-ordinates in PDB-format can be downloaded, displayed and analysed locally using 3D visualisation tools like RasMol, Chime or WebMolecule.

The current version of our approach is designed to explore the conformational space for N-glycans. Other types of complex carbohydrate structures like O-glycans or lipopolysaccharides can be as well handled by this approach: only the basic data base has to be expanded with fragments contained in these structures.

The work presented is part of a larger project [25] trying to establish bioinformatics tools for complex carbohydrate structures and to cross-reference sugar structures with existing data collections in the proteomics field.

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