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Recognition of Protease Binding Site in Bovine Pancreatic RNase B: Role of Thr45 Modification by *O*-GlcNAc, Phosphate and their Interplay

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Abstract.- Protein functions are modulated by primary structure. Certain dynamic modifications in primary structural molecules affect the structure-functions temporarily. The dynamic modifications such as *O*-GlcNAc and phosphate substitutions and their interplay regulate functions in diverse manner. Ribonuclease (RNase) B is a form of RNase A that differs in having an oligosaccharide chain at Asn34 that is lacking in RNase A. This oligisoccharide chain by hydrogen bonding with Lys37 moderately stabilizes the RNase B as compared to RNase A. Due to H-bond interaction and resulting stability the protease recognition site is shifted from Asn34-Leu35 to the Thr45-Phe46 in the case of RNase B. We propose modification of Thr45 by GlcNAc and phosphate and their interplay play crucial role in recognition of protease and in degradation pathway of RNase B.

Key words: RNase-B, glycosylation, phosphorylation, protease recognition.

INTRODUCTION

Glycosylation, an important modification of proteins, is known to affect the stability of the protein structure toward proteolytic degradation (Rudd *et al*., 1994) as well as toward denaturants (Jaenicke, 1991). The proteolytic degradation of proteins strongly depends on their structural flexibility (Hubbard, 1998) and is mainly induced by unfolding of the molecules. Unfolding of proteins involves destabilization of non-covalent interactions among different amino acids. Phosphorylation is one of the covalent modifications that affect the non-covalent interactions of the protein by interfering with the non-covalent interactions involving negative charges. Modifications on amino acid side chain by negatively charged groups may result in different effects, either resulting in promotion of proteolysis (Elorza *et al*., 2003) or resistance to proteolysis, as in the case of carboxyl or sulfate groups in glycoproteins (Nasir-ud-Din *et al*., 2003).

Ribonuclease B (RNase B), an isoform of Ribonuclease A (RNase A), is more stable in spite of the similar primary, secondary and tertiary structure (Williams *et al*., 1987) but differ only in having an *N*-linked oligosaccharide chain on Asn 34. The carbohydrate chain ($GlcNAc_2Man_{5-9}$) contains two *N*-acetylglucosamine and 5–9 mannose residues with relative molar proportions of 57% (Man_5), 31% (Man_6), 4% (Man_7), 7% (Man_8) and 1% (Man_9) (Fu *et al*., 1994). Interestingly, the glycosylation site of RNase B is located in the region of proteases binding such as thermolysin and trypsin (Arnold *et al*., 1996). The position of this unfolding region is not altered due to the attachment of the carbohydrate moiety in RNase B (Arnold *et al*., 1998), whereas the kinetic as well as the thermodynamic thermal stabilities of RNase B are higher than those of RNase A (Arnold *et al*., 1997). Arnold *et al*. (1999) have also shown that stability of RNase B depends on first GlcNAc attached to Asn 34 only but the degradation region is shifted from Asn34-Leu35 (that is in RNase A) to the Thr45-Phe46 (that is in RNase B) peptide bond due to increasing shielding effects offered by oligosaccharide chain.

Similarly, we propose here that the involvement of Thr45 modification by alternative modification by

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GlcNAc and phosphate results in delaying or favouring the unfolding of protease binding site at RNase B.

MATERIALS AND METHODS

The sequence data

The sequence data of RNase A/B of *Bos taurus* used for predicting phosphorylation and glycosylation sites was taken from Entrez protein database, the NCBI sequence viewer and SWISSPROT protein database. The sequence of Pancreatic RNase A/B was described very early (Plummer and Hirs, 1964). The RNase A/B sequence with Entrez database No 1RBBA/1RBBB and SWISSPROT entry name RNAS1_BOVIN and primary accession number P61823, was retrieved. The sequence data retrieved from both of the sources were aligned using BLAST algorithm for two sequences. This aligned sequence was used for prediction of potential glycosylation and phosphorylation sites.

Prediction methods

Glycosylation prediction methods

The methods used for defining potential glycosylation sites involved prediction of both *O*- and *N*-linked sites. The three methods for predicting *O*-linked glycosylation sites include *NetOGlyc* 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/), that predicts *O*-glycosylation sites in mucin- type proteins (*i.e.,* for *O*-GalNAc sites), *DictyOGlyc* 1.1 (http://www.cbs.dtu.dk/services/DictyOGlyc/) that predicts *O*-α-GlcNAc sites in eukaryotic proteins and YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/), that predicts *O*-β-GlcNAc sites in eukaryotic proteins. *NetNGlyc* 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) was used for predicting *N*-glycosylation sites. The above-mentioned four methods for predicting glycosylation sites are neural network based.

Phosphorylation prediction method

For prediction of phosphorylation sites in RNase B, *NetPhos* 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) was utilized. *NetPhos* 2.0 is also a neural network based program designed by training the neural networks through protein phosphorylation data from phosphoBase 2.0.

RESULTS

Prediction results of O-glycosylation sites

The results for *O*-glycosylation sites predicted by three methods include methods *NetOGlyc* 3.1 (for prediction of *O*-GalNAc sites), *DictyOGlyc* 1.1 (for prediction of *O*-α-GlcNAc sites), and *YinOYang* 1.2 (for prediction of *O*-β-GlcNAc sites)

O-GalNAc sites by NetOGlyc 3.1

This method is for predicting the protein modification by *O*-GalNAc in mammalian proteins and has been developed by training the artificial neural networks through the sequence context of glycosylated and non-glycosylated serines and threonines. The results obtained for the prediction of *O*-GalNAc sites in the RNase B show that there is no appreciable potential for GalNAc addition.

O-α-GlcNAc sites by DictyOGlyc 1.0

The prediction results obtained by *DictyOGlyc* 1.0 for *O*-α-GlcNAc sites in bovine RNAse B show the same as that for *O*-α-GalNAc.

O-β-GlcNAc sites by YinOYang 1.2

The prediction results obtained for *O*-β-GlcNAc sites by *YinOYang* 1.2 show that the number of potential *O*-β-GlcNAc sites among all three types of *O*-linked modifications is highest. There have been 13 potential sites for *O*-β-GlcNAc modifications off which 8 are at Ser; 15*, 16, 18*, 21*, 22, 23, 59, and 89 and 4 are at Thr; 03, 17, 87*, and 100, with four Yin Yang sites marked by an asterisk. There are some other residues close to the threshold level as shown by the wavy threshold line in Figure 1, which are likely to be modified by GlcNAc.

Prediction results of N-glycosylation sites by NetNGlyc 1.0

There are total 10 Asn residues in RNase B of bovine with only one within sequon Asn-Xaa-Ser/Thr at Asn34 that is correctly predicted as *N*-linked glycosylated site.

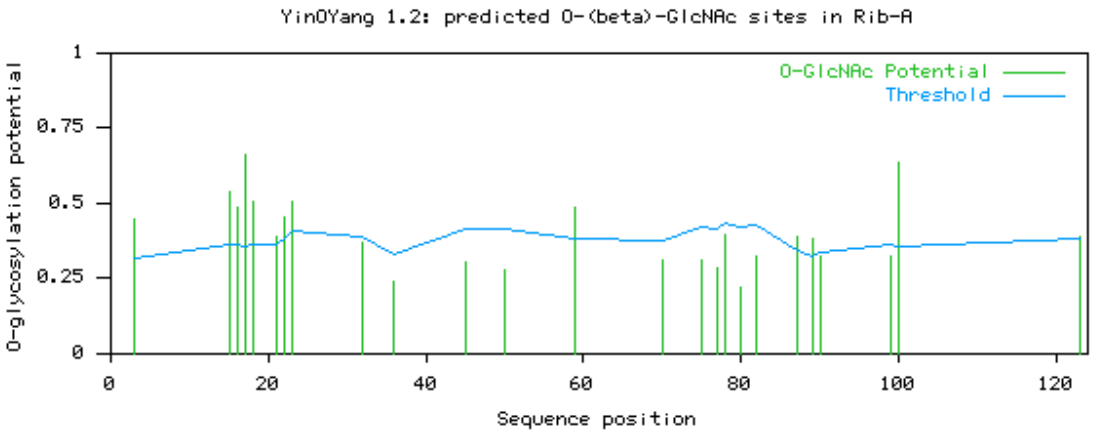

Fig. 1. Graphic representation for potential or all Ser and Thr residues for GlcNAc modification.

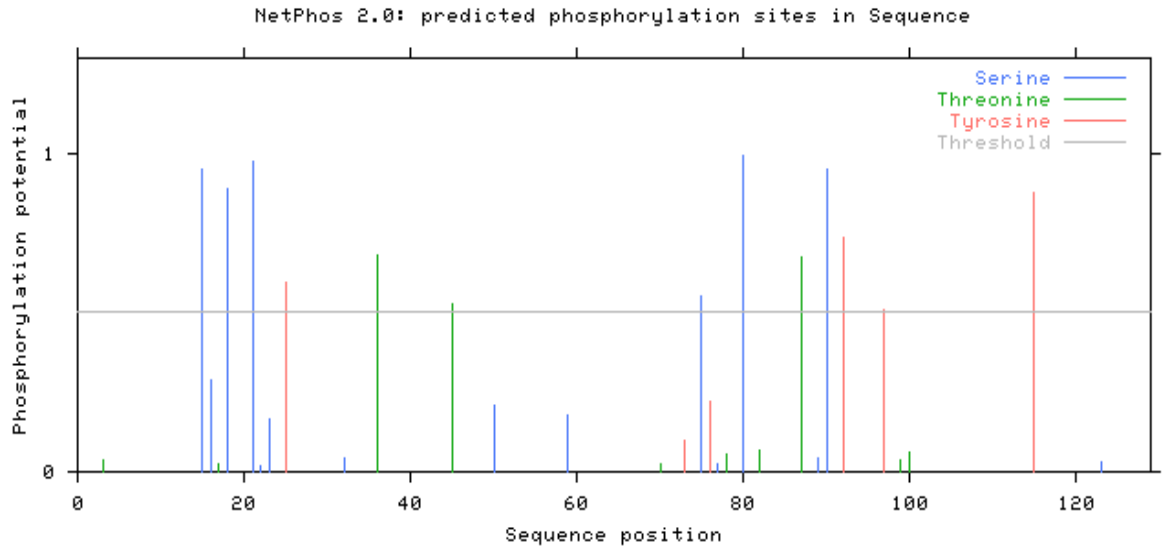

Fig. 2. Graphic potential of all Ser, Thr, and Tyr modifications by phosphate group.

Prediction results for phosphorylation by NetPhos 2.0

Utilizing *NetPhos* 2.0, *O*-linked phosphorylation at serine and threonine was predicted. There are total 13 residues which are predicted to be potential for phosphate modification. Out off these 13 sites 6 are at Ser: 15, 18, 21, 75, 80, and 90; 3 are at Thr: 46, 45, and 87; and 4 at Tyr: 25, 92, 97, and 115 (Fig. 2).

DISCUSSION

Protein translation releases non-functional polypeptides. These polypeptides after translation are subjected to a number of post-translational modifications including glycosylation and phosphorylation before and after folding of protein into a functional unit. These protein modifications are actually determined by the primary structure or

sequence of protein in the same way as sequence of protein is determined by genetic code on mRNA that was transcribed by DNA portion, the gene. So the structure-function relation of protein is analogous to sequence-structure relation. But most often a number of proteins present in cytosol and nucleus are subjected to certain momentary conformational changes induced by alternative *O*-GlcNAc and phosphate modification responsible for regulating diverse functions (Shafi *et al*., 2000; Roquemore *et al*., 1996).

Pancreatic ribonuclease A and its glycosylated form, ribonuclease B are enzymes specific for the degradation of ribonucleic acid, cleaving the polynucleotide chain after pyrimidine residues**.** The enzymes bind, but do not sever, deoxyribonucleotide chains, at least not at perceptible rates (Brayers and McPherson, 1982). Thus, ribonucleases A and B are single stranded DNA binding proteins, in spite of the absence of cooperative protein-protein interactions, and DNA helix-destabilizing proteins. In this way, they are somewhat similar in nature to the gene 5 protein from bacteriophage fd and the DNA unwinding protein gene 32 product from bacteriophage. As such, they offer opportunity for direct visualization, using x-ray diffraction of complexes between a protein and single-stranded DNA.

The differences in the rate of proteolytic degradation of RNase A and RNase B were reported for trypsin, chymotrypsin, pepsin and elastase (Birkeland and Christensen, 1975) as well as for Pronase (Rudd *et al*., 1994) where RNase B was more resistant than RNase A. The comparison of the proteolytical susceptibility of RNase A, RNase B, and two chemically glycosylated RNase species to trypsin revealed that RNase B was degraded more slowly than the other species. While both chemically glycosylated species renatured better after heat treatment, no protection against proteolytic degradation was observed. Arnold *et al.* (1994) showed that the difference of thermal stability between RNase A and RNase B can be attributed to a hydrogen bond between the side-chain of Lys37 and an oxygen atom in the first GlcNAc at Asn34 (Woods *et al*., 1994). Thus, due to the increased shielding effects of oligosaccharide chain in RNase B, the main degradation pathway is shifted from the Asn34-Leu35 to the Thr45-Phe46 peptide bond.

The *O*-linked glycosylation in RNase A has been evidenced by mutating Asn34 to Ala in heterologous system expression studies (Chatani *et al*., 2000). The bond Thr45 – Phe46, acts as a degrading pathway for the RNase B. According to the prediction results by YinOYang 1.2 and NetPhos 2.0, Thr45 is predicted as a potential site for phosphate modification (Fig. 2). On the other hand, Thr-45 shows a potential very close to the threshold for *O*-GlcNAc modification (Fig.1). Thus Thr45 is most likely to be modified by phosphate and *O*-GlcNAc alternatively and may act as potential Yin Yang site. Figures 3 and 4 openly show that during or before unfolding of protein, the –OH group of Thr45 lying in a pocket, somewhat far from Asn34, is easily accessible by any enzyme such as OGT or kinase. We propose that when Thr45 is modified by *O*-GlcNAc it may possibly favour binding of protease to RNase B resulting in its quick unfolding and degradation. But phosphate may hinder protease binding due to its much probable H-bond involving its oxygen to the side chain hydrogen of positively charged amino group of Arg85 (Figs. 3 and 4). This may lead to delay in protease action until replaced by *O*-GlcNAc. No other charged residues in the vicinity of Thr45 in 3-D form of molecule are expected to be potential to interact covalently or non-covalently with incoming phosphate on Thr45 (Fig. 3.)

The H-bond formation by oxygen of phosphate with positively charged NH_2 group of Arg85 is also strengthened by contact surface area calculation using MOLMOL (MOLecule analysis and MOLecule display). Contact surface area calculations show that Thr45 lies in a pocket with oxygen outside of the total dotted contact surface area (Fig. 4) and solvent accessible surface for Thr45 is 5.4% whereas that for Arg85 is 36.6%. Figure 4 clearly depicts that positively charged NH_2 group of Arg85 is also outside of the surface area thus not involved in any non-covalent interaction with any neighboring residue, so can easily be engaged in H-bonding with oxygen of incoming phosphate on Thr45. This H-bonding is expected to make the molecule more compact especially narrowing of the pocket in which Thr45-Phe46 is present ultimately making this peptide bond

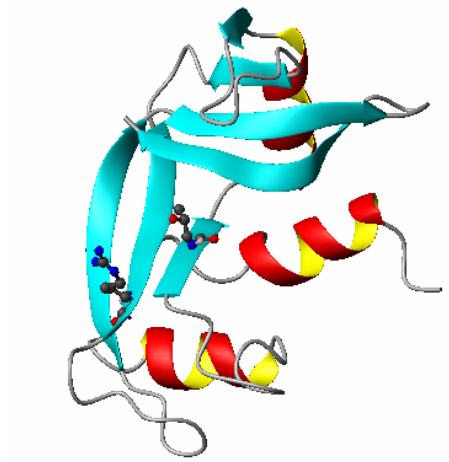

Fig. 3. This figure shows a chain of RNase B with Thr45 and Arg85 shown in ball stick style. The oxygen of Thr45 (red in colour) when modified by phosphate group the oxygen of this phosphate group is proposed to make a H-bond with positively charged NH_2 group (in blue colour) blocking the Thr45-Phe46 bond from attack of protease. But when this site is occupied by GlcNAc no such non-covalent interaction is expected by the environmental residues thus favours protease binding. This figure is prepared by modifying the PDB entry 1RBB using MOLMOL.

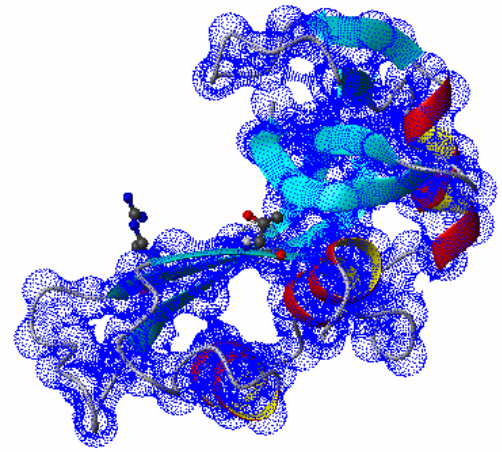

Fig. 4. Contact surface calculation by MOLMOL shows a pocket with oxygen of Thr45 outside the surface area so easily surface accessible by other molecules such as OGT or kinases. Moreover positively charged NH_2 group of Arg85 is also outside the surface area of molecule that also increases the chances of the H-bond formation by oxygen of phosphate with positively charged NH_2 group of Arg85.

inaccessible by protease and delaying its degradation until a phosphatase removes the phosphate from Thr45 and is blocked by GlcNAc. Figure 4 also shows that protease action on Asn34 – Leu35 requires unfolding of molecule, whereas for Thr45 – Phe46. OGT or kinase action on oxygen of Thr45 do not require unfolding of molecule. This in inspite of the fact that only 5.4% of the total Thr45 surface area is solvent accessible and the other portion with Phe46 and bond between them are embedded inside.

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