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Structural basis of the 14-3-3 protein-dependent activation of yeast neutral trehalase Nth1

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ABSTRACT

Background: Trehalases are highly conserved enzymes catalyzing the hydrolysis of trehalose in a wide range of organisms. The activity of yeast neutral trehalase Nth1 is regulated in a 14-3-3- and a calcium-dependent manner. The Bmh proteins (the yeast 14-3-3 isoforms) recognize phosphorylated Nth1 and enhance its enzymatic activity through an unknown mechanism.
Methods: To investigate the structural basis of interaction between Nth1 and Bmh1, we used hydrogen/deuterium exchange coupled to mass spectrometry, circular dichroism spectroscopy and homology modeling to identify structural changes occurring upon the complex formation.
Results: Our results show that the Bmh1 protein binding affects structural properties of several regions of phosphorylated Nth1: the N-terminal segment containing phosphorylation sites responsible for Nth1 binding to Bmh, the region containing the calcium binding domain, and segments surrounding the active site of the catalytic trehalase domain. The complex formation between Bmh1 and phosphorylated Nth1, however, is not accompanied by the change in the secondary structure composition but rather the change in the tertiary structure.
Conclusions: The 14-3-3 protein-dependent activation of Nth1 is based on the structural change of both the calcium binding domain and the catalytic trehalase domain. These changes likely increase the accessibility of the active site, thus resulting in Nth1 activation.
General significance: The results presented here provide a structural view of the 14-3-3 protein-dependent activation of yeast neutral trehalase Nth1, which might be relevant to understand the process of Nth1 activity regulation as well as the role of the 14-3-3 proteins in the regulation of other enzymes.

1. Introduction

The neutral trehalase Nth1 has been widely studied since its isolation and characterization in the 1990s [1–3]. Nth1 belongs to the glycoside hydrolase family 37 (EC 3.2.1.28) of O-glycosyl hydrolases (EC 3.2.1) which comprises enzymes with a common trehalase activity. The biological function of trehalase consists of the control of trehalase concentration via the degradation of trehalose [α-D-glucopyranosyl-(1-1)-α-D-glucopyranoside] into two molecules of glucose by hydrolyzing one of the two glycosidic bonds in trehalose with the inversion of the anomeric configuration. Trehalose is a naturally occurring non-reducing sugar found in a wide variety of organisms where it protects proteins and membranes from various stress conditions like dehydration, heat, cold, oxidation and desiccation and serves as a carbon and energy source. Trehalose is particularly important for insects as the hydrolyzed glucose is crucial for insect flight. It has also been suggested that in yeast and plants it serves as a regulatory and signaling molecule to direct certain metabolic pathways or to affect growth [4]. Recent studies have revealed that the enzyme activity of *Saccharomyces cerevisiae* Nth1 is regulated through an interesting mechanism involving phosphorylation of several sites by cAMP-dependent protein kinase (PKA), Ca^{2+} and the Bmh protein binding [5–7].

The yeast Bmh proteins belong to the 14-3-3 protein family, a eukaryotic family of highly conserved regulatory molecules, playing an important role in the regulation of signal transduction, apoptosis, cell cycle control, and nutrient-sensing pathways (reviewed in [8–10]). They fold into U-shaped homo- or heterodimers with a 40 Å-wide channel containing two amphipathic binding grooves, by which they bind their ligands mostly in a phosphorylation-dependent manner

Abbreviations: HDX, H/D exchange; HDX–MS, H/D exchange coupled to mass spectrometry; DSS, disuccinimidyl suberate; DSG, disuccinimidyl glutarate; WT, wild type; Nth1, neutral trehalase; pNth1, phosphorylated neutral trehalase; DMSO, dimethyl sulfoxide; TCEP, tris (2-carboxyethyl)phosphine; VDM, validoxylamine; CD, circular dichroism

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[11–14]. Through these binding interactions, the members of the 14-3-3 protein family serve as molecular chaperons that modulate the subcellular localization, the structure or the stability of hundreds of other proteins. Notably, the catalytic activity of several enzymes, including tryptophan and tyrosine hydroxylases, serotonin N-acetyltransferase (AANAT), Raf kinases, ASK1 kinase, plant plasma membrane H^+-ATPase, plant nitrate reductase, plant mitochondrial and chloroplast adenosine 5-triphosphate (ATP) synthases and more, have been shown to be regulated in a 14-3-3 protein-dependent manner (reviewed in [9,10,15]). However, the mechanistic understanding of these regulations is mostly elusive. The 14-3-3 protein-dependent activation of AANAT is one of the few cases where available structural data enabled insight into the mechanism of the 14-3-3 protein action [16]. Regulation of this enzyme is based on a direct structural change where the 14-3-3 protein forces AANAT to adopt a conformation that allows optimal substrate binding.

Recently, we have performed a detailed biochemical characterization of the 14-3-3 protein-dependent activation of *S. cerevisiae* Nth1 and showed that Bmh proteins bind tightly to the phosphorylated N-terminal segment of Nth1 with residues Ser60 and Ser83 being sites primarily responsible for this interaction [6]. The close proximity of these two phosphorylated recognition sites also suggests a synergistic effect on binding as has been shown previously [17,18]. The complex formation strongly enhances the enzymatic activity of Nth1 and this activation is significantly more potent compared to Ca^{2+}-dependent one. Although we have obtained valuable information concerning the activation of Nth1, that study has not yielded sufficient structural insights into these mechanisms of Nth1 activation.

Thus, in this work we employed hydrogen/deuterium exchange coupled with mass spectrometry (HDX–MS) and circular dichroism spectroscopy to perform a structural analysis of a complex formed between the *S. cerevisiae* Nth1 and Bmh1. The HDX–MS method is commonly used to probe interactions within protein–protein and protein–ligand complexes since changes in HDX rates allow identification of binding surfaces and detection of conformational changes [19–21]. Our results show that Bmh1 binding to the phosphorylated Nth1 induces substantial changes in the deuteration kinetics of several Nth1 regions including the N-terminal segment where the 14-3-3 binding motifs are located, the Ca^{2+}-binding domain, and the catalytic trehalase domain. This suggests that these regions form the interaction surface of Nth1. In addition, our data also indicate that the Bmh1 protein binding affects the structural properties of segments surrounding the buried active site of Nth1. This might enable easier substrate and product entry and departure, respectively, and thus results in Nth1 activation. The interaction surface of Bmh1 includes not only the surface of the ligand binding groove where the phosphorylated N-terminal segment of Nth1 binds but also surfaces outside the central cavity of Bmh1 dimer. Circular dichroism measurements confirmed that the interaction between Bmh1 and Nth1 affects their tertiary structure but without the change in their secondary structure composition.

2. Materials and methods

2.1. Expression and purification of Bmh1 protein

DNA encoding *S. cerevisiae* Bmh1 protein was ligated into pET-15b (Novagen) using the NdeI and BamHI sites. The entire coding region was checked by sequencing. The Bmh proteins were expressed and purified as described previously [22].

2.2. Expression, purification, phosphorylation and activity measurement of Nth1

DNA encoding *S. cerevisiae* Nth1 protein was ligated into pET-32b (Novagen) with deletion of 81 base pairs after the sequence (6 × H) SSGLVPRGS using the NcoI and BamHI sites. The entire coding region was checked by sequencing. The Nth1 protein was expressed, purified and phosphorylated as described previously [6]. The enzyme kinetics of the hydrolysis of trehalose by Nth1 was measured using a stopped assay as described previously [6]. Specific activity was determined in μmol of glucose liberated per min per mg of protein.

2.3. Circular dichroism spectroscopy

The far-UV CD spectra were measured in a quartz cuvette with an optical path length of 1 mm (Starna, USA) using a J-810 spectropolarimeter (Jasco, Japan). The conditions of the measurements were as follows: a spectral region of 200–260 nm, a scanning speed of 10 nm/min, a response time of 8 s, a resolution of 1 nm, a bandwidth of 1 nm and a sensitivity of 100 mdeg. The final spectrum was obtained as an average of 5 accumulations. The spectra were corrected for a baseline by subtracting the spectra of the corresponding polypeptide-free solution. The CD measurements were conducted at room temperature (23 °C) in the buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM 2-mercaptoethanol, 10% (w/v) glycerol buffer. The Bmh1 concentration was 0.089 $mg \cdot mL^{-1}$; the concentration of pNth1 and Nth1 was 0.145 $mg \cdot mL^{-1}$. After baseline correction, the final spectra were expressed as a mean residue elipticities Q_{MRW} ($deg \cdot cm^2 \cdot dmol^{-1}$) and were calculated using the equation

$$[Q]_{MRW} = \frac{\theta_{obs} \times 100 \times M_W}{c \times l \times N_R}, \quad (1)$$

where θ_{obs} is the observed ellipticity in mdeg, c is the protein concentration in $mg \cdot mL^{-1}$, l is the path length in cm, M_w is the protein molecular weight and N_R is the number of amino acids in the protein. Secondary structure content was estimated using tools available at Dichroweb website [23].

The near-UV CD spectra were measured in a quartz cuvette with an optical path length of 1 cm (Starna, USA) in a spectral region of 250–320 nm. The Bmh1 concentration was 0.447 $mg \cdot mL^{-1}$; the concentration of pNth1 and was 0.69 $mg \cdot mL^{-1}$.

2.4. Molecular modeling

Three-dimensional model of the catalytic domain of yeast neutral trehalase Nth1 (sequence 295–721) was generated using the DeepView v4.0.4, the SWISS-MODEL server [24,25] and the crystal structure of trehalase Tre37A from *Escherichia coli* (PDB code 2JF4, sequence 145–533) as a template [26]. The sequence identity and similarity of Nth1 catalytic domain to trehalase Tre37A are 27 and 42%, respectively. The final model was validated by PROCHECK, and bad contacts were corrected manually by Coot program [27]. The three-dimensional model of Bmh1 (sequence 4–236) was generated employing the same procedure and using the crystal structures of several known 14-3-3 protein isoforms as templates: PDB codes 1A4O [11], 1YZ5 [28], 2B05, 2BTP, 2C63 and 2BR9 [29]. Both models were further experimentally validated using the distance constraints derived from chemical cross-linking with homo-bifunctional cross-linkers DSS and DSG (amine–amine coupling) which covalently modify lysine residues.

2.5. Hydrogen/deuterium exchange kinetics coupled to mass spectrometry (HDX–MS)

HDX of the Bmh1 protein, phosphorylated Nth1 (pNth1) protein, and pNth1 in the presence of the Bmh1 protein was initiated by a 10-fold dilution in a deuterated buffer containing 20 mM Tris–HCl (pH/pD 7.5), 1 mM EDTA, 3 mM DTT, 200 mM NaCl and 10% (w/v) glycerol. The final protein concentrations were 3.16 μM for Bmh1 and 1.6 μM for phosphorylated Nth1. The molar ratio between Bmh1 and Nth1 was therefore 2:1. Aliquots (80 μL) were taken after 30 s, 1 min, 3 min,