Role of the EF-hand-like Motif in the 14-3-3 Protein-mediated Activation of Yeast Neutral Trehalase Nth1*

Miroslava Kopecka†**, Dalibor Kosek†**, Zdenek Kukacka†**, Lenka Rezabkova†**, Petr Man†**, Petr Novak†**, Tomas Obsil†*, and Veronika Obsilova††

From the †Institute of Physiology and the ‡Institute of Microbiology, Academy of Sciences of the Czech Republic v.v.i., Videnska 1083, 14220 Prague, Czech Republic, the ††Second Faculty of Medicine, Charles University, V Uvalu 84, 150 06 Prague, Czech Republic, and the Departments of †§Physical and Macromolecular Chemistry and **Biochemistry, Faculty of Science, Charles University, Hlavova 3020, 12843 Prague, Czech Republic.

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Background: The yeast neutral trehalase Nth1 is activated by the 14-3-3 protein binding.

Results: The 14-3-3 protein induces a structural rearrangement of Nth1 with changes within the EF-hand-like motif being essential for the activation process.

Conclusion: The EF-hand-like motif-containing domain is crucial for the 14-3-3-dependent activation of Nth1.

Significance: Structural basis of the mechanism of Nth1 activation.

Trehalose (α-D-glucopyranosyl-(1-1)-α-D-glucopyranoside) is a non-reducing disaccharide of glucose found in a broad variety of organisms, including bacteria, yeast, fungi, insects, and plants, with the exception of mammalian cells. The generation of trehalose is triggered by stresses, such as heat, drying, or oxidative stress, indicating that the accumulated trehalose protects proteins and membranes from these stress conditions. Moreover, it can also act as a signaling or regulatory molecule in some cells, connecting the trehalose metabolism to glucose transport and glycolysis (1).

Hydrolysis of trehalose into two glucose subunits is carried out by trehalases (2). Trehalase was first described in Aspergillus niger and then in Saccharomyces cerevisiae and subsequently in many other organisms, including plants and animals (3–6). It has been shown that the yeast S. cerevisiae possesses several different trehalases: the vacuolar acid trehalase Ath1 with a lower pH optimum of about 4.5, and the cytoplasmic neutral trehalases Nth1 and Nth2 with a pH optimum of about 7 (7–10). The sequence comparison revealed that neutral trehalases from yeast S. cerevisiae and Kluyveromyces lactis possess, compared with other organisms, an N-terminal extension that contains several protein kinase A (PKA) phosphorylation sites as well as the EF-hand-like calcium binding motif, suggesting that this region is involved in the regulation of these enzymes’ activity (11–13). Indeed, it has recently been shown that the activation of S. cerevisiae Nth1 is regulated by PKA phosphorylation, Ca2+, and the 14-3-3 protein binding (14–16).

In yeast S. cerevisiae, two 14-3-3 protein isoforms (Bmh1 and Bmh2) with a great degree of homology have been identified (17). Bmh1 and Bmh2 were shown to be essential in most laboratory yeast strains (18). As in higher eukaryotes, yeast 14-3-3 proteins bind to and modulate the activity of plenty of proteins involved in crucial cellular processes (19). In our previous study, we identified two key phosphorylation sites within the N-terminal segment of S. cerevisiae Nth1 that are responsible for the 14-3-3 protein-mediated activation of Nth1 (15). This activation is significantly more potent compared with the Ca2+ only-dependent activation, which is more common among trehalases from other organisms. Subsequently, we showed that the 14-3-3 protein binding affects the conformation of both the region containing the EF-hand-like motif and the catalytic trehalase domain (Fig. 1), with changes in the EF-hand-like motif being, surprisingly, most profound (20). Thus, these data sug-

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†To whom correspondence should be addressed. Tel.: 420-241062191; Fax: 420-244472269; E-mail: obsilova@biomed.cas.cz.
gested that this motif plays an important, although unclear, role in the activation of *S. cerevisiae* Nth1.

In this work, the structure of the Nth1-14-3-3 complex and the importance of the EF-hand-like motif located between residues 114 and 125 in the activation of Nth1 were investigated using the site-directed mutagenesis, the hydrogen/deuterium exchange (HDX) coupled to mass spectrometry (HDX-MS), chemical cross-linking, and small angle x-ray scattering (SAXS). The low resolution structural views of Nth1 alone and the Nth1-14-3-3 complex show that the 14-3-3 protein binding induces a significant structural rearrangement of the whole Nth1 molecule. The EF-hand-like motif-containing region forms a separate domain that interacts with both the 14-3-3 protein and the catalytic trehalase domain. The structural integrity of the EF-hand-like motif is essential for the 14-3-3 protein-mediated activation of Nth1, and calcium binding, although not required for the activation, facilitates this process by affecting its structure. Our data suggest that the EF-hand-like motif-containing domain functions as the intermediary through which the 14-3-3 protein modulates the function of the catalytic domain of Nth1.

**EXPERIMENTAL PROCEDURES**

*Expression and Purification of Bmh1—DNA encoding* *S. cerevisiae* Bmh1 protein was ligated into pET-15b (Novagen) using the NdeI and BamHI sites (21). The histidine-tagged protein was expressed by isopropyl 1-thio-β-D-galactopyranoside induction for 5 h at 37°C and purified from *E. coli* BL21(DE3) using chelating Sepharose® Fast Flow (GE Healthcare) using the standard protocol. Next, Bmh1 was purified by anion exchange chromatography using Q Sepharose® Fast Flow (GE Healthcare). The protein was eluted using a linear gradient of NaCl (50–1000 mM). Fractions containing Bmh1 were concentrated and further purified using size exclusion chromatography on a Superdex 75 10/300 GL column (GE Healthcare) in a buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% (w/v) glycerol. The protein concentration of purified Bmh1 was determined from UV absorption at 280 nm using an extinction coefficient value of 28,880 M⁻¹·cm⁻¹ (22).

*Expression, Purification, and Phosphorylation of Nth1—Nth1 from* *S. cerevisiae* was expressed, purified, and phosphorylated as described previously (15). To ensure that prepared Nth1 is calcium-free, the final purification step (the size exclusion chromatography) was done in the presence of either 1 mM EDTA or EGTA (in a buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA or 1 mM EGTA, 1 mM DTT, and 10% (w/v) glycerol). The protein concentration of purified Nth1 was determined from UV absorption at 280 nm using an extinction coefficient value of 142,560 M⁻¹·cm⁻¹ (22).

Mutants of Nth1 (D103L, D114L, D114E, D116L, K117L, N118L, I121L, D125L, D125E, and D173L) were created by using the QuikChange™ approach (Stratagene). All mutations were confirmed by sequencing, and phosphorylation was checked by mass spectrometry.

**Differential Scanning Fluorimetry**—The thermofluor assay was performed using a real-time PCR LightCycler 480 II (Roche Applied Science). The proteins at a concentration of 0.2 mg/ml were tested in the presence of 8× concentrated Sypro Orange (Sigma-Aldrich) in a total reaction volume of 25 μl in the LightCycler 480 Multiwell Plate 96 (Roche Applied Science). The plate was sealed with the LightCycler 480 Sealing Foil (Roche Applied Science), and a temperature gradient from 20 to 95°C with a rate of 0.01 °C/s was applied. The wavelengths for fluorescence excitation and emission were 465 and 580 nm, respectively. The melting temperature values, Tₘ, corresponding to the inflection points of the melting curves, were determined as the minima of the negative first derivative using the Roche LightCycler 480 SW 1.5 software (23, 24).

**Enzyme Activity Measurements**—The trehalase activity of phosphorylated Nth1 (pNth1) WT and mutants was measured by estimating the glucose produced by hydrolysis of trehalose using a stopped assay as described previously (15, 25). Specific trehalase activity of pNth1 was measured in the presence and in the absence of Bmh1 and/or Ca²⁺. The final concentrations of pNth1, Bmh1, and Ca²⁺ were 100 nM, 15 μM, and 10 μM, respectively. The calcium was added to the 50 μl of reaction mixture from the 200 mM stock solution of CaCl₂. The assay was performed at 30 °C in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (w/v) glycerol, and 30 mM trehalose. Experiments performed in the absence of Ca²⁺ also contained 1 mM EDTA or EGTA. The production of glucose was detected using the Amplex® Red glucose/gluco oxidase assay kit (Invitrogen). The specific activity of trehalase was determined as μmol of glucose liberated/min/mg of protein at 571 nm.

**Near-UV Circular Dichroism (CD) Spectroscopy**—The near-UV ECD spectra were measured in a quartz cuvette with an optical path length of 1 cm (Starna) using a J-810 spectropolarimeter (Jasco, Japan). The conditions of the measurements were as follows: a spectral region of 250–320 nm, a scanning speed of 10 nm/min⁻¹, a response time of 8 s, a resolution of 1 nm, a bandwidth of 1 nm, and a sensitivity of 100 millidegrees. The final spectrum was obtained as an average of five accumulations. The spectra were corrected for a baseline by subtracting the spectra of the corresponding polypeptide-free solution. The ECD measurements were conducted at room temperature (23 °C) in 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 0.45 mg/ml⁻¹, and the concentration of pNth1 WT and mutants was 0.69 mg/ml⁻¹. After baseline correction, the final spectra were expressed as mean residue ellipticities, Q_MWR (degrees cm² dmol⁻¹ number of residues⁻¹) and were calculated using the equation,

\[
\theta_{222} = \frac{\theta_{\text{obs}} M_r}{c d N_{\lambda} 10}
\]  
(Eq. 1)
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where $\theta_{\text{obs}}$ is the observed ellipticity in millidegrees, $c$ is the protein concentration in mg ml$^{-1}$, $l$ is the path length in cm, $M_r$ is the protein molecular weight, and $N_A$ is the number of amino acids in the protein (26).

**Analytical Ultracentrifugation Measurements**—Sedimentation velocity (SV) experiments were performed using a ProteomLab™ XL-1 analytical ultracentrifuge (Beckman Coulter). SV experiments of Bmh1 and pNth1 were conducted at loading concentrations of 0.2–20 $\mu$M, 20 °C, and 42,000 or 48,000 revolutions/min rotor speed (An-50 Ti rotor, Beckman Coulter). All data were collected with absorbance optics at 280 nm. Samples were dialyzed against the buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2 mM 2-mercaptoethanol before analysis. To study the effect of Ca$^{2+}$ on the interaction, the dilution series of Bmh1 with constant concentration of pNth1 were analyzed with and without 10 mM CaCl$_2$ in the buffer solution. The $c(S)$ distributions were calculated from the raw absorbance data using the software package SEDFIT followed by fitting the chemical equilibrium using the Lamm equation modeling implemented in the software package SEDPHAT with the previously known values of each component (27, 28). Loading concentrations were slightly corrected in the process of fitting.

**Hydrogen/Deuterium Exchange Kinetics Coupled to Mass Spectrometry (HDX-MS)—** HDX of the Bmh1 protein, pNth1 protein, both proteins in the presence and in the absence of 10 mM Ca$^{2+}$, and pNth1 in the presence of the Bmh1 protein and/or 10 mM Ca$^{2+}$ 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, 150 mM NaCl, and 10% (w/v) glycerol. The final protein concentrations were 3.16 $\mu$M for Bmh1 and 1.6 $\mu$M for phosphorylated Nth1. The molar ratio between Bmh1 and Nth1 was therefore 2:1. Aliquots (80 $\mu$l) were taken after 30 s, 1 min, 3 min, 10 min, 30 min, 1 h, 3 h, and 5 h of exchange. The exchange was quenched by adding 20 $\mu$l of 0.1 M HCl and rapid freezing in liquid nitrogen. Analysis was done as described previously (20, 29).

**Chemical Cross-linking Combined with Mass Spectrometry**—Both Bmh1 and pNth1 alone and pNth1 in the complex with Bmh1 were cross-linked using cross-linkers disuccinimidyl suberate (DSS) or disuccinimidyl glutarate (DSG). For the cross-linking reaction, all proteins were dialyzed against buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EGTA, and the protein concentrations were as follows: pNth1, 0.25 mg ml$^{-1}$; Bmh1, 0.25 mg ml$^{-1}$. All proteins were cross-linked in the presence of 10 mM Ca$^{2+}$ using non-deuterated cross-linkers (DSSD0 and DSGD0) and in the absence of Ca$^{2+}$ using four-times deuterated cross-linkers (DSSD4 and DSGD4). Freshly prepared stock solutions of cross-linkers (5 mg ml$^{-1}$ in DMSO) were added in a $15 \times 30$ molar excess to each protein alone or in a $50 \times 100$ molar excess to the pNth1•Bmh1 complex. The reaction mixtures were incubated for 2 h at room temperature. After that, samples that were cross-linked in the presence of Ca$^{2+}$ with non-deuterated compounds were mixed with identical samples that were cross-linked in the absence of Ca$^{2+}$ with deuterated compounds in a 1:1 molar ratio, and their analysis was performed as described previously (20, 30).

**SAXS**—SAXS data were collected on the European Molecular Biology Laboratory P12 beamline on the storage ring DORIS III (Deutsches Elektronen Synchrotron, Hamburg, Germany). The pNth1•Bmh1 protein complex and Nth1 were measured in a concentration range of 1.8–15 mg ml$^{-1}$. Bmh1 was measured in a concentration range of 2.2–16.3 mg ml$^{-1}$. Data analysis was performed using the ATSAS software suite (31). The data were averaged after normalization to the intensity of the transmitted beam, and the scattering of the buffer was subtracted using PRIMUS (32). The forward scattering ($I(0)$) and the radius of gyration ($R_g$) were evaluated using the Guinier approximation. The distance distribution function ($P(r)$) and the maximum particle dimension ($D_{\text{max}}$) were determined by the indirect Fourier transformation of the scattering data ($I(s)$) using GNOM (33). The solute apparent molecular mass ($MM_{\text{app}}$) was estimated by comparison of the forward scattering with that from reference solutions of bovine serum albumin (molecular mass 66 kDa). Ab initio molecular envelopes were computed using DAMMIN (34), which represents the protein by a collection of dummy atoms in a constrained volume with a maximum diameter defined experimentally by $D_{\text{max}}$. For each protein, 10 surfaces were generated and averaged using DAMAVER (35). The averaged surfaces were then used as the final SAXS three-dimensional structure.

**RESULTS**

The Integrity of the EF-hand-like Motif-containing Region Is Crucial for the 14-3-3 Protein-dependent Activation of Nth1—

The catalytic activity of *S. cerevisiae* Nth1 is regulated by PKA-mediated phosphorylation followed by 14-3-3 binding, with Ca$^{2+}$ playing an unclear regulatory role (14–16). Fig. 2A shows the activity of phosphorylated Nth1 (pNth1) in the presence of Ca$^{2+}$, Mg$^{2+}$, and Bmh1 (yeast 14-3-3 protein isofrom). Samples with Bmh1 only also contained additional 1 mM EDTA or EGTA to ensure that no traces of metals were present. As can be seen, the Ca$^{2+}$-only dependent activity of pNth1 is very small, whereas Bmh1- and Bmh1 + Ca$^{2+}$-dependent activities are significantly higher, with the last one being a little bit more profound. No significant difference was observed for the Bmh1 only-dependent activity of pNth1 in the presence of either 1 mM EDTA or 1 mM EGTA; thus, only the activity in the presence of EDTA is shown. This activity (54 ± 1 μmol min$^{-1}$·mg$^{-1}$) is somewhat lower compared with the activity measured in the absence of EDTA (64–66 μmol min$^{-1}$·mg$^{-1}$ (20)). In addition, no significant activation was observed in the presence of Mg$^{2+}$ alone, and the effect of Bmh1 + Mg$^{2+}$ on pNth1 activity was similar to that of Bmh1 alone.

Franco *et al.* (13) showed that the Ca$^{2+}$-dependent activation of Nth1 from *Schizosaccharomyces pombe* is mediated by a conserved Ca$^{2+}$-binding EF-hand-like motif that is also present in *S. cerevisiae* Nth1 (sequence $^{114}$DTDKNQITTED$^{125}$). To investigate the importance of this motif in *S. cerevisiae* pNth1 activation, we performed site-directed mutagenesis of several residues that correspond to both conserved and non-conserved positions from EF-hand motifs of numerous Ca$^{2+}$-binding proteins (Fig. 1B) (36, 37). We mutated residues Asp$^{114}$, Asp$^{116}$, Asn$^{118}$, and Asp$^{125}$, which correspond to conserved positions 1, 3, 5, and 12 in EF-hand motifs participating in metal coordina-
tion, and residues Lys^117 and Ile^121 at non-conserved positions 4 and 8, respectively, which are not involved in metal coordination (36, 37). In addition, we also mutated residues Asp^103 and Asp^173 from regions bordering the EF-hand-like motif.

To verify that the introduced mutations did not result in an overall destabilization of the Nth1 structure, the stability of all prepared mutants was checked by measuring the thermally induced protein denaturation using differential scanning fluorimetry. No significant differences in the temperature of the unfolding transition ($T_{m}$) were observed for all Nth1 mutants with the exception of the I121L variant (Table 1). The slightly lower $T_{m}$ of I121L mutant might reflect different conformation of the EF-hand-like motif because this residue (position) is known to be important for the proper conformation of the motif (36). The binding of selected phosphorylated Nth1 mutants to Bmh1 was also checked by using analytical ultracentrifugation, and no significant differences compared with pNth1 WT were observed (data not shown). Thus, all prepared Nth1 mutants were found to be suitable for trehalase activity measurements.

Next, the Bmh1-mediated activity of prepared pNth1 mutants in the absence and the presence of Ca^2+ was measured (Fig. 2B). Although all mutants, with the exception of D116L, showed either significantly or totally suppressed Bmh1-mediated activity in the absence of Ca^2+ (Fig. 2B, white bars), the presence of Ca^2+ (Fig. 2B, gray bars) rescued the activity of all but the D114L, N118L, and D125L mutants. These three residues are located at positions crucial for metal coordination, and their replacement with Leu had the most profound effect on pNth1 activity both in the absence and the presence of Ca^2+. On the other hand, mutations D114E and D125E, which should rescue the Ca^2+ binding, showed high Bmh1-mediated activity but only in the presence of Ca^2+. These data suggested not only that the structural integrity of the EF-hand-like motif is essential for the Bmh1-mediated activation of pNth1 but also that calcium binding helps to mediate the activation process, probably through the structural stabilization of the EF-hand-like motif.

### TABLE 1

| Nth1 variant | $T_{m}$ °C |
|-------------|-----------|
| WT          | 53.6 ± 0.3 |
| D103L       | 52.3 ± 0.2 |
| D114L       | 52.7 ± 0.2 |
| D114E       | 53.4 ± 0.2 |
| D116L       | 53.1 ± 0.1 |
| K117L       | 53.6 ± 0.1 |
| N118L       | 52.8 ± 0.5 |
| I121L       | 50.4 ± 0.3 |
| D125L       | 52.6 ± 0.2 |
| D125E       | 53.3 ± 0.1 |
| D173L       | 52.5 ± 0.1 |

Midpoint temperatures of the protein-unfolding transition ($T_{m}$) for Nth1 WT and mutants as determined using differential scanning fluorimetry

Uncertainties are the S.E. values calculated from three experiments.

FIGURE 1. A domain structure of S. cerevisiae Nth1. Relative positions of the 14-3-3 protein binding sites (Ser^60 and Ser^83), the EF-hand like motif (sequence 114–125), and the catalytic domain (sequence 295–721) are shown. B, the sequence and the model of the EF-hand like motif (sequence 114–125) of Nth1. Mutated residues important for metal coordination at positions 1, 3, 5, and 12 are shown in red. The structural model of the EF-hand like motif of Nth1 was created using Modeler version 9.12 (46) and the structure of the EF-hand motif of calmodulin (Protein Data Bank code 1EXR) as a template (47). C, three-dimensional model of the catalytic domain of yeast neutral trehalase Nth1 (sequence 295–721) was generated as described previously using the crystal structure of trehalase Tre37A from E. coli (Protein Data Bank code 2JF4, sequence 145–533) as a template. The active site contains trehalase inhibitor validoxylamine (shown as spheres), which was present in the structure of the template (20, 40).
Ca\textsuperscript{2+} Ions Do Not Affect the Dissociation Constant of the pNth1-Bmh1 Complex—Trehalase activity measurements revealed that several pNth1 mutants exhibit significantly higher activity in the presence of Bmh1 + Ca\textsuperscript{2+} compared with the presence of Bmh1 only (Fig. 2B). Therefore, we checked whether the presence of Ca\textsuperscript{2+} increases the stability of the pNth1-Bmh1 complex using analytical ultracentrifugation (sedimentation velocity method). Continuous distributions of sedimentation coefficients, c(s), for mixtures of pNth1 and Bmh1 at five different molar ratios (from 5:1 to 1:20) both in the absence and the presence of Ca\textsuperscript{2+} are shown in Fig. 4. These distributions (normalized on the peak height) showed that Bmh1 and pNth1 form a complex with a weight-averaged sedimentation coefficient (s\textsubscript{w,20}) of 7.2 S, whereas Bmh1 and pNth1 alone show single peaks with s\textsubscript{w,20} values of 3.6 and 5.1 S, respectively. The low abundance of the complex formation at 7.2 S for samples containing the lowest and the highest concentration of either pNth1 or Bmh1 in these mixtures (the concentration of pNth1 was 1 μM).

The analysis of sedimentation velocity data revealed no significant effect of Ca\textsuperscript{2+} on the apparent equilibrium dissociation constant (K\textsubscript{d}) of the pNth1-Bmh1 complex because K\textsubscript{d} values of 10 × 10\textsuperscript{-9} were determined both in the presence and the absence of 10 mM Ca\textsuperscript{2+}. Thus, the more potent activation of pNth1 WT and mutants in the presence of Bmh1 + Ca\textsuperscript{2+} compared with that with Bmh1 only (Fig. 2B) cannot be explained by the increase in the binding affinity of pNth1 for Bmh1.

The EF-hand-like Motif of pNth1 Adopts Different Conformations in the Presence of Ca\textsuperscript{2+}, Bmh1, and Bmh1 + Ca\textsuperscript{2+}—Many proteins containing the EF-hand motif undergo a conformational change upon the Ca\textsuperscript{2+} binding (37). To investigate whether the same also holds true for pNth1, HDX-MS measurements were performed. HDX-MS experiments are based on monitoring the deuteration kinetics of backbone amides and enable characterization of protein dynamics and conformational changes because the rate of exchange of deuterium for hydrogen depends on both the solvent exposure and the hydrophobic hydrogen.

Structural Changes within the EF-hand-like Motif-containing Region—The comparison of HDX-MS data for pNth1 in the presence of Ca\textsuperscript{2+}, Bmh1, and Bmh1 + Ca\textsuperscript{2+} revealed large differences in the deuteration kinetics for five peptides from the region containing the EF-hand-like motif under all conditions.
Low Resolution Structure of the Bmh1-pNth1 Complex

Structural Changes within the Catalytic Trehalase Domain of pNth1—Exchange kinetics for four peptides from the vicinity of the pNth1 active site whose deuteration was moderately but significantly decreased upon the Bmh1 protein binding is shown in Fig. 5B (20). It can be noticed that only the peptide 665–698 showed some decrease in the deuteration kinetics in the presence of Ca$^{2+}$ (compare black and red lines). In addition, the presence of Ca$^{2+}$ had no significant effect on isotope exchange kinetics of these four peptides in the presence of Bmh1 (compare blue and green lines).

Thus, HDX-MS data suggested that the Ca$^{2+}$ binding affects mainly the structure of the EF-hand-like motif-containing region, whereas its effect on the catalytic domain is less profound.

Ca$^{2+}$-mediated Structural Changes of Bmh1 Molecule—We also investigated whether the presence of Ca$^{2+}$ affects the deuterium exchange kinetics of Bmh1 peptides. A significant decrease in the deuteration level in the presence of Ca$^{2+}$ was observed for several peptides, with the strongest effect being observed for helices H3 (peptides 39–47 and 48–61), H8 (peptide 184–207), and H9 (peptide 227–232) (compare black and red lines in Fig. 6). Only two peptides (184–207 and 222–232 from helices H8 and H9, respectively) showed significantly decreased exchange kinetics when comparing peptides from the pNth1-Bmh1 complex with or without Ca$^{2+}$ (compare green and blue lines in Fig. 6), suggesting that these helices might interact with and thus be affected by changes within the EF-hand-like motif of bound pNth1.

Binding to Bmh1 Affects the Relative Position of the N-terminal Region and the Catalytic Domain of pNth1—Site-directed mutagenesis and HDX-MS suggested that the structural integrity of the EF-hand-like motif is crucial for pNth1 activation and that its conformational change is an integral part of the activation process. This also implied that this region might be adjacent to the catalytic domain and that its conformational changes affect the structure (or the accessibility) of the active site and hence enable the activation. The crystallographic structural data are available only for trehalase Tre37A from E. coli (40) which shows homology with the catalytic domain of S. cerevisiae Nth1 (sequence 295–721). We used this homology to build a structural model of the catalytic domain of Nth1, which, however, does not include either the region containing the EF-hand-like motif or the N-terminal segment containing PKA phosphorylation sites (and the 14-3-3 protein-binding motifs) (20). Therefore, we used chemical cross-linking combined with mass spectrometry and SAXS to obtain structural information concerning the relative position of the region containing the EF-hand-like motif and the catalytic domain as well as additional information about structural changes induced by Ca$^{2+}$ and the 14-3-3 protein binding.

To enable easier distinction of changes induced by Ca$^{2+}$, both pNth1 alone and the pNth1-Bmh1 complex were cross-linked by non-deuterated cross-linking agents (DSS and DSG) in the presence of Ca$^{2+}$ and by four-times deuterated agents in

tested. Peptides 102–110, 110–124, and 156–172 exhibit significantly slower deuteration in the presence of Ca$^{2+}$ (compare black and red lines in Fig. 5A), with the peptide 110–124 (which contains the EF-hand-like motif) showing the slower isotope exchange only in short incubation times. In addition, an even more profound decrease in the rate of deuteration was observed for all pNth1 peptides between residues 102 and 185 in the presence of Bmh1 (blue line) or Bmh1 + Ca$^{2+}$ (green line). Observed changes in HDX kinetics might reflect additional conformational change and/or decreased accessibility to the solvent. Interestingly, only the peptide 110–124 showed significant differences in isotope exchange kinetics when comparing peptides from the pNth1-Bmh1 complex with or without Ca$^{2+}$. These results suggest that the region 102–185 of pNth1, especially the peptide 110–124 (the EF-hand like motif), adopts three different structural states (and/or positions) in the presence of Ca$^{2+}$, Bmh1, and Bmh1 + Ca$^{2+}$.
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A

Peptide 102-110

% HDX

log time (s)

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

0 10 20 30 40 50 60

Peptide 110-124

% HDX

log time (s)

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

0 10 20 30 40 50 60

Peptide 121-151

% HDX

log time (s)

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

0 10 20 30 40 50 60

Peptide 156-172

% HDX

log time (s)

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

0 10 20 30 40 50 60

Peptide 173-185

% HDX

log time (s)

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

0 10 20 30 40 50 60

Peptide 263-275

% HDX

log time (s)

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

0 10 20 30 40 50 60

B

Peptide 666-698

% HDX

log time (s)

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

0 10 20 30 40 50 60

Peptide 713-737

% HDX

log time (s)

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

0 10 20 30 40 50 60

Peptide 607-636

% HDX

log time (s)

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

0 10 20 30 40 50 60

Peptide 466-489

% HDX

log time (s)

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

0 10 20 30 40 50 60
the absence of Ca$^{2+}$ (see “Experimental Procedures” for details). The cross-linking experiments with pNth1 alone revealed 33 intramolecular distance constraints (Table 2), from which 17 can be compared with Ca–Ca distance constraints derived from the homology model of the catalytic domain (data not shown). No cross-links between the N-terminal region (sequence 1–250) and the catalytic domain (sequence 300–720) were observed, suggesting that in the absence of Bmh1, these two domains are not in contact with one another. Quantification of obtained cross-links (last two columns in Table 2) revealed that the ratio between non-deuterated and deuterated cross-links is close to 1:1 for all but two of them. The two exceptions are for the peptide from the region containing the EF-hand like motif whose residues Lys-132 and Lys-142 are cross-linked only in the presence of Ca$^{2+}$ (the abundances of DSG and DSS cross-links are ~78 and ~90%, respectively). This suggests that in the presence of Ca$^{2+}$, these two lysines are close enough to form a cross-link. However, in the absence of Ca$^{2+}$, this region possesses different conformation and/or flexibility, and the distance between these two residues is too large to form a cross-link.

The list of pNth1 cross-links from the pNth1-Bmh1 complex is shown in Table 3. In this case, the presence of Ca$^{2+}$ changed the abundances of significantly more cross-links compared with pNth1 alone. This confirmed that pNth1 (when bound to Bmh1) adopts a different conformation that is more sensitive to Ca$^{2+}$ binding compared with free pNth1. The most profound changes were observed for intramolecular cross-links Lys$^{211}$–Lys$^{214}$, Lys$^{214}$–Lys$^{563}$, Lys$^{257}$–Lys$^{258}$, Lys$^{258}$–Lys$^{393}$, Lys$^{385}$–Lys$^{387}$, Lys$^{517}$, Lys$^{546}$–Lys$^{548}$, and Lys$^{589}$–Lys$^{593}$. The presence of the cross-link Lys$^{214}$–Lys$^{563}$, which was not observed for pNth1 alone, suggested that the part containing residue Lys$^{214}$ and the catalytic domain (containing Lys$^{563}$) of pNth1 are much closer to one another in the Bmh1-bound form. In addition, the differences between conformations of pNth1 bound to Bmh1 in the presence and the absence of Ca$^{2+}$ are also supported by intermolecular cross-links between pNth1 and Bmh1 peptides (Table 4). Although in the presence of Ca$^{2+}$ the intramolecular cross-link between pNth1 residues Lys$^{258}$ and Lys$^{563}$ is preferentially formed (Table 3), in the absence of Ca$^{2+}$, these two residues preferentially form intermolecular cross-links with Bmh1 residues Lys$^{127}$ and Lys$^{76}$, respectively (Table 4). A similar effect was also observed for pNth1 residue Lys$^{393}$, which forms in the presence of a Ca$^{2+}$ intramolecular cross-link with Lys$^{258}$, whereas in the absence of Ca$^{2+}$, prefers an intermolecular cross-link with Bmh1 residue Lys$^{145}$ (Tables 3 and 4). The results of chemical cross-linking for the Bmh1 alone correspond well with the distance restraints derived from the homology model of the Bmh1 molecule (data not shown).

**Low Resolution Structure of the Bmh1-pNth1 Complex**

SAXS offers information about the dimension and shape of a protein in solution and was thus used here to gain visual insight into the global architecture of Nth1, Bmh1, and their complex. The experimental SAXS curves from Nth1, Bmh1, and the pNth1-Bmh1 complex are shown in Fig. 7A. The apparent molecular mass of the pNth1-Bmh1 protein complex was estimated by comparison of the forward scattering intensity $I(0)$ with that from reference solutions of bovine serum albumin. The estimated molecular mass of ~147 kDa corresponds well to 2:1 molar stoichiometry, in good agreement with our previously published results (15). The Guinier analysis revealed that Nth1 alone has a significantly larger radius of gyration ($R_g$ of 52.0 ± 0.4 Å) compared with Bmh1 alone ($R_g$ of 32.6 ± 0.1 Å) and the pNth1-Bmh1 complex ($R_g$ of 40.5 ± 0.1 Å), suggesting that the complex is a more compact particle than Nth1 alone.

This was further confirmed by the distance distribution function, $P(r)$, which revealed maximal dimensions ($D_{max}$) of Bmh1, Nth1, and the pNth1-Bmh1 complex to be of 92, 183, and 127 Å, respectively (Fig. 7B). These values of $D_{max}$ corroborated a more extended and asymmetric shape of free Nth1 compared with the complex.

The calculated low resolution ab initio envelopes for Nth1 alone, Bmh1 alone, and the pNth1-Bmh1 complex are shown in Fig. 7, C–E. The envelope of Bmh1 alone shows a characteristic cuplike shape of the 14–3–3 dimer molecule and agrees well with the theoretical model of Bmh1 dimer (Fig. 7C). The envelope for Nth1 alone (Fig. 7D) shows that the enzyme adopts an extended rodlike conformation, in good agreement with the results of cross-linking experiments, where no cross-links between the N-terminal region and the catalytic domain were observed (Table 2). The narrower half probably represents the flexible and unstructured N-terminal segment containing all PKA phosphorylation sites and the 14–3–3-binding motifs, whereas the thicker half would correspond to the rest of the enzyme (the EF-hand like motif-containing region and the catalytic domain).

The envelope of the complex is, as expected, more spherical and shows that pNth1 adopts significantly different conformation when bound to Bmh1 (Fig. 7E). The shape of the envelope suggests that the cuplike-shaped Bmh1 dimer is located within the wide central part of the particle. The rigid body modeling of the pNth1-Bmh1 complex was performed using homology models of Bmh1 and the catalytic domain of Nth1 (sequence 295–721). The rigid body model of the Nth1(295–721)Bmh1 complex displayed good agreement with both the low resolution molecular envelope and the results of cross-linking experiments (Table 4). The inset in Fig. 7E shows the detailed view of the binding interface between Nth1(295–721) and Bmh1, where two of three observed intermolecular cross-links
Low Resolution Structure of the Bmh1·pNth1 Complex
TABLE 2

Intramolecular distance constraints of pNth1 derived from the cross-linking experiments in the presence and the absence of Ca$^{2+}$ and their comparison with distance constraints derived from the homology model of the catalytic domain of Nth1

| Cross-linker | Cross-linked residues | Co-Co distance from the homology model | Co-Co distance constraint from the cross-linking experiments$^a$ |
|--------------|----------------------|--------------------------------------|-------------------------------------------------------------|
|              |                      | $\AA$                                | $\AA$                                                      |
| DSG/DSGD4    | Lys$^{49}$–Lys$^{584}$ | $\leq 20$                            |                                                             |
| DSG/DSGD4    | Lys$^{62}$–Lys$^{104}$ | $\leq 20$                            |                                                             |
| DSG/DSGD4    | Lys$^{357}$–Lys$^{528}$ | $\leq 24$                            |                                                             |
| DSG/DSGD4    | Lys$^{558}$–Lys$^{63}$ | $\leq 20$                            |                                                             |
| DSG/DSGD4    | Lys$^{558}$–Lys$^{343}$ | $\leq 20$                            |                                                             |
| DSG/DSGD4    | Lys$^{558}$–Lys$^{31}$ | $\leq 24$                            |                                                             |
| DSG/DSGD4    | Lys$^{528}$–Lys$^{371}$ | $\leq 20$                            |                                                             |
| DSG/DSGD4    | Lys$^{528}$–Lys$^{113}$ | $\leq 20$                            |                                                             |
| DSG/DSGD4    | Lys$^{528}$–Lys$^{343}$ | $\leq 20$                            |                                                             |
| DSG/DSGD4    | Lys$^{528}$–Lys$^{371}$ | $\leq 20$                            |                                                             |
| DSG/DSGD4    | Lys$^{537}$–Lys$^{584}$ | $\leq 16.4$                          |                                                             |
| DSG/DSGD4    | Lys$^{561}$–Lys$^{563}$ | $\leq 5.6$                            |                                                             |
| DSG/DSGD4    | Lys$^{589}$–Lys$^{593}$ | $\leq 6.1$                            |                                                             |
| DSG/DSGD4    | Lys$^{589}$–Lys$^{593}$ | $\leq 24$                            |                                                             |
| DSG/DSGD4    | Lys$^{589}$–Lys$^{593}$ | $\leq 24$                            |                                                             |
| DSG/DSGD4    | Lys$^{589}$–Lys$^{593}$ | $\leq 24$                            |                                                             |
| DSG/DSGD4    | Lys$^{593}$–Lys$^{597}$ | $\leq 6.3$                            |                                                             |
|              |                      | $\%$                                | $\%$                                                      |
| DSG/DSGD4    | $49.8$                | $50.2$                               |                                                             |
| DSG/DSGD4    | $55.7$                | $44.3$                               |                                                             |
| DSG/DSGD4    | $42.7$                | $57.8$                               |                                                             |
| DSG/DSGD4    | $48.8$                | $51.6$                               |                                                             |
| DSG/DSGD4    | $45.1$                | $54.9$                               |                                                             |
| DSG/DSGD4    | $53.4$                | $46.6$                               |                                                             |
| DSG/DSGD4    | $77.6$                | $22.4$                               |                                                             |
| DSG/DSGD4    | $89.8$                | $10.2$                               |                                                             |
| DSG/DSGD4    | $51$                  | $49$                                 |                                                             |
| DSG/DSGD4    | $45.4$                | $54.6$                               |                                                             |
| DSG/DSGD4    | $47$                  | $53$                                 |                                                             |
| DSG/DSGD4    | $50.3$                | $49.7$                               |                                                             |
| DSG/DSGD4    | $47.8$                | $52.2$                               |                                                             |
| DSG/DSGD4    | $55.6$                | $44.4$                               |                                                             |
| DSG/DSGD4    | $51$                  | $49$                                 |                                                             |
| DSG/DSGD4    | $51.3$                | $48.7$                               |                                                             |
| DSG/DSGD4    | $49.7$                | $50.3$                               |                                                             |
| DSG/DSGD4    | $45.4$                | $54.6$                               |                                                             |
| DSG/DSGD4    | $50.1$                | $49.9$                               |                                                             |
| DSG/DSGD4    | $48.9$                | $51.1$                               |                                                             |
| DSG/DSGD4    | $48.8$                | $51.2$                               |                                                             |
| DSG/DSGD4    | $45.5$                | $55.4$                               |                                                             |
| DSG/DSGD4    | $46.4$                | $53.6$                               |                                                             |
| DSG/DSGD4    | $51.4$                | $48.6$                               |                                                             |
| DSG/DSGD4    | $41.3$                | $58.7$                               |                                                             |
| DSG/DSGD4    | $48.1$                | $51.9$                               |                                                             |
| DSG/DSGD4    | $48.8$                | $51.2$                               |                                                             |
| DSG/DSGD4    | $44.9$                | $55.1$                               |                                                             |
| DSG/DSGD4    | $52.4$                | $47.6$                               |                                                             |
| DSG/DSGD4    | $46.2$                | $53.8$                               |                                                             |
| DSG/DSGD4    | $46.9$                | $53.1$                               |                                                             |
| DSG/DSGD4    | $46.9$                | $53.1$                               |                                                             |
| DSG/DSGD4    | $47.7$                | $52.3$                               |                                                             |

$^a$ The Co–Co interresidue distance constraints used were based on the length of the spacer arm, which is 7.7 Å for DSG and 11.4 Å for DSS. Concerning the flexibility of the lysine side chains, the following cut-offs are generally used: 20 Å for α-carbons of lysine cross-linked with DSG and 24 Å for α-carbons of lysine cross-linked with DSS (30).

$^b$ Representation (%) of individual cross-link isofrom (the ratio between the abundance of non-deuterated and deuterated cross-links). pNth1 was cross-linked by non-deuterated cross-linking agents (DSS and DSSG) in the presence of Ca$^{2+}$ and by four-times deuterated agents in the absence of Ca$^{2+}$.

DISCUSSION

The helix-loop-helix EF-hand Ca$^{2+}$-binding motif is a widespread and versatile sequence found in a large number of protein families (36, 37). The N-terminal part of S. cerevisiae contains sequence that closely resembles such an EF-hand motif (Fig. 1), suggesting the possibility that this sequence and the calcium binding play a role in the regulation of this enzyme activity (13). In this work, various techniques of structural biology, including HDX,
changes not only within the catalytic trehalase domain but mainly in the mechanism of the 14-3-3 protein-mediated activation of Nth1 because it enabled activation of mutants that are catalytically inactive in the absence of Ca\(^{2+}\).

The key role of the EF-hand like motif-containing region in the activation of pNth1 because it enabled activation of mutants that are catalytically inactive in the absence of Ca\(^{2+}\).

Site-directed mutagenesis of residues located at important positions within the EF-hand-like motif significantly affected the Bmh1-mediated activation of pNth1 (Fig. 2B, white bars), thus suggesting the essential role of this region in the activation process. This is in a good agreement with our previous HDX-MS experiments that revealed significant 14-3-3 protein-mediated structural changes not only within the catalytic trehalase domain but mainly in this region (20). Interestingly, the presence of Ca\(^{2+}\) recovered the Bmh1-mediated trehalase activity of most of the studied mutants (Fig. 2B, gray bars), with the exception of those where we mutated conserved positions 1, 5, and 12 of the EF-hand motif (mutants D114L, N118L, and D125L), which are directly involved in metal coordination (36, 37). The inability of the D114L and D125L mutants to become activated was not due to the lack of the Bmh1-mediated structural change, as documented by near-UV CD spectra (Fig. 3), but rather resulted from different conformational changes of the EF-hand-like motif. In support of that, Ca\(^{2+}\)-binding rescue mutants D114E and D125E showed high Bmh1-mediated activity but only in the presence of Ca\(^{2+}\). These data suggested that the calcium binding to the EF-hand like motif facilitates the 14-3-3 protein-mediated activation of pNth1 because it enabled activation of mutants that are catalytically inactive in the absence of Ca\(^{2+}\).

MS, chemical cross-linking, and SAXS, were used to investigate the mechanism of the 14-3-3 protein-mediated activation of Nth1 and, especially, the role of EF-hand like motif in this process.

The catalytic domain of Nth1 is a homodimer of two subunits and the EF-hand like-containing region of Nth1 forms a separate domain that interacts with both the outer surface of the Bmh1 dimer and the molecular envelopes of both Nth1 alone and the pNth1 complex.

### Table 3

| Cross-linker | Cross-linked residues | Ca–Ca distance from the homology model | Ca–Ca distance constraint from the cross-linking experiments | pNth1 (DSS(G)/DSS(G)D4) | +Ca\(^{2+}\) | −Ca\(^{2+}\) |
|--------------|-----------------------|----------------------------------------|-------------------------------------------------------------|--------------------------|----------------|----------------|
| DSG/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 55.5 | 44.5  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 49.0 | 51.0  |
| DSG/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 56.8 | 43.2  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 52.9 | 47.1  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 65.3 | 34.7  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 58.8 | 41.2  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 80.0 | 20   |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 60.1 | 39.9  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 66.7 | 33.3  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 59.3 | 40.7  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 68.3 | 31.7  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 71.2 | 28.8  |

### Table 4

| Cross-linker | Cross-linked residues | Ca–Ca distance constraint from the cross-linking experiments | Bmh1:pNth1 (DSS(G)/DSS(G)D4) | +Ca\(^{2+}\) | −Ca\(^{2+}\) |
|--------------|-----------------------|-------------------------------------------------------------|--------------------------------|----------------|----------------|
| DSG/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | 28.8 | 71.2  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | 40.2 | 59.8  |
| DSG/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | 34.7 | 65.3  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | 42.2 | 57.8  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | 31.3 | 68.7  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | 30.7 | 69.3  |

The Ca–Ca interresidue distance constraints used were based on the length of the spacer arm, which is 7.7 Å for DSG and 11.4 Å for DSS. Concerning the flexibility of the lysine side chains, the following cut-offs are generally used: 20 Å for Ca\(^{2+}\)–carbons of lysine cross-linked with DSG and 24 Å for Ca\(^{2+}\)–carbons of lysine cross-linked with DSS.

### Table 3

| Cross-linker | Cross-linked residues | Ca–Ca distance from the homology model | Ca–Ca distance constraint from the cross-linking experiments | pNth1 (DSS(G)/DSS(G)D4) | +Ca\(^{2+}\) | −Ca\(^{2+}\) |
|--------------|-----------------------|----------------------------------------|-------------------------------------------------------------|--------------------------|----------------|----------------|
| DSG/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 55.5 | 44.5  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 49.0 | 51.0  |
| DSG/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 56.8 | 43.2  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 52.9 | 47.1  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 65.3 | 34.7  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 58.8 | 41.2  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 80.0 | 20   |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 60.1 | 39.9  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 66.7 | 33.3  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 59.3 | 40.7  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤20 | ≤20 | 68.3 | 31.7  |
| DSS/DSDG4   | Lys\(^{270}\)–Lys\(^{275}\) | ≤24 | ≤24 | 71.2 | 28.8  |

The representation (%) of individual cross-link isoform (the ratio between the abundance of non-deuterated and deuterated cross-links) pNth1 bound to Bmh1 was cross-linked with non-deuterated cross-linking agents (DSS and DSG) in the presence of Ca\(^{2+}\) and by four-times deuterated agents in the absence of Ca\(^{2+}\).

Theabsence of Ca\(^{2+}\) derived from the cross-linking experiments in the presence and the absence of Ca\(^{2+}\) and their comparison with distance constraints derived from the homology model of the catalytic domain of Nth1

The EF-hand like-containing region of Nth1 forms a separate domain that interacts with both the outer surface of the Bmh1 dimer and the molecular envelopes of both Nth1 alone and the pNth1 complex.
catalytic trehalase domain, thus supporting our hypothesis that the conformation of this region modulates the 14-3-3-mediated structural changes within the catalytic trehalase domain and thus the resulting enzyme activity.

Therefore, based on our data, we suggest the following model of Nth1 activation. In the absence of the 14-3-3 protein, Nth1 adopts an extended rodlike conformation, and the trehalase activity is very small, probably as a result of the inaccessibility of the active site, as shown by the crystal structure of the homologous domain of the trehalase Tre37A from *E. coli* (Fig. 5B) (40). The 14-3-3 protein binding to the phosphorylated N-terminal segment of pNth1 induces a significant structural rearrangement of the whole Nth1 molecule. This conformational change probably increases the accessibility of the active site and thus activates the enzyme. The EF-hand-like motif-containing region forms a separate domain that interacts with both the 14-3-3 protein and the catalytic trehalase domain. The structural integrity of the EF-hand-like motif is essential for the 14-3-3 protein-mediated activation of Nth1, and calcium binding, although not required for the activation, facilitates this process by affecting its structure. Our data suggest that the EF-hand-like motif-containing domain functions as the intermediary through which the 14-3-3 protein modulates the function of the catalytic domain of Nth1.

FIGURE 7. SAXS scattering data and the low resolution structure of Nth1, Bmh1, and pNth1-Bmh1 complex. A, solution scattering pattern for Nth1, Bmh1, and the pNth1-Bmh1 complex. Scattering intensity *I* is plotted in relation to the scattering vector *s* (*s* = 4πsin*θ*/λ, where *2θ* is the scattering angle and *λ* is the wavelength). B, plot of the distance distribution functions P(r) with the maximum particle dimensions (*D* max) of 92, 183, and 127 Å for Bmh1, Nth1, and the pNth1-Bmh1 complex, respectively. C, superposition of the SAXS-based envelope (spheres represent the dummy residues) of Bmh1 with the theoretical model of Bmh1 (sequence 4–236). D, SAXS-based envelope of Nth1 alone. E, overlay of the rigid body model of the Nth1 (295–721)-Bmh1 complex with SAXS-based envelope. The envelope is shown in gray, the catalytic domain of Nth1 (sequence 295–721) is shown in magenta, and Bmh1 dimer (sequence 4–236) is shown in cyan. A rigid body model was prepared using homology models of the catalytic domain of Nth1 (295–721) and Bmh1 (20). The inset shows the binding interface between Nth1 (295–721) and Bmh1, where two of three observed intermolecular cross-links (Lys76(Bmh1)–Lys563(Nth1) and Lys145(Bmh1)–Lys393(Nth1)) are located.
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