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Crystallographic studies of a novel DNA-binding domain from the yeast transcriptional activator Ndt80

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The Ndt80 protein is a transcriptional activator that plays a key role in the progression of the meiotic divisions in the yeast *Saccharomyces cerevisiae*. Ndt80 is strongly induced during the middle stages of the sporulation pathway and binds specifically to a promoter element called the MSE to activate transcription of genes required for the meiotic divisions. Here, the preliminary structural and functional studies to characterize the DNA-binding activity of this protein are reported. Through deletion analysis and limited proteolysis studies of Ndt80, a novel 32 kDa DNA-binding domain that is sufficient for DNA-binding *in vitro* has been defined. Crystals of the DNA-binding domain of Ndt80 in two distinct lattices have been obtained, for which diffraction data extend to 2.3 Å resolution.

1. Introduction

The process of meiosis and sporulation in the yeast *Saccharomyces cerevisiae* is carried out through a highly regulated pathway (for a review, see Kupiec *et al.*, 1997). In many cases, the failure to express genes at the proper time and level will block the pathway and result in the failure of the cells to complete the process. It is therefore essential that the genes required for meiosis and sporulation be coordinately regulated. One key step in the pathway is the progression out of pachytene and initiation of the meiotic divisions. Up to this point, the cells have not committed to meiosis and can return to mitotic growth if switched to favorable environmental conditions. However, once the cells have initiated the meiotic divisions they must complete the process and form spores. Ndt80 is a transcriptional activator and is required for the transition into meiosis and activation of genes during the middle stages of sporulation (Xu *et al.*, 1995; Chu *et al.*, 1998; Hepworth *et al.*, 1998; Tung *et al.*, 2000). The Ndt80 protein binds specifically to a promoter element termed MSE (middle sporulation element; Chu & Herskowitz, 1998). This element is found in the promoters of many middle sporulation genes and is required for their Ndt80-dependent induction (Hepworth *et al.*, 1995; Ozsarac *et al.*, 1997; Chu *et al.*, 1998).

Although the Ndt80 protein binds specifically to the MSEs, it has no sequence similarity to any known DNA-binding proteins (Xu *et al.*, 1995). This suggests that Ndt80 may use a novel DNA-binding motif to bind to MSE DNA. To map the functional domains of Ndt80, we have constructed a series of deletions in the protein, purified these proteins to homogeneity and assayed the ability of these proteins to bind DNA. Further domain mapping by limited proteolytic digestion has defined a minimal Ndt80 domain that is required for MSE DNA-binding activity *in vitro*. We report here the overexpression, purification, characterization, crystallization and preliminary X-ray crystallographic analysis of the DNA-binding domain from Ndt80.

2. Experimental methods

2.1. Cloning

The C-terminally tagged Ndt80-hexa-His truncation plasmids were constructed by cloning a PCR-generated fragment containing the *NDT80* open reading frame coding for the desired amino acids into the *Nde*I and *Xho*I sites of pET21c (Novagen). The N-terminally tagged hexa-His-Ndt80 truncation plasmids were constructed similarly using pET15b (Novagen). Positive clones were identified by restriction analysis and protein expression in BL21 Codon Plus *Escherichia coli* cells (Stratagene), in some cases verified by Western blot analysis using anti-hexa-His antibodies (Sigma). The following amino-acid substitutions were introduced during PCR into the N-terminally tagged $Ndt80_{59–330}$ protein: M213T, D225N and S267F. None of these substitutions affects DNA binding *in vitro* when compared with the wild-type sequence in the context of $Ndt80_{1–409}$. Of the aforementioned substitutions, S267F was present in the C-terminally tagged $Ndt80_{1–409}$. This substitu-

tion in $Ndt80_{1-409}$ does not affect DNA binding *in vitro* when compared with the wild-type sequence.

2.2. Protein purification

Proteins used in the EMSA analysis were prepared from BL21 Codon Plus cells transformed with *E. coli* expression vectors expressing truncated forms of hexa-His-tagged Ndt80. Cells were grown in LB (Luria–Bertani media) with ampicillin and chloramphenicol to mid-log phase. Protein expression was induced by the addition of IPTG to a final concentration of 1 m*M* and cells were allowed to grow at 310 K for 3 h. Cells were harvested by centrifugation at 4500 rev min^{-1} for 30 min at 277 K. Cell pellets were suspended in 50 m*M* NaH_2PO_4/Na_2HPO_4 pH 7.8 buffer containing 1 *M* NaCl and 10 m*M* imidazole and then lysed in two passes through a French pressure cell at 6.9 MPa. Crude extracts were obtained by centrifugation of lysates at 35 000 rev min^{-1} for 35 min at 377 K. The lysates were filtered through 0.45 µm filters and passed over an Ni–NTA column (Qiagen). The protein was eluted with an imidazole gradient ranging from 10 to 250 m*M*. Fractions containing the desired protein were then combined and assayed for DNA-binding activity by electrophoretic mobility shift assay (EMSA).

Protein used for crystallization studies was further purified using a HiTrap Heparin column (Amersham Pharmacia Biotech). The protein was loaded in 50 m*M* HEPES pH 7.0, 0.5 *M* NaCl and 1 m*M* DTT and eluted by fractionation with a 0.5–2.0 *M* NaCl gradient. Ndt80-containing fractions of N-terminally hexa-His-tagged proteins were then combined, digested overnight at 277 K with thrombin in order to remove the N-terminal hexa-His tag and subjected to a final chromatographic separation on a HiTrap Heparin column using the same buffering conditions as for the first separation. The protein was concentrated using Centricons (Millipore). The concentrated protein was dialyzed against 50 m*M* HEPES pH 7.0, 0.2 *M* NaCl and 1 m*M* DTT and the protein concentration was determined using the Micro BCA Protein Assay (Pierce).

2.3. Limited proteolysis of Ndt80

Purified $Ndt80_{1-409}$ (50 µ*M*) was incubated with and without double-stranded DNA (70 µ*M*) containing the *SMK1* (sporulation-specific mitogen-activated protein kinase) MSE before subjecting the protein to cleavage by trypsin, chymotrypsin, elastase and V8 proteases (3, 8 or 24 µg ml^{-1}). The 10 µl reactions were incubated for 1 h at 297 K in 12 m*M* Tris–HCl pH 8.0, 0.5 m*M* EDTA, 0.1 *M* NaCl, 3 m*M* DTT and 20 m*M* HEPES pH 7.0. The samples were then subjected to SDS–PAGE. A comparison of the trypsin-digested products in the presence and absence of the MSE revealed an ∼30 kDa fragment that was clearly protected by the DNA. Samples were prepared for N-terminal sequence analysis by SDS–PAGE followed by electroblotting on a PVDF membrane, which was then stained with Coomassie. N-terminal sequence analysis was performed by the Protein Core Facility at Columbia University.

2.4. Electrophoretic mobility shift assays (EMSA)

Double-stranded oligonucleotides containing wild-type *SMK1* MSE were end-labeled with γ^{32}-P-ATP using polynucleotide kinase and purified by nucleotide-removal columns (Qiagen) according to the manufacturer's instructions. All binding reactions were carried out in 10 m*M* Tris–HCl pH 7.5, 40 m*M* NaCl, 4 m*M* $MgCl_2$, 6%(*w*/*v*) glycerol, 10 mg ml^{-1} BSA, 10 µg ml^{-1} of sonicated salmon sperm DNA and ^{32}P-labeled oligonucleotide pairs (3000 counts min^{-1}) in a total volume of 24 µl at room temperature for 30 min. All protein dilutions were made in 20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 1 m*M* EDTA, 1 mg ml^{-1} BSA, 5 m*M* β-mercaptoethanol and 1 m*M* phenylmethylsulfonyl fluoride. Samples were analyzed on a 6% polyacrylamide gel (run in 0.5× TBE buffer for 60 min at 200 V). Gels were dried after electrophoresis, exposed to a phosphor screen and scanned on a Model 425E Molecular Dynamics phosphorimager.

2.5. Crystallization of Ndt80

Form I crystals of $Ndt80_{59-330}$ were obtained by the hanging-drop vapor-diffusion method. Microseeding was used to obtain larger single crystals suitable for data collection. 0.5 µl of a solution containing finely crushed crystals stabilized in 1.6 *M* ammonium sulfate, 0.1 *M* sodium citrate pH 5.6, 50 m*M* HEPES pH 7.0 and 0.2 *M* NaCl was added to pre-equilibrated drops. The drops were then incubated at 294 K for crystal growth. The precipitant solution for form I crystals contains 1.6 *M* ammonium sulfate buffered at pH 5.6 using sodium citrate.

For the form II crystals, microseeding was also used to improve the quality of the crystals. In this case, 1 µl of microseeds suspended in the precipitant solution was added to 1 µl of the protein solution (1.9 m*M*) to form the hanging drop, which was then suspended over the precipitant solution. Drops were incubated at 298 K. The precipitant solution contained 1.85–1.9 *M* lithium sulfate buffered in 0.1 *M* sodium citrate at pH 5.6. The protein concentration used for both crystal forms was 1.9 m*M*.

Table 1
Summary of the data-collection statistics.

Values in parentheses were obtained in the highest resolution shell (2.9–2.8 Å for form I and 2.38–2.3 Å for form II).

	Form I	Form II
Wavelength (Å)	1.0716	1.5418
Resolution limit† (Å)	2.8	2.3
Space group	$P3_121$ or $P3_221$	$C2$
Unit-cell parameters		
a (Å)	63.96	101.28
b (Å)	63.96	64.90
c (Å)	285.16	92.42
β (°)	—	107.52
R_{sym}‡	0.067 (0.21)	0.084 (0.24)
$I/\sigma(I)$	18.6 (6.80)	15.8 (3.40)
Completeness (%)	98.7 (99.9)	95.7 (84.9)
No. of crystals used	1	1
Size of crystal used (mm)	0.05 × 0.12 × 0.12	0.2 × 0.08 × 0.02
Crystal-to-detector distance (mm)	200.0	148.2
Detector 2θ (°)	0	0
Oscillation step per image (°)	0.3	1.0
No. of images collected	330	181
Exposure time	10 s	30 min
No. of reflections rejected	2084	699
No. of molecules per a.u.	2	2
Calculated V_M (Å^3 Da^{-1})	2.4	2.8
Calculated solvent content (%)	47	54

† Although diffraction data extended to 2.3 Å in form I and 2.2 Å in form II, usable data extended to the resolution limit listed. ‡ $R_{sym} = \sum|I - \langle I\rangle|/\sum I$, where $\langle I\rangle$ refers to the average intensity of multiple measurements of the same reflection.

2.6. X-ray data collection

Form I crystals were characterized at the National Synchrotron Light Source, beamline X25. The crystals were soaked momentarily in a precipitant solution including 20% ethylene glycol as the cryoprotectant and then plunged into liquid nitrogen. A single cryoprotected crystal was used for each data set collected. Diffraction images were collected at 100 K on the Brandeis B4 CCD detector (204.8 × 204.8 mm, record length 2048 pixels). A total of 110° of data at a detector distance of 200 mm were collected using an exposure time of 10 s per oscillation angle of 0.3°. It was necessary to use a small oscillation angle and move the detector back to 200 mm in

order to resolve the closely spaced reflections resulting from the very long c cell axis (285.16 Å). We had previously observed that the crystals suffered from radiation damage and therefore did not attempt to collect data beyond 2.8 Å resolution, which would have required several different 2θ settings for the detector and an oscillation angle per image of 0.2°.

X-ray diffraction data for the form II crystals were collected on an R-AXIS IV image-plate detector. Data were collected at 108 K after soaking the crystals in precipitant solution containing 20% ethylene glycol as the cryoprotectant. A total of 181° were collected at a crystal-to-detector distance of 148.2 mm. Exposure times were 30 min for each 1.0° oscillation image. All X-ray diffraction images were indexed and processed using the *HKL* package (v. 1.97.2) (Otwinowski & Minor, 1997). See Table 1 for additional information regarding data collection.

3. Results and discussion

Ndt80 contains 627 residues. Initial deletion analysis of Ndt80 revealed that the DNA-binding domain resides in the N-terminal part of the protein (Fig. 1*c*). We then initiated purification of a fragment comprising residues 1–409. No apparent degradation was observed for the sample after the first chromatographic separation on a Ni–NTA affinity column. However, following subsequent ion-exchange chromatographic separations, we noticed that the protein was degraded to several shorter fragments. In an EMSA analysis, smaller fragments of the partially degraded $Ndt80_{1-409}$ retained DNA-binding activity (Fig. 1*b*; lanes 6–10).

To further delineate the DNA-binding domain of the protein, we performed limited proteolysis of $Ndt80_{1-409}$ with several different proteases in the presence and absence of MSE DNA. Limited digestion of the protein with trypsin in the absence of the MSE yielded a stable ∼30 kDa fragment (Fig. 1*a*). In the presence of DNA, there was a significant enhancement in the amount of this fragment. These results suggest that the ∼30 kDa fragment of Ndt80 forms a stable domain that is relatively resistant to proteolytic digestion and is further protected from digestion by the presence of the DNA. N-terminal sequence analysis of this fragment revealed that the N-terminus was residue 59 of Ndt80. To corroborate this result, we deleted the N-terminal 58 residues in the context of the $Ndt80_{1-409}$ fragment. The $Ndt80_{59-409}$ protein still retained its ability to bind the MSE *in vitro* (data not shown). We also prepared a protein containing residues 59–330 that would approximate the molecular weight of the fragment produced by limited proteolysis. When this protein was used in an EMSA with *SMK1* MSE DNA, we observed a shift with similar mobility to the most rapidly migrating band for complexes with the partially degraded $Ndt80_{1-409}$ (Fig. 1*b*). Moreover, $Ndt80_{59-330}$ binds to the MSE with similar affinity to the $Ndt80_{1-409}$ and $Ndt80_{59-409}$ fragments based on the quantification of the intensities of the shifted bands. This result suggests that $Ndt80_{59-330}$ is a minimal DNA-binding domain.

To determine the three-dimensional structure of the DNA-binding domain of Ndt80, we have pursued X-ray crystallographic studies. Crystallization experiments were performed using $Ndt80_{1-409}$, $Ndt80_{59-409}$ and $Ndt80_{59-330}$. No crystals were obtained for any of these proteins in an initial screen using Hampton Research Crystal Screens 1 and 2. However, inspection of the $Ndt80_{59-330}$ drops suggested that high ammonium sulfate conditions were the most promising. An ammonium sulfate screen (0.8–3.2 *M* in 0.8 *M* increments) was then performed at pH 4.6, 5.6, 6.5, 7.5 and 8.4. Crystals were obtained in this screen for 1.6 *M* ammonium sulfate at pH 5.6. Crystals grew at 294 K in 2–3 d in hanging drops containing 1.9 m*M* $Ndt80_{59-330}$, 50 m*M* HEPES pH 7.0 and 0.2 *M* sodium chloride mixed with an equal volume of a reservoir solution containing 1.6 *M* ammonium sulfate and 0.1 *M* sodium citrate pH 5.6, over which they were suspended. The size of the crystals was improved by microseeding as described in §2.

A second crystal form was obtained from the attempts to crystallize $Ndt80_{59-330}$ in a complex with DNA. In a Natrix screen (Hampton Research) of Ndt80 precomplexed with DNA, a promising condition was identified which included lithium sulfate in vapor hanging drops. Addition of 1 µl of

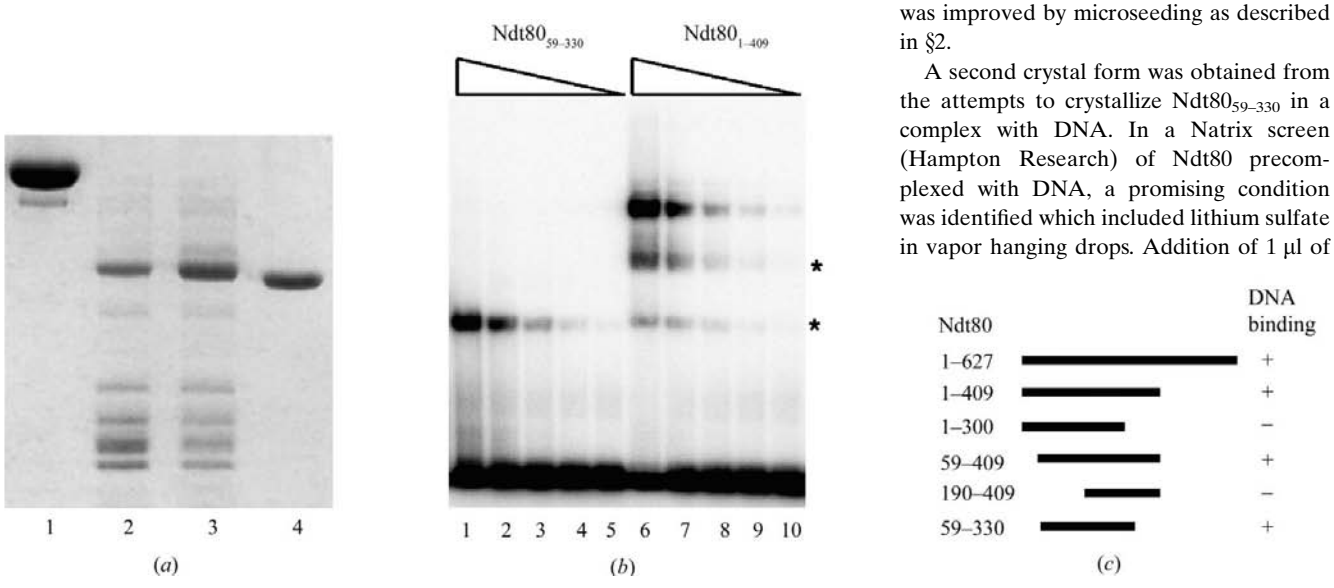

Figure 1
Deletion analysis of Ndt80. (*a*) Limited proteolytic digestion of Ndt80 resulted in a stable fragment. An SDS gel is shown including purified $Ndt80_{1-409}$ (lane 1), which was subjected to limited proteolysis with trypsin in the absence of DNA (lane 2) or in the presence of DNA (lane 3) and purified $Ndt80_{59-330}$ (lane 4). (*b*) Analysis of the DNA-binding activity of truncated Ndt80 proteins. An EMSA of purified $Ndt80_{59-330}$ (lanes 1–5) and $Ndt80_{1-409}$ (lanes 6–10) binding to the *SMK1* MSE. Lanes 1 and 6 contain approximately the same concentration of protein and subsequent lanes show fivefold serial dilutions of each of the protein samples. The asterisks show the position of the shifts produced by the proteolytic degradation products of the $Ndt80_{1-409}$ fragment. (*c*) A summary of the deletions in Ndt80 on DNA binding is shown. Deletions were created by PCR amplification of the *NDT80* coding sequence for the indicated amino acids followed by cloning into hexa-His-tagged bacterial expression vectors. After purification, the relative binding affinity of each of the truncated proteins was measured by EMSA using the *SMK1* MSE as shown in (*b*). A '+' indicates that an Ndt80-dependent shift was observed. A '–' indicates that a specific shift was not observed in the EMSA at the concentrations tested.

an ammonium sulfate containing solution to this drop resulted in the growth of single large crystals at 298 K. The resulting high-salt condition for the drop suggested that the protein alone had crystallized. In order to confirm that the crystals included protein and not the protein–DNA complex, a systematic screen was then performed for the protein alone, varying the lithium sulfate concentration from 1.0 to 1.9 *M* lithium sulfate buffered in 0.1 *M* sodium citrate pH 5.6. Crystals were obtained for conditions including 1.85–1.9 *M* lithium sulfate in the precipitant solution at 298 K. Microseeding was used in order to improve the size of the crystals.

The diffraction patterns for each of the crystal forms extend to high resolution, suggesting that the crystals are well ordered and suitable for structural analysis. Diffraction data extended to 2.3 Å resolution for form I crystals and 2.2 Å resolution for form II crystals. Based on the systematic absences, the space group of the form I crystals was determined to be either $P3_121$ or $P3_221$. The form II space group was determined to be *C*2. A summary of the data-collection statistics of both forms is shown in Table 1. Only one lattice spacing is found in both of the crystal forms, corresponding to the ∼64 Å cell constant. The fact that crystals were only obtained for $Ndt80_{59–330}$ suggests that this fragment is more homogeneous (perhaps conformationally as well as chemically) than the larger fragments that we prepared.

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