Yeast N-terminal Amidase

A NEW ENZYME AND COMPONENT OF THE N-END RULE PATHWAY*

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The N-end rule relates the in vivo half-life of a protein to the identity of its N-terminal residue. Tertiary destabilizing N-terminal residues asparagine and glutamine function through their conversion, by enzymatic deamidation, into the secondary destabilizing N-terminal glutamate, whose activity requires their enzymatic conjugation to arginine, of one primary the N-terminal destabilizing residues. We isolated a Saccharomyces cerevisiae gene, termed NTA1, that encodes an amidase (NT-amidase) specific for N-terminal asparagine and glutamine. Alterations at the putative active-site cysteine of the 52-kDa NT-amidase inactive the enzyme. Null nta1 mutants are viable but unable to degrade N-end rule substrates that bear N-terminal asparagine or glutamate. The effects of overexpressing NT-amidase and other components of the N-end rule pathway suggest interactions between these components and the existence of a multienzyme targeting complex.

The in vivo half-lives of damaged or otherwise abnormal proteins are often shorter than half-lives of their normal counterparts. Many regulatory proteins are also metabolically unstable. Short lifetimes of regulatory proteins allow for rapid adjustments of their concentrations through changes in the rates of their synthesis or degradation. Features of proteins that confer metabolic instability are called degradation signals, or degrons (Varshavsky, 1992). The essential component of one degradation signal, termed the N-degron, is a destabilizing N-terminal residue of a protein (Bachmair et al., 1986). A set of N-degrons containing different destabilizing residues is referred to as the N-end rule, which regulates the in vivo half-life of a protein to the identity of its N-terminal residue (for review, see Varshavsky, 1992)). The N-degron comprises two determinants: a destabilizing N-terminal residue and an internal lysine (or lysines) of a substrate (Bachmair and Varshavsky, 1989; Johnson et al., 1990; Hill et al., 1993; Dohmen et al., 1994). The Lys residue is the site of formation of a multienzyme targeting complex whose components include Nt-amidase (NTAl), N-recognin (UBR1), and Ubc2p, one of the uh-conjugating enzymes in yeast (Varshavsky, 1992). In S. cerevisiae, N-recognin is a 225-kDa protein (encoded by UBR1) that selects potential N-end rule substrates by binding to their N-dP residues Phe, Leu, Trp, Tyr, Ile, Arg, Lys, or His (Bartel et al., 1990; Baker and Varshavsky, 1991; Madura et al., 1993).

An apparently enzymatic conversion of N-terminal Asn and Gln in cytosolic proteins into Asp and Gln was demonstrated in both yeast and mammalian cells (Gonda et al., 1989), but the postulated amidase(s) responsible for this conversion remained unknown. Stewart et al. (1994, 1995) described purification, cDNA isolation, and analysis of porcine N-terminal amidase (NT-amidase) that deamidates N-terminal Asn but not Gln. We report a S. cerevisiae gene NTA1 that encodes an enzymatically distinct Nt-amidase; it deamidates either N-terminal Asn or Gln and is essential for the in vivo degradation of proteins bearing N-terminal Asn or Gln residues. NTA1p has previously been referred to as Dea1p (Varshavsky, 1992). In addition, we examine functional interactions between targeting components of the N-end rule pathway and consider a multienzyme targeting complex whose components include Nt-amidase (NTA1p), R-transferase (Ate1p), N-recognin (UBR1p), and Ubc2p, one of at least 11 ub-conjugating enzymes in S. cerevisiae.
Asn-/β-gal but not Asp-/β-gal or Arg-/β-gal (six mutants). All mutants had wt levels of Met-/β-gal and Ub-Pro-/β-gal. Mutants of the third class were in a single complementation group. One mutant, RBY56, was crossed to the parental strain BWG9a-1; sporulation and tetrad analysis of the resulting diploid showed a 2:2 segregation of high Asn-/β-gal levels. Two back-crosses of RBY56 to BWG9a-1 and BWG1-7a yielded the haploid RBY561 carrying the original mutation. Curing RBY561 of pUB23-N, transforming it with pUB23-X plasmids expressing different Ub-X-β-gal (Bachmair and Varshavsky, 1989; X = Asn, Gln, Asp, Glu, Leu, and Arg), and measuring β-gal activity in the transformants (Fig. 1B) showed that among the normally short-lived β-gal, only those bearing N-linked residues (Asn and Gln) accumulated to high levels in RBY561. Pulse-chase analyses of X-β-gals (Fig. 2) confirmed these results. The gene a mutation in which produced the phenotype of RBY561 was termed NTA1.

Isolation of NTA1—The nta1-1 strain RBY561, carrying pNL, a derivative of pUB23-N (expressing Ub-Asn-/β-gal) in which the UR3 marker had been replaced by LEU2, was transformed with S. cerevisiae genomic DNA library carried in the UR3, CEN4-based vector YCP50 (Rose et al., 1987). About 4 × 10^6 transformants were screened for white colonies (low levels of β-gal) on SG-X-gal plates; these results were confirmed using the o-nitrophenyl β-D-galactoside assay for β-gal. When the two transformants were cured of their library-derived plasmids on 5-fluoroacetic acid plates and retransformed on SG-X-gal plates, only one isolate yielded blue colonies (high levels of β-gal). Plasmid DNA (Hoffman and Winston, 1987) from these cells was used to transform E. coli MC1061 to ampicillin resistance. Transformants carrying YCP50 library-derived plasmids were distinguished from those carrying pNL by picking white E. coli colonies on LB/ampicillin/X-gal plates. A ~14.3-kb insert in the plasmid thus obtained (termed pRB8) complemented the defect in degradation of Asn-/β-gal in RBY561 and the other five nta1 mutants. This subclone was sequenced, revealing two complete open reading frames (ORFs) and a portion of a third (Fig. 3A). Comparisons of the amino acid sequences encoded by these ORFs with sequences in GenBank showed that one of the two complete ORFs had similarities to known amidotransferases. A low copy plasmid expressing only the putative NTAl portion of the ~4.3-kb insert (Fig. 3A) was constructed and found to complement all six nta1 mutants. Conversely, the low copy plasmid pBB82.5 that expressed only the smaller complete ORF was unable to complement nta1 mutants.

The nta1-Δ Allele—To construct nta1-Δ, the ~3.7-kb XhoI-SalI fragment of pRB8515 (Fig. 3A) that contained NTAl was subcloned into SacI-cut pUC9, yielding pUC9.7. The yeast UR3 gene, isolated as a 1.1-kb XbaI-KpnI fragment of pRB1 (Tobias and Varshavsky, 1991),
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Fig. 2. Metabolic stabilization of Gln-(β-gal) in the nta-1 mutant. A, lanes a–c, BWG1-7a (wt) cells that expressed Gln-(β-gal) (Ub-Gln-β-gal) were labeled with Translabel for 5 min at 30 °C, followed by a chase for 0, 10, and 30 min, respectively; extraction and immunoblotting. The EcoRI fragment of pRB8S13 that contained the rest of NTA1 was ligated into Smal/EcoRI-cut pRS316 (a vector; Sikorski and Hieter, 1989), yielding p195NTA1-CA, with the same fragment being confirmed by sequencing as well. Two diagnostic restriction fragments of the resulting plasmids were ligated into BamHI/SalI-cut YEplac195, yielding p195NTA1-CS and p195NTA1-CA, which were expressed, respectively, Nta1p-C187S and Nta1p-C187A from the PpTAJ promoter. The ha tag was linked to Nta1p-C187S and Nta1p-C187A by ligation of the 472-bp SpeI-HindIII fragment of p195NTA1-ha, together with either the 897-bp HindIII-XbaI fragment of p195NTA1-CS or an analogous fragment of p195NTA1-CA, into SpeI/XbaI-cut p195NTA1-ha, yielding, respectively, p195NTA1-CS-ha and p195NTA1-CA-ha.

Plasmids expressing S. cerevisiae Ate1p either alone or together with Nta1p were constructed as follows. The ~4-kb HindIII fragment of pRB8S13 containing NTAl was isolated (Lindquist et al., 1990) and subcloned into the HindIII-cut high copy plasmid YEplac195, yielding p195ATE1. The same fragment was also subcloned into the low copy plasmid YCplac33 (Gietz and Sugino, 1988), yielding p33ATE1. The 4.3-kb fragment that overexpressed both Nta1p and Ate1p, was constructed by inserting the above HindIII fragment into pY150NTAl that had been partially digested with HindIII, and screening the products in which the insert was located at the polylinker HindIII site.

Southern Hybridization and DNA Sequencing—DNA was isolated from S. cerevisiae as described by Hoffman and Winston (1987), digested with restriction endonucleases, and processed for electrophoresis and hybridization as described by Kurtz et al. (1990). Restriction fragments of the NTAl-containing a 4.3-kb fragment of pRB8S13 (Fig. 3A) were subcloned into Mlu13mp18 and M13mp19 (Ausubel et al., 1992) and were sequenced using the Sequenase kit (U. S. Biochemical Corp.). The entire 4.3-kb fragment was sequenced on both strands. The nucleotide sequence of NTAl (GenBank accession L55664) and the predicted amino acid sequence of Nta1p (Fig. 3B) were compared with sequences in the GenBank©EMBL data base using the GCG program (Deveraux et al., 1984) version 7.2, Genetics Computer Group, Madison, WI.

5′ Mapping of NTAl mRNAs—For mapping by primer extension, RNA was isolated from an exponential culture of BWG1-7a in YPD, using the phenol/chloroform/phenol procedure of Sparr et al. (1983). Poly(A)+ RNA was isolated from total RNA by chromatography on oligo(dT)-agarose (Pharmacia Biotech Inc.). Primer extension analysis was carried out as described by Teem and Rosbash (1983), using 3 μg of poly(A)+ RNA sample. The oligodeoxynucleotide primer was complementary to the coding strand of NTAl between nucleotide positions +34 and +67. For mapping by RNA-nuclease, the procedure described by Nyunoya and Lusty (1984) was used, with 50 μg of total RNA or 2 μg of poly(A)+ RNA sample. The probe was a single-stranded DNA from an M13 subclone containing the 400-bp Scal-SpeI fragment of pRB8S13 (NTAl nucleotide positions +336 to +63) uniformly labeled with 32P (Baker et al., 1992).

Phenotypic Characterization of the nta-1Δ Mutant—Assays measuring sensitivity of yeast cells to chronic heat stress (at 39 °C), sensitivity to canavanine, and survival in stationary phase were carried out as described by Finley et al. (1987). Sensitivity to acute heat stress was determined by exposing cells (which have been growing exponentially in YPD at 30 °C) to YPD at 50 °C for 0–15 min prior to plating on YPD to assay colony formation at 30 °C. Ability to use glycerol as a carbon source was assayed on YPD plates lacking glucose and containing 3% (v/v) glycerol. Ability to utilize Asn or Gln as a nitrogen source was assayed on synthetic media plates containing 0.17% yeast nitrogen base (YNB) and amino acids or ammonium sulfate (Difco). 3% glucose, 5% yeast extract, and other nutrients at standard concentrations (Sherman et al., 1986), and 0.1% (w/v) of either Asn or Gln as a major nitrogen source. Control plates lacked Asn and Gln, and in addition either contained or lacked 0.1% (w/v) ammonium sulfate.

We did not detect a significant difference between the S. cerevisiae nta-1Δ mutant and a congenic wt strain in their sensitivity to acute or chronic heat stress; in their survival at stationary phase after growth in rich or poor media, or upon starvation for either carbon or nitrogen; in their sensitivity to canavanine (a toxic arginine analog); in their ability...
Fig. 3. The NTA1 locus. A, ORFs are shown as arrow-shaped boxes indicating the direction of transcription. The incompletely sequenced ORFa is shown as a jagged-end box. Subcloned regions are also indicated, with (+) or (-) denoting their ability to complement the nta1-1 phenotype. Dashed lines delineate the region of NTA1 that has been replaced with URA3 in the nta1-Δ1 allele. Restriction sites: E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SalI; Sc, SacI; Spe, SpeI; X, XbaI. The scale (in kb) is above the map, with zero denoting the end of yeast genomic DNA insert in pRB8. Nucleotide sequence encompassing NTA1 (a 3747-bp region from the Xhol to the SalI site) has been submitted to GenBank (accession number L35564). An ORF located 346 bp upstream of NTA1 and oriented in the opposite direction has been identified as the RRN4 gene encoding the 125-residue A12.2 subunit of S. cerevisiae RNA polymerase I (Nogi et al., 1993). A partially sequenced ORFa 156 bp downstream of NTA1 is oriented in the opposite direction and encodes a protein of at least 740 residues. ORFa is transcriptionally active (data not shown) and encodes a protein highly similar to the product of a putative S. cerevisiae ORF on chromosome XI (GenBank accession numbers Z28200 and Z28201). The functions of either of these ORFs are unknown. B, deduced amino acid sequence of the Nta1p. Amino acid residues are numbered on the right. The sequence Ile-Gly-Ile-Cys-Met that matches a portion of the consensus active-site region of several amidotransferases and contains an essential Cys residue is doubly underlined. A black rectangle indicates the position of the 12-residue ha epitope tag in the Nta1p-ha derivative of Nta1. C, alignment of the Nta1p sequence with the 11-residue consensus sequence encompassing the putative active-site Cys residue of glutamine amidotransferases. Alternate amino acids in the consensus among these enzymes are shown above the consensus sequence. The region of identity between Nta1p and the consensus sequence is boxed. The essential Cys-187 of Nta1p is doubly underlined. D, nucleotide sequence at the 5′ region of NTA1. Positions of the major and minor 5′ ends of NTA1 mRNAs are indicated by closed and open circles, respectively. ATG codons are boxed. A start codon at position +1 was inferred in part from the 5′ mapping data. The in-frame ATG codon at position +31 is likely to be used as an in vivo translation initiation site as well (see text). The ATG codon at position +51 is indicated by an asterisk. This in-frame ATG is absent from the presently detectable NTA1 mRNAs; it is followed by two in-frame (underlined) stop codons. Motifs that are present in the promoter regions of genes encoding components of the N-end rule pathway (Fig. 6) are doubly underlined.
to grow on glycerol as a carbon source; and in their ability to utilize either Asn or Gin as a source of nitrogen. No short-lived yeast proteins detectable by a pulse-chase and two-dimensional electrophoresis were significantly stabilized in the nta1-1 mutant (data not shown). urb1Δ mutants (in which normally short-lived N-end rule substrates are metabolically stable) grow slightly (~3%) slower than wt cells and have a defect in sporulation, increased fraction of ascii with fewer than four spores (Bartel et al., 1990). The growth rate phenotype was not observed with the nta1-1 mutant (data not shown), while the sporulation of an nta1Δ/nta1Δ strain has yet to be investigated.

Protein Labeling, Pulse-Chase Analysis, and Immunoblotting—Pulse-labeling with Tran35 S-label (ICN), a chase in the presence of cycloheximide, preparation of cell extracts, immunoprecipitation with a polyclonal antibody to βgal (Sigma), and electrophoretic analysis of X-β-galgs by SDS-PAGE in 6% gels were carried out as described by Bachmaier et al. (1986), with slight modifications (Baker and Varshavsky, 1991). Immunoblotting of extracts from the nta1-1 strain RBY561 that has been transformed with either p195NTA1, p195NTA1- ha-CS, or p195NTA1-ha-CA was carried out after SDS-PAGE in a 10% gel, using a monoclonal anti-ha antibody (Bartel et al., 1990; Madura et al., 1993), a phosphatase-linked second antibody, a chromogenic phosphatase substrate, and procedures described by Tobias and Varshavsky (1991) and Baker et al. (1992).

RESULTS AND DISCUSSION

Isolation of nta1 Mutants and Cloning of the NTAl Gene—To screen for S. cerevisiae mutants defective in the N-end rule pathway, we used a strain carrying a plasmid that expressed Ub-Asn-βgal. Ub fusions are rapidly cleaved in vivo after the last residue of Ub, making possible the production of otherwise identical proteins bearing different N-terminal residues (Bachmaier et al., 1986; Baker et al., 1992). Since Asn-βgal is short-lived in wt cells (t½ of ~3 min at 30 °C; Bachmaier and Varshavsky (1989)), its steady-state level is low, and the corresponding yeast colonies are white on plates containing the chromogenic βgal substrate X-gal (Bartel et al., 1990). By contrast, cells that express long-lived X-β-gals such as Met-βgal (t½ > 30 h) have high βgal activity and form blue colonies on X-gal plates. Cells expressing Asn-βgal were mutagenized, plated on X-gal plates, and screened for blue colonies. These were tested further, and the putative nta1 mutants among them were identified as described under "Experimental Procedures."

The NTAl gene was cloned by complementation (see "Experimental Procedures"). The position of the start (ATG) codon of the NTAl ORF was inferred as to yield the largest ORF (Fig. 3). The 1,371-bp NTAl encodes an acidic (calculated pl of 4.9), 457-residue (51.8 kDa) protein. The codon adaptation index of NTAl (calculated according to Sharp and Li (1987)) is 0.125, characteristic of weakly expressed yeast genes.

An Essential Cytochrome in Nta1p—Weak sequence similarities were detected between Nta1p and an aliphatic amidase from Pseudomonas aeruginosa (Ambler et al., 1987) as well as several other amidotransferases. The substrates of aliphatic amidase (acetamide and propionamide) are the side chains of Asn and Gln, which are the substrates of Nta1p when these residues are present at the proteins' N termini. Nyunoya and Lusty (1986) identified an 11-residue region conserved among 7 glutamine amidotransferases from five species, including E. coli, S. cerevisiae, and Neospora crassa (Fig. 3C). The conserved region contains a Cys residue that could be labeled with reactive glutamine analogs in two of these enzymes, suggesting that this cysteine is a part of the active center (Nyunoya and Lusty, 1986). Nta1p contains a 5-residue sequence that is identical to the sequence in the middle of the 11-residue consensus stretch and includes the conserved cysteine (Cys-187) (Fig. 3C). The position of the start (ATG) codon but with two stop codons in between (Fig. 3D). If NTAl mRNA were to contain the ~51 ATG codon, initiation of translation at this codon would result in the synthesis of an 8-residue peptide and might also interfere with initiation at the downstream (+1) ATG codon. A short translated ORF is present in the 5' leader region of the yeast GCN4 mRNA, which encodes transcriptional activator of the regulon for amino acid biosynthesis (Hinnebusch and Liebman, 1991). A potentially more relevant example is CPAl, which encodes glutamine

![Fig. 4. Nt-amidase contains an essential cysteine residue.](image-url)
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**Fig. 5. The Nta1p protein.** Immunoblotting of extracts from cells that expressed Nta1p-ha containing either the wt Cys-187 (Nta1p-ha), Ser-187 (Nta1p-ha-CS), or Ala-187 (Nta1p-ha-CA). Equal amounts of total protein in extracts from the nta1-1 strain RBY561 that has been transformed with plasmids expressing Nta1p-ha-CS (lane b), Nta1p-ha-CA (lane c), or the untagged (control) Nta1p (lane d) were fractionated by SDS-PAGE and analyzed by immunoblotting with anti-ha antibody (see “Experimental Procedures”). Note the presence of two Nta-ha species (~53 kDa and ~52 kDa; see text).

amidotransferase, a subunit of carbamoyl-phosphate synthetase and component of the arginine biosynthetic pathway. In vivo translation of the upstream ORF in CPA1 mRNA yields a 25-residue peptide that down-regulates translation of the major CPA1 ORF in the presence of arginine (Werner et al., 1987). To determine whether NTA1 mRNA contains the -51 start codon, we used primer extension and S1 nuclease mapping (see “Experimental Procedures”). Both tests identified major 5' ends of NTA1 mRNA at positions -14 and -15 relative to the inferred (+1) start codon, with less abundant 5' ends at -15 and -13 (Fig. 3D). No transcripts extending beyond -19 were detected, indicating that most of NTA1 mRNAs lack the (-51) start codon. Thus, NTA1 appears not to be regulated in a way observed with CPA1. The 5' mapping also showed that in a minor but significant fraction of NTA1 mRNAs, their 5' ends are located immediately upstream, or even downstream of the inferred (+1) NTA1 start codon. Specifically, S1 mapping detected minor 5' ends largely at positions -4, -3, and +1, while primer extension detected sites at +2, +3, +4, +5, and also at +13 relative to the inferred (+1) start codon (the 5' end at +13 was not detected by S1 mapping) (Fig. 3D and data not shown). Thus, there exist NTA1 mRNAs that either lack the inferred (+1) start codon or contain it too close to the 5' end of the message for efficient initiation of translation at that position. An in-frame ATG is present 30 bp downstream of the inferred (+1) NTA1 start codon (Fig. 3D). Initiation of translation at this (+31) ATG should yield a 50.8-kDa protein lacking the first 10 residues of the inferred Nta1p (51.8 kDa). The (+31) ATG lies within a relatively favorable context for translation initiation (Kozak, 1992), with As in positions -3 and +4 (AA-GATGA), whereas the (+1) ATG is located in a less favorable context, with pyrimidines at -3 and +4 (TGAATGC).

**Immunoblot Analysis of Nta1p-ha**—Two nearly comigrating, Nta1p-specific, ha-containing species of ~52 and ~53 kDa, were observed upon immunoblot analysis of Nta1p-ha, the smaller species being less abundant and partially obscured by the band of the ~53-kDa Nta1p-ha (Fig. 5 and data not shown). The ~1-kDa difference between these species of Nta1p is consistent with the possibility that the translation start site of the larger (~53 kDa) Nta1p-ha is at the inferred (+1) ATG codon of NTA1 (predicted Nta1p-ha of 53.2 kDa, including the ha tag), whereas the smaller (~52 kDa) Nta1p-ha is initiated at the (+31) ATG codon (predicted Nta1p-ha of 52.2 kDa, including the ha tag) (Fig. 3D). The results of mRNA mapping are consistent with this interpretation, inasmuch as the set of NTA1 mRNAs contains both the species whose 5' ends encompass the (+1) ATG and the species whose 5' ends are located between the (+1) and the (+31) ATG (Fig. 3D). The 29-residue region between Asp-4 and Asp-34 in the larger Nta1p (Fig. 3, B and D) resembles mitochondrial translocation signals (von Heijne, 1986). However, more extensive testing will be required to verify the conjecture that the larger Nta1p species might be a mitochondrial protein.

A Null nta1 Mutant and Biochemical Aspects of Nt-amidase—A deletion/disruption allele of NTA1 (Fig. 3A) was used to produce the nta1-Δ1 mutant (see “Experimental Procedures”). As expected from the phenotype of the original nta1 mutants (Figs. 1B and 2B), Asn-βgal and Gln-βgal but not the other normally short-lived X-βgals were long-lived in the nta1-Δ1 mutant (t½ > 10 h) (data not shown), whereas they were short-lived in the congenic NTA1 strain (t½ of ~ 3 and 10 min, respectively (Varshavsky, 1992)). The normally long-lived X-βgals (bearing stabilizing N-terminal residues) remained long-lived in the nta1-Δ1 mutant. These results supported the conjecture that NTA1 encodes an amidase specific for N-terminal Asn and Gln. These data also indicated that Nta1p is the only such amidase in *S. cerevisiae*.

In a biochemical test, purified, 35S-labeled Asn-dihydrofolate reductase or Gln-dihydrofolate reductase (dihydrofolate reductase-based N-end rule substrates (Bachmair and Varshavsky, 1989)) were incubated with extracts prepared from *E. coli* that either expressed or lacked Nta1p, and then fractionated by isoelectric focusing in a polyacrylamide gel. Isoelectric points of both substrates became more acidic after incubation with the Nta1p-containing *E. coli* extract but not after incubation with the control (Nta1p-lacking) extract. Moreover, the isoelectric point of Met-dihydrofolate reductase, an otherwise identical protein bearing a non-amide N-terminal residue, was not altered by incubation with either of *E. coli* extracts. These findings confirmed the inferred deamidating activity of Nta1p and the confinement of this activity to N-terminal Asn and Gln.

Stewart et al. (1994) have purified a distinct Nt-amidase from porcine liver. They also isolated a cDNA encoding this enzyme (Stewart et al., 1995). In contrast to the 52-kDa yeast Nta1p, which deamidates either N-terminal Asn or N-terminal Gln, the activity of the 33-kDa porcine Nt-amidase is confined to N-terminal Asn. This finding (Stewart et al., 1994) suggests the existence of yet another mammalian Nt-amidase (the one specific for N-terminal Gln) and hence a bifurcation at the deamidation step in the N-end rule pathway of mammals but not of yeast. The amino acid sequence of the Asn-specific mouse Nt-amidase, deduced from sequences of the corresponding cDNA and the gene, is highly similar to the sequence of porcine Nt-amidase but lacks similarities to the sequence of yeast Nta1p.

No significant phenotypic differences (other than the metabolic stabilization of Asn-βgal and Gln-βgal) were observed between the nta1-Δ1 and congenic wt strains (see “Experimental Procedures”). Ubr1p (N-recognin) was recently found to be required for the peptide import in *S. cerevisiae*; ubr1Δ mutants do not express PTR2, which encodes a peptide transporter, and are unable to import peptides from the medium (Alagramam et al., 1995). It is unknown whether this function of Ubr1p is mediated by the N-end rule pathway or another Ubr1p-dependent mechanism. Unlike ubr1Δ mutants, the nta1-Δ1 mutant is able to import peptides.


4 S. Grigoryev, A. Stewart, S. Arfin, B. Bradshaw, and A. Varshavsky, unpublished data.

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al. (1990) found that overexpression of S. cerevisiae N-recognin (Ubr1p) accelerated the degradation of N-end rule substrates. We asked whether R-transferase (Ate1p) and Nt-amidase (Nta1p) are also rate limiting for certain classes of these substrates. Overexpression of R-transferase from a high copy plasmid in cells expressing either Glu-βgal or Gln-βgal decreased the levels of βgal activity by -2- and -3-fold, respectively (Fig. 6A). Thus, the arginylation of N-terminal Glu in Glu-βgal by R-transferase appears to be rate-limiting for the degradation of Glu-βgal and Gln-βgal. Even a weaker overexpression of R-transferase (from a low copy plasmid) resulted in a small but significant decrease of Glu-βgal (Fig. 6A). Previous work (Bartel et al., 1990; Balzi et al., 1990; Baker and Varshavsky, 1991; Dohmen et al., 1991; Madura et al., 1993) has shown that the concentration of an X-βgal test protein in yeast cells is a sensitive indicator of its metabolic stability.

Surprisingly, overexpression of Nt-amidase in cells expressing Glu-βgal increased the level of Gln-βgal by ~5-fold (Fig. 6A). In other words, overexpression of Nt-amidase inhibited the degradation of Gln-βgal. This result is likely to be related to an earlier finding that Gln-βgal, which bears an N-dt residue and therefore requires two modifications (deamidation and arginylation) prior to its binding by N-recognin, has a shorter half-life (t½ of ~10 min at 30 °C) than Glu-βgal (t½ of ~30 min), which bears an N-dp residue and is therefore only one step (arginylation) away from its binding by N-recognin (Bachmair and Varshavsky, 1989; Gonda et al., 1989). No such "inverse" order of half-lives was observed with Asn-βgal and Asp-βgal (t½ of ~3 min for both substrates) (op. cit.). The following assumptions are sufficient to account for these apparently paradoxical findings: (i) R-transferase arginylates Asp-βgal significantly faster than Glu-βgal; (ii) Nt-amidase is about equally effective in deamidating Asn-βgal and Gln-βgal; (iii) in wt cells, Nt-amidase exists largely as an Nt-amidase-R-transferase complex. Specifically, the R-transferase-mediated arginylation of Glu-βgal that has been produced from Gln-βgal by the Nt-amidase-R-transferase complex is presumed to occur kinetically in preference to the arginylation of Gln-βgal that reaches this complex directly from the bulk solvent, a feature known as "substrate channeling" in other multistage enzymatic reactions (Srere, 1987; Ovádi, 1991; Knowles, 1991; Negrutskii and Deutscher, 1989; Knighton et al., 1994).

This model accounts for the observed stabilization of Gln-βgal upon overexpression of Nt-amidase. Indeed, under these conditions, a greater fraction of Gln-βgal is converted into Glu-βgal by the free (overexpressed) Nt-amidase. As a result, a greater fraction of the deamidation-produced Glu-βgal will have to reach the Nt-amidase-R-transferase complex directly from the bulk solvent, a kinetically inefficient route to arginylation. The resulting delay in formation of Arg-Glu-βgal (which can be bound by N-recognin) would cause the observed stabilization of Gln-βgal in cells that overexpress Nt-amidase (Fig. 6A). Note that overexpression of Nt-amidase raised the level of Glu-βgal to that of Gln-βgal (Fig. 6A). This result is also predicted by the model, because the bulk of Gln-βgal in cells that overexpress Nt-amidase is deamidated by the free (overexpressed) Nt-amidase rather than by the less abundant form of Nt-amidase that exists in the complex with R-transferase. Another prediction of the model is that expression of R-transferase and Nt-amidase is likely to be coregulated in wt cells to maintain optimal ratios of these apparently interacting enzymes. This conjecture is consistent with the presence of common sequence motifs in the 5' regions of genes that encode components of the N-end rule pathway (Fig. 7 and below).

Overexpression of R-transferase Perturbs the Function of N-Recognin: Evidence for a Targeting Complex—Overexpression of R-transferase accelerated the degradation of Glu-βgal and Gln-βgal, which bear, respectively, an N-dt and an N-dp residue (Fig. 6A). However, the same overexpression inhibited the degradation of N-end rule substrates bearing a type 1 N-dp residue (Fig. 6B). The yeast N-recognin and its mammalian counterparts contain a binding site for type 1 (basic) N-dp residues Arg, Lys, and His, and another physically distinct binding site for type 2 (bulky hydrophobic) N-dp residues Phe, Leu, Trp, Tyr, and Ile (Reiss et al., 1988; Gonda et al., 1989; Baker and Varshavsky, 1991; Varshavsky, 1992). Overexpression of R-transferase increased by ~2-fold the steady state level of His-βgal (bearing a type 1 N-dp residue), and slightly but reproducibly decreased the level of Tyr-βgal (bearing a type 2 N-dp residue) (Fig. 6B). Overexpression of Nt-amidase resulted in a slight inhibition of His-βgal degradation, whereas overexpression of both Nt-amidase and R-transferase caused a stronger inhibition of His-βgal degradation equal to the one observed upon overexpression of R-transferase alone (Fig. 6B). Thus,
overexpression of R-transferase interferes with the function of the type 1 binding site in N-recognin, but slightly stimulates its type 2 binding site. The latter effect is consistent with the data indicating that an occupation of the type 1 site in either yeast or mammalian N-recognins with dipeptides bearing a type 1 N-dP residue stimulates the activity of the other (type 2) site in N-recognin (Gonda et al., 1989; Baker and Varshavsky, 1991).

These findings (Fig. 6B) are especially illuminating in conjunction with the data suggesting the existence of an Nt-amidase-R-transferase complex (Fig. 6A). Taken together, our results suggest that the 58-kDa R-transferase is physically associated with the 225-kDa N-recognin in proximity to its type 1 binding site. The “proximity” aspect of the postulated complex is invoked to account for the markedly different effects of overexpressed R-transferase on the functions of type 1 and type 2 binding sites in N-recognin (Fig. 6B). Specifically, a physical proximity of the bound R-transferase to the type 1 site in N-recognin is presumed to decrease the steric accessibility of this site to an N-end rule substrate bearing a type 1 N-dP residue that approaches the type 1 site directly from the bulk solvent. Conversely, a substrate that acquired Arg (a type 1 N-dP residue) through the arginylation by N-recognin-bound R-transferase would get access to the (nearby) type 1 binding site of N-recognin in kinetic preference to an otherwise identical substrate that has to reach the type 1 site directly from the bulk solvent.

This model (Fig. 8) postulates an Nt-amidase-R-transferase-N-recognin complex in which the access to the type 1 binding site of N-recognin directly from the bulk solvent may be partially obstructed by the bound Nt-amidase-R-transferase complex. Similarly, the access to the active site of R-transferase from the bulk solvent is presumed to be at least partially obstructed by the bound Nt-amidase. In this view, which accounts for the entire set of otherwise paradoxical interference data in Fig. 6, the spatially distinct type 2 binding site of N-recognin would not be inhibited by the presence of the Nt-amidase-R-transferase complex near the type 1 binding site of N-recognin, thereby explaining the observed dichotomy between the effects of overexpressed R-transferase on the reactions mediated by the type 1 and type 2 binding sites of N-recognin (Fig. 6B). The postulated targeting complex in Fig. 8 includes the Ubc2p Ub-conjugating enzyme, whose physical association with N-recognin was demonstrated directly (Dohmen et al., 1991; Madura et al., 1993).

N-recognin may partition in vivo between R-transferase-bound and free states. In this view, the free N-recognin would bind substrates bearing either type 1 or type 2 N-dP residues directly from the bulk solvent, whereas the R-transferase-bound form of N-recognin would be preferentially accessible to substrates bearing N-dR, N-dD, or type 2 N-dP residues (in comparison to substrates bearing type 1 N-dP residues). Substrates bearing N-dR or N-dD residues would be “channeled” to the type 1 binding site of N-recognin after their modification by the Nt-amidase-bound N-recognin-R-transferase complex (Fig. 8). The mechanics of channeling may involve diffusion of an N-end rule substrate in proximity to surfaces of the targeting complex, similar to the channeling mechanism described for the bifunctional enzyme dihydrofolate reductase-thymidylate synthetase, where the channeling of dihydrofolate results from its movement across the surface of the protein (Knighton et al., 1994). Overexpression of R-transferase would partition more of N-recognin into an R-transferase-bound form that is less active toward substrates bearing type 1 N-dP residues, resulting in a slower degradation of these substrates, as observed (Fig. 6B).

Common Sequence Motifs in Promoters of Genes That Encode Components of the N-end Rule Pathway—We examined 5′ regions of NTA1, ATE1, and UBR1 for common sequence elements and found two of them, 11- and 14-bp long, at different distances from the (inferred) start codons in each of these loci (Fig. 7). The 11-bp sequence TTTCAATGCTA (motif 1) is present in both UBR1 and ATE1; a single-mismatch variant of this sequence is also present in NTA1. Variants of the 14-bp consensus sequence CTTTTAATTTCAT (motif 2) are also present in NTA1, ATE1, and UBR1 (Fig. 7). No other S. cerevisiae gene in data bases contains both of these motifs, suggesting that these sequences are recognized by transcriptional regulators whose combination is specific for genes encoding targeting components of the N-end rule pathway. The 5′ regions of about 15 S. cerevisiae genes in data bases contain one or the other but not both of the two motifs. Three of these genes, UBI1, UBI2, and UBC2, encode components of the Ub system. UBI1 and UBI2 encode identical precursors of Ub (Özkaynak et al., 1987).
UBC2 (Fig. 7) encodes the Ubc2p Ub-conjugating (E2) enzyme that is physically associated with the UBR1-encoded N-recognin (Dohmen et al., 1991; Madura et al., 1993) (Fig. 8). However, unlike Ubr1p, Atel1p, and Nta1p, which appear to have no functions outside of the N-end rule pathway, Ubc2p has other functions as well, mediated by complexes of Ubc2p with recognins distinct from N-recognin (Sung et al., 1990; Sharon et al., 1991; Ellison et al., 1991).

Concluding Remarks—The NTA1-encoded N-terminal amidase (N-amidase) of the yeast S. cerevisiae mediates the conversion of tertiary (Asn or Gln) into secondary (Asp or Glu) destabilizing N-terminal residues in a substrate of the N-end rule pathway. The hierarchical organization of N-end rule, with its tertiary (N-d³) and secondary (N-d²) and primary (N-d⁰) destabilizing residues, is a feature that is more conserved in evolution than the Ub dependence of N-end rule pathways or the precise identity of enzymatic reactions that mediate the hierarchy of destabilizing amino acids in an N-end rule. For example, in bacteria such as E. coli, which lack Ub and Ubp-specific enzymes, the N-end rule has both N-d⁰ and N-d² residues (it lacks N-d¹ residues) (Tobias et al., 1991). However, the identities of N-d² residues in E. coli (Arg and Lys) are different from those in eukaryotes (Asp and Glu in S. cerevisiae, Asp, Glu, and Cys in rabbit reticulocytes) (Varshavsky, 1992). Bacterial and eukaryotic enzymes that implement the coupling between N-d³ and N-d² residues are also different: Leu, Phe-tRNA-protein transferase in E. coli and R-transferase in eukaryotes (Shrader et al., 1993; Balzi et al., 1990; Ciechanover et al., 1988). Nonetheless, both bacterial Leu, Phe-tRNA-protein transferase and eukaryotic R-transferase catalyze reactions of the same type (conjugation of an amino acid to an N-terminal residue of a polypeptide) and use the same source of activated amino acid (aminoacyl-tRNA).

Hierarchical organization of the N-end rule "distributes" domains that recognize specific destabilizing N-terminal residues among several proteins such as Nt-amidase, R-transferase, and N-recognin. It is likely that cells can produce different N-recognins and can also regulate either synthesis or activity of Nt-amidase and R-transferase. The resulting changes of N-end rule may occur in response to physiologically relevant alterations in the state of a cell, for example, during cell differentiation. A change of the N-end rule may provide a way to destroy a set of previously long-lived proteins or to stabilize a set of previously short-lived proteins. A variety of indirect evidence supports this conjecture (Varshavsky, 1992) but a definitive test remains to be done. Physiological substrates of Nt-amidase and R-transferase remain to be identified as well.

Experiments in which Nt-amidase, R-transferase, or both of these enzymes were overexpressed in S. cerevisiae suggested a substrate channeling in the N-end rule pathway and a specific organization of its multienzyme targeting complex (Figs. 6 and 8 and above). These ideas are consistent with the presence of two distinct sequence motifs in the promoter regions of genes encoding Nt-amidase, R-transferase, and N-recognin (Fig. 7).

No other S. cerevisiae gene in data bases contains both of these motifs, suggesting that they are recognized by transcriptional regulators whose combination is specific for genes encoding targeting components of the N-end rule pathway.

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REFERENCES

Amidase and the N-end Rule