Minireview

N\textsuperscript{x}-terminal Acetylation of Eukaryotic Proteins\textsuperscript{*}

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The two cotranslational processes, cleavage of N-terminal methionine residues and N-terminal\textsuperscript{1} acetylation, are by far the most common modifications, occurring on the vast majority of eukaryotic proteins. Studies with the yeast Saccharomyces cerevisiae revealed three N-terminal acetyltransferases, NatA, NatB, and NatC, that acted on groups of substrates, each containing degenerate motifs. Orthologous genes encoding the three N-terminal acetyltransferases and the patterns of N-terminal acetylation suggest that eukaryotes generally use the same systems for N-terminal acetylation. The biological significance of the N-terminal modification varies with the particular protein, with some proteins requiring acetylation for function, whereas others do not.

**Methionine Cleavage**

Cleavage of N-terminal methionine residues is by far the most common modification, occurring on the vast majority of proteins. Proteins from prokaryotes, mitochondria, and chloroplasts initiate with formylmethionine, whereas proteins from the cytosol of eukaryotes initiate with methionine. The formyl group is usually removed from prokaryotic proteins by a deformylase, resulting in methionine at N termini. The methionine at N termini is cleaved from nascent chains of most prokaryotic and eukaryotic proteins. Studies with the yeast Saccharomyces cerevisiae revealed three N-terminal acetyltransferases, NatA, NatB, and NatC, that acted on groups of substrates, each containing degenerate motifs. Orthologous genes encoding the three N-terminal acetyltransferases and the patterns of N-terminal acetylation suggest that eukaryotes generally use the same systems for N-terminal acetylation. The biological significance of the N-terminal modification varies with the particular protein, with some proteins requiring acetylation for function, whereas others do not.

Methionine excision occurs before completion of the nascent chain and before other N-terminal processing events, such as N-terminal acetylation (16, 17). S. cerevisiae contains two types of methionine aminopeptidases, Map1p and Map2p (18). Mutants containing either map1 or map2 null mutations are viable, but the map1 map2 double mutants are nonviable (19). Thus, removal of N-terminal methionine is an essential function in yeast, as in prokaryotes, but the process can be carried out by either of two enzymes. There are probably a number of reasons why N-terminal methionine removal is required for viability. For example, N-myristoylation is essential for growth, and N-myristoyltransferase requires a free N-terminal glycine (19). Also, N-terminal residues can be important for the activity of a variety of diverse proteins, such as actin (20, 21) and proteasome subunits (22, 23).

**Cotranslational N-terminal Acetylation**

N-terminal acetylation of proteins is catalyzed by NATs that transfer acetyl groups from acetyl-CoA to termini of α-amino groups. Similar to N-terminal methionine cleavage, N-terminal acetylation is one of the most common protein modifications in eukaryotes, occurring on approximately 85% of the different varieties of eukaryotic proteins but rarely on prokaryotic proteins (16). In vitro studies indicated that N-terminal acetylation of eukaryotic proteins occurs cotranslationally when there are between 20 and 50 residues extruding from the ribosome (16, 17).

N-terminal acetylation also can occur at internal sites after specific proteolytic processing of the completely translated protein, as in the cases of peptide hormones (24). Posttranslational acetylation of each of the different proteins occurs with different NATs having different specificities, and these differ from the set of NATs carrying out cotranslational acetylation.

Interestingly, internal threonine residues were acetylated when the propeptide region of several proteasome subunits was replaced by a ubiquitin sequence (22). When such artificial protein fusions are expressed in yeast, ubiquitin is rapidly cleaved by debiquitinating enzymes, presumably on the growing nascent chain, and N termini can serve as substrates for co-translational acetylation. On the other hand, after translation of normal mRNA containing the propeptide region and after near completion of proteasome assembly, subunits are processed at a conserved Gly-Thr motif, exposing the catalytic N-terminal Thr residues, which are not acetylated. Interestingly, internal N-terminal acetylation did not occur when the appropriate N-terminus was formed posttranslationally. Furthermore, artificial constructs with a Met-Thr terminus have the expected cleavage of methionine and N-terminal acetylation of the penultimate threonine residue, resulting in the lack of function.

Eukaryotic proteins susceptible to N-terminal acetylation have a variety of different N-terminal sequences with no simple consensus motif and with no dependence on a single type of residue. Eukaryotic proteins with serine and alanine termini are the most frequently acetylated, and these residues along with methionine, glycine, and threonine account for over 95% of the N-terminal acetylated residues (16, 17, 25, 26). However, only subsets of proteins with any of these N-terminal residues are acetylated, and none of these N-terminal residues guarantee acetylation.

Three Different NATs

Ard1p, Nat3p, and Mak3p are related to each other by amino acid sequence and are believed to be the catalytic subunits of three different NATs, designated NatA, NatB, and NatC, respectively, each acting on different sets of proteins having different N-terminal regions (27–29) (Fig. 1). Ard1p activity requires two subunits, Ard1p itself and Nat1p. A nat1 mutant was originally uncovered by screening a collection of heavily mutagenized strains for protein acetyltransferase activity in vitro (30). The previously identified

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The abbreviations used are: N-terminal, NH\textsubscript{2}-terminal, N\textsuperscript{-}terminal; NAT, N-terminal acetyltransferase.
ard1 mutant was first suspected to be related to nat1 because of certain similar phenotypes (30). Overexpression of both Ard1p and Nat1p subunits is required for increased NAT activity in vivo (30), and both interact with each other to form an active complex in vitro (31). The MAK3 gene encodes a NAT that is required for the N-terminal acetylation of the killer viral major coat protein, gag, with an N-terminal Met-Leu-Arg-Phe terminus. MAK3 was first identified from makk3-deficient mutants that did not assemble or maintain the L-A double-stranded RNA viral particle (28, 32). The co-purification of Mak3p, Mak10p, and Mak31p suggests that these three subunits form a complex that is required for N-terminal acetylation (33). Nat3p was originally identified on the basis of similarities of its amino acid sequence to those of Ard1p and Mak3p (27).

**Sequences Required for N-terminal Acetylation**

Previous attempts to predict N-terminal acetylation based on the properties of amino acid residues distributed along the N-terminal region were unsuccessful (16, 26, 34, 35). However, new insights on this problem have been provided by using yeast mutants deleted in one or another of these NAT genes. The substrate specificities for each of the Ard1p, Nat3p, and Mak3p enzymes were deduced from considering the lack of acetylation of the following groups of protein containing one or another of the Ard1p, Nat3p, and Mak3p acetyltransferases, respectively. In addition, acetylation of the NatD substrates requires all three NAs. Except for possibly the Met-Glu and Met-Asp NatB substrates, not all proteins with the designated N-termini are acetylated.

Primary amino acid sequence. Only the NatB substrates have common sequences that can be easily deciphered, and these are composed of Ac-Met-Glu, Ac-Met-Asp, Ac-Met-Asn-Asn, and possibly Ac-Met-Asn-Asn-Asn sequences. As emphasized by Moerschel et al. (3), all seven eukaryotic Met-Glu and Met-Asp proteins uncovered in literature and data base searches were N-terminally acetylated, but not any of the six prokaryotic proteins with the same N-terminal residues. Furthermore, all 11 normal yeast proteins having Met-Glu and Met-Asp were acetylated (3, 12, 27, 32). However, there are Met-Glu and Met-Asp iso-1-cytotrichomes with reduced efficiency of acetylation, including CYC1–838 (55%) and CYC1–878 (67%) (Table II). We suggest that all the NatB substrates contain any one of these required sequences, but acetylation is diminished by inhibitory residues. For example, from the result with the Ac-Met-Asp-Pro iso-1 (CYC1–878) having only 67% acetylation, one can suggest that adjacent proline residues diminish the action of Nat3p. Similarly, Moerschel et al. (3) demonstrated that antepenultimate proline residues can inhibit methionine cleavage from certain residues.

An alignment of the N-terminal region of NatD substrates and related sequences reveals an obvious requirement, but not sufficient, for acidic residues, Glu or Asp, at the antepenultimate position (Table I).

We suggest that NATs act on substrates with specific but degenerate sequences and that the activities can be diminished by suboptimal residues. We further suggest that acetylation can be diminished by the inhibitory residues situated anywhere on the nascent chain at the time of this addition. Thus, the degree of acetylation is the net effect of positive optimal or suboptimal residues and negative inhibitory residues. Furthermore, this lack of acetylation could be because of the absence of required residues or the presence of inhibitory residues. For example, the antepenultimate residue, Asn, in the CYC1–872 sequence Ac-Met-Asn-Asn can be considered optimal, allowing complete acetylation, whereas the antepenultimate residue, Phe, in the CYC1–849 sequence Ac-Met-Asn-Phe can be considered suboptimal, resulting in only 79% acetylation (Table II). On the other hand, the antepenultimate residues, Gin, in the CYC1–9-CB sequence Met-Asn-Gln prevents acetylation because it is not part of a required sequence. From the other point of view, the lysine residue in the CYC1–838 sequence Ac-Met-Glu-Phe-Lys and the Pro residue in the CYC1–878 Ac-Met-Asp-Pro-Leu can be considered inhibitory residues. Because the identities of required and inhibitory residues are unknown, the ability of a protein to be acetylated cannot be definitively predicted from the primary sequence. Also, it is unclear if an amino acid position is occupied by required or inhibitory residues. For example, in the NatC series Met-Leu-Arg-Any, represented by the CYC1–1201, L-A gag, JC33B, and Jv5p proteins (Table II), it is unknown whether the "Any" residue is part of the required sequence or if it depicts residues inhibiting the action of the Met-Leu-Arg sequence. Other examples of NatC substrates and related nonacetylated proteins are also presented in Table II.

The NatA substrates appear to be the most degenerate, encompassing a wide range of sequences, especially those with N-terminal residues of serine or alanine. In fact, approximately 50% of the Ser and Ala proteins, respectively, are acetylated (16). Examples of related acetylated and nonacetylated proteins having serine, alanine, or threonine termini are presented in Table II. Whereas the reason for the lack of acetylation of most of these proteins is unclear, the N-terminal region of many of the nonacetylated proteins related to both NatA and NatB substrates contains basic residues, lysine, arginine, and histidine, as well as proline residues, whereas some nonacetylated proteins related to NatC substrates contain acidic residues, such as glutamic acid. Because Erg7p but not the CYC1–1371 iso-1-cytotrichome is acetylated (Table II), one can suggest that inhibitory residues could occupy sites further than five amino acid residues from the terminus. Because the required and inhibitory residues may affect acetylation to various degrees and because inhibitory residues may possibly occupy various sites in the nascent chain, predicting acetylated and nonacetylated sequences is still unreliable.

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**FIG. 1. The pathways of N-terminal processing.** The two methionine aminopeptidases, Map1p and Map2p, cleave N-terminal methionine residues that have small side chains (glycine, alanine, serine, cysteine, threonine, proline, and valine), although methionine is retained on some proteins having penultimate residues of valine. Subsequently, N termini of NatA, NatB, and NatC substrates are acetylated by the Ard1p, Nat3p, and Mak3p acetyltransferases, respectively. In addition, acetylation of the NatD substrates requires all three NAs. Except for possibly the Met-Glu and Met-Asp NatB substrates, not all proteins with the designated N-termini are acetylated. 

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**Examples of N-terminal acetylation of NatD and other substrates**

| Type | Gene | Sequence | Normal | NatA ard1, nat1 | NatB nat3 | NatC mak3 | Ref. |
|------|------|----------|--------|----------------|----------|-----------|------|
| NatA | CYC1-785 | Thr-Glu-Phe-Leu-Ala- | 0 | 0 | 100 | 100 | 27 |
| NatB | CYC1-1383 | Ac-Ser-Glu-Ile-Thr-Ala- | 100 | 100 | 0 | 100 | 27 |
| NatC | CYC1-853 | Ac-Met-Glu-Phe-Leu-Ala- | 100 | 100 | 0 | 100 | 27 |
| NatD | CYC1-1162 | Ac-Met-Ile-Arg-Leu-Lys- | 94 | 100 | 100 | 0 | 27 |
| NatE | CYC1-963 | Ac-Gly-Glu-Phe-Leu-Ala- | 100 | 0 | 50 | 60 | 2 |
| NatF | CYC1-962 | Ac-Ala-Glu-Phe-Leu-Ala- | 100 | 0 | 10 | 40 | 2 |
| NatG | RPS20 | Ac-Ser-Asp-Phe-Glu-Lys- | 100 | 0 | 90 | 60 | 12 |
| NatH | RPS24A | Ac-Ser-Asp-Ala-Val-Thr- | 100 | 0 | 100 | 40 | 12 |

* B. Polevoda and F. Sherman, unpublished result.

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**Biological Importance of N-terminal Acetylation**

The finding that N-terminal acetylation, occurring posttranslationaly, causes increased melanotropic effects of α-melanocyte-stimulating hormone while it reduces the analgesic action of β-endorphin is the clearest example of the biological importance of this modification (39, 40). However, there are surprisingly few examples demonstrating the biological importance of N-terminal acetylation occurring cotranslationally.

Alterations at N-termini, including loss of acetylation, decreased thermal stabilities of NADP-specific glutamate dehydrogenase from *Neurospora crassa* (41) and the *E. coli* ribosomal 5 S protein (42). Nonacetylated cytoplasmic actin from cultured *Drosophila* cells is less efficient in the assembly of microfilaments than the acetylated form (43). Herskho et al. (44) observed that N-terminal-acetylated cytochrome *c* and enolase from mammalian sources were not degraded in *vitro*, in contrast to the nonacetylated correspondents from yeast, which were good substrates. Also, Matsuura et al. (45) suggested that N-terminal acetylation protected apo-cytochrome *c* from degradation in *vitro*. It should be emphasized that in these and other examples, the proteins lacking acetylated termini also had other differences in amino acid sequences. In contrast, R. E. Cohen et al. used NAT1* and nat1* yeast strains to prepare acetylated and unacetylated pairs of rat and yeast cytochrome *c*, respectively, and observed equal extents of ubiquitin conjugation within each pair, although both yeast forms were more highly ubiquitinated than both of the rat forms. Thus, the difference in ubiquitination of mammalian and yeast cytochrome *c* is because of differences other than N-terminal acetylation. Furthermore, Mayer et al. (46) observed ubiquitin-dependent degradation of N-terminal acetylated proteins in a crude reticulocyte lysate.

A significant means for assessing the general importance of N-terminal acetylation comes from the phenotypic defects in the ard1Δ (or nat1Δ), mak3Δ, and nat3Δ mutants. As described above, the silent mating loci, particularly HMLα, are partially derepressed in nat1− and ard1− mutants, leading to a partial mating defect of *MATα* strains. In addition nat1 and ard1 mutants exhibit defects of slow growth; inability of homoygous diploid strains to sporulate; and the failure to enter G0 when limited for nutrients (30). Presumably, these multiple defects are because of the lack of N-terminal acetylation of one or more specific proteins requiring acetylation for function. Diminished function by the lack of acetylation of the SIR3 protein, for example, can explain the partial derepression of HML (47), whereas diminished function of any one of a number of proteins in the cAMP pathway can explain the failure to enter G0 and the inability of homoygous diploids to sporulate (30). Also, as described above, the lack of N-terminal acetylation of the viral major coat protein, gag, in mak3Δ strains prevents assembly or maintenance of the viral particle (28). Also, mak3Δ strains do not utilize nonfermentable carbon sources at 37 °C, probably because of the lack of acetylation of a still unidentified protein (27, 28). Similar to the other mutants, nat3-Δ mutants exhibit multiple defective phenotypes, including lack of growth on YPG medium at 37 °C, reduced growth on medium containing NaCl, and reduced mating of the *MATα* cells. Such defects could arise from the lack of acetylation of any of a number of proteins essential for different processes. Whereas the unacetylated proteins responsible for these defects are not easily identified, the temperature and NaCl sensitivity could be attributed to lack of acetylation of act1p, which contains a normal N-terminal sequence, Ac-Met-Asp-Ser-Glu. Many actin mutants are temperature- and NaCl-sensitive, including act1-136, which has D2A replacement (48). Furthermore, nonacetylated actin of *Dictyostelium discoideum* prepared in *vitro* weakened the interaction with actinomyosin (20).

Not only can the lack of acetylation result in various defects, but abnormal acetylation also can prevent normal functions. The acetylation of the N-terminal catalytic threonine residue of various 20 S proteasome subunits causes the loss of specific peptidase activities (22). Obviously, both N-terminal acetylation and the lack of N-terminal acetylation have evolved to meet the individual requirements of specific enzymes.

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**Examples of similar sequences that are completely, partially or not acetylated**

The percentage acetylation values are estimates. Residues that appear to be interfering with acetylation are underlined; certain residues that appear to require acetylation are highlighted in black.
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Interestingly, the Met-Glu-Ile N-terminal sequence of Mak3p is characteristic of a NatB substrate. The acetylation of NatC substrates in the NatB mutant, characterizes a NatB substrate. The acetylation of NatC sub-

3 B. Polevoda and F. Sherman, unpublished results.

REFERENCES

1. Tsunasawa, S., Stewart, J. W., and Sherman, F. (1985) J. Biol. Chem. 260, 5382–5391
2. Sherman, F., Stewart, J. W., and Tsunasawa, S. (1985) Biosci. Rep. 3, 27–31
3. Moerschell, R. P., Hosokawa, Y., Tsunasawa, S., and Sherman, F. (1990) J. Biol. Chem. 265, 19638–19643
4. Hiei, H.-P., Schmitter, J.-M., Dessen, P., Fayat, G., and Blanquet, S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8247–8251
5. Dalluge, H., Bayen, S., and Pedersen, J. (1990) FEBS Lett. 266, 1–3
6. Ben-Bassat, A., Bauer, K., Chang, S.-Y., Myame, K., Boosman, A., and Chang, S. (1987) J. Bacteriol. 169, 751–757
7. Wingfield, P., Gruber, P., Turgut, M., Movva, N. R., Pelletier, M., Craig, S., Rose, K., and Miller, G. (1989) Eur. J. Biochem. 180, 23–32
8. Huang, S., Elliott, R. C., Liu, P. S., Koduri, K. W., Weckmann, J. L., Lee, J. H., Blair, L. C., Goh-Dastidar, P., Bradshaw, R. A., Bryan, K. M., Emason, B., Kendall, R. L., Kolacs, K. H., and Saito, K. (1987) Biochemistry 26, 8242–8246
9. Boissel, J. P., Kasper, T. J., and Bunn, H. F. (1988) J. Biol. Chem. 263, 8443–8449
10. Chang, Y.-H., Teichert, U., and Smith, J. A. (1990) J. Biol. Chem. 265, 19892–19897
11. Lowther, W. T., Orville, A. M., Madden, D. T., Lim, S., Rich, D. H., and Matthews, B. W. (1999) Biochemistry 38, 7678–7688
12. Arnold, R., Polevoda, B., Reilly, J. P., and Sherman, F. (1999) J. Biol. Chem. 274, 37005–37014
13. Prechtl, J. T., Cashman, D. P., and Kan, Y. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 24–27
14. Yamada, T., Kato, K., Kawahara, K., and Nishimura, O. (1986) Biochem. Biophys. Res. Commun. 135, 837–843
15. Devlin, P. E., Drummond, R. J., Toy, P., Mark, D. F., Watt, K. W., and Devlin, J. J. (1988) Gene (Amst.) 56, 13–22
16. Driessen, H. P., de Jong, W. W., Tesser, G. I., and Bloemendal, H. (1985) CRC Crit. Rev. Biochem. 18, 281–325
17. Kendall, R. L., Yamada, R., and Bradshaw, R. A. (1990) Methods Enzymol. 185, 398–407
18. Li, X. and Chang, Y.-H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12357–12361
19. Towler, D. A., Rubanks, S. R., Towery, D. S., Adams, S. P., and Glaser, L. (1987) J. Biol. Chem. 262, 1030–1036
20. Abe, A., Saeki, K., Yasunaga, T., and Wakabayashi, T. (2000) Biochem. Biophys. Res. Commun. 268, 14–19
21. Hansen, J. E., Marner, J., Pavlov, D., Rubenstein, P. A., and Reisler, E. (2000) Biochemistry 39, 1792–1799
22. Arend, N. S., and Hochstrasser, M. (1999) EMBO J. 18, 3575–3585
23. Kroll, M., Heinemeyer, W., Jager, S., Ulrich, T., Bochtler, M., Wolf, D. H., and Huber, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10976–10983
24. Dore, S. M., McDonald, R. K., Steveson, T. C., and Seel, C. A. (1990) Brain Res. Bull. 26, 30–99
25. Bradshaw, R. A., Brickey, W. W., and Walker, K. W. (1998) Trends Biochem. Sci. 23, 263–267
26. Persson, B., Flinta, C., von Heijne, G., and Jornvall, H. (1985) Eur. J. Biochem. 152, 520–527
27. Polevoda, B., Norbeck, J., Takakura, H., Blomberg, A., and Sherman, F. (1999) EMBO J. 18, 6155–6168
28. Tercero, J. C., and Wickner, R. B. (1992) J. Biol. Chem. 267, 20277–20281
29. Tercero, J. C., Riles, L. E., and Wickner, R. B. (1992) J. Biol. Chem. 267, 20270–20276
30. Tercero, J. C., Riles, L. E., and Wickner, R. B. (1993) J. Biol. Chem. 268, 3192–3194
31. Rigaut, G., Shevchenko, A., Rutz, B., Wilms, M., Mann, M., and Seraphin, B. (1999) Nature Biotechnol. 17, 1030–1032
32. Flinta, C., Persson, B., Jornvall, H., and von Heijne, G. (1986) Eur. J. Biochem. 153, 195–196
33. Augen, J., and Wold, P. (1986) Trends Biochem. Sci. 11, 494–497
34. Perrot, M., Saglio, F., Mini, T., Musirot, C., Schneider, U., Shevchenko, A., Mann, M., Jero, P., and Boucherie, H. (1999) Electrophoresis 20, 2280–2298
35. Garrels, J. I., McLaughlin, C. S., Warner, J. R., Fuchs, B., Litten, G., I., Kobayashi, R., Schwender, B., Vele, T., Anderson, D. S., Mesquita-Fuentes, R., and Payne, W. E. (1997) Electrophoresis 18, 1347–1360
36. Kimura, Y., Takakau, M., Tanaka, S., Sassa, H., Tanaka, K., Polevoda, B., Sherman, F., and Hirano, H. (2000) J. Biol. Chem. 275, 4635–4639
37. Symth, D. G., Massey, D. E., Zacharian, S., and Finnie, D. A. (1979) Nature 279, 252–254
38. Symth, D. G., and Zacharian, S. (1980) Nature 288, 613–615
39. Siddig, M. A. M., Kinsey, J. A., Fincham, J. R. S., and Keighren, M. (1980) J. Mol. Biol. 137, 125–135
40. Cumberlidge, A. G., and Isono, K. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 321–324
41. Hersko, A., Heller, H., Eytan, E., Kaklij, G., and Rose, I. A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7051–7055
42. Matsuura, S., Arpin, M., Hannum, C., Margoliash, E., Sabatini, D. D., and Morimoto, T. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4368–4372
43. Mayer, A., Siegel, N. R., Schwartz, A. L., and Ciechanover, A. (1989) Science 244, 1480–1483
44. Stone, E. M., Swanson, M. J., Romeo, A. M., Hicks, J. B., and Sternglanz, R. (1991) Mol. Cell. Biol. 11, 2253–2262
45. Wertman, R. F., Drubin, D. G., and Bostein, D. (1992) Genes 132, 337–350

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