Specificity of Cotranslational Amino-Terminal Processing of Proteins in Yeast†

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ABSTRACT: Polypeptides synthesized in the cytoplasm of eukaryotes are generally initiated with methionine, but N-terminal methionine is absent from most mature proteins. Many proteins are also N^{α}-acetylated. The removal of N-terminal methionine and N^{α}-acetylation are catalyzed by two enzymes during translation. The substrate preferences of the methionine aminopeptidase (EC 3.4.11.x) and N^{α}-acetyltransferase (EC 2.3.1.x) have been partially inferred from the distribution of amino-terminal residues and/or mutations found for appropriate mature proteins, but with some contradictions. In this study, a synthetic gene corresponding to the mature amino acid sequence of the plant protein thaumatin, expressed in yeast as a nonexported protein, i.e., lacking a signal peptide, has been used to delineate the specificities of these enzymes with respect to the penultimate amino acid. Site-directed mutagenesis, employing synthetic oligonucleotides, was utilized to construct genes encoding each of the 20 amino acids following the initiation methionine codon, and each protein derivative was isolated and characterized with respect to its amino-terminal structure. All four possible N-terminal variants—those with and without methionine and those with and without N^{α}-acetylation—were obtained. These results define the specificity of these enzymes in situ and suggest that the nature of the penultimate amino-terminal residue is the major determinant of their selectivity.

Depending on cellular location and function, the amino-terminal structure of mature eukaryotic proteins varies considerably. The amino acid sequence, however, rarely reveals the exact genetic instructions for this region of the molecule. In those proteins intended for export or for translocation to the inner membrane or matrix of the mitochondrion (Blobel, 1980; Shatz & Butow, 1983), amino terminal extension peptides involved in directing these proteins to their final locations are removed, obscuring the fate of the methionine residue. Most proteins found in other intracellular locations, including the cytoplasm, are also devoid of N-terminal methionine. Comparisons of mature protein sequences to genomic (or cDNA) sequences have established that in most of these cases only the methionine is removed. The specificity of the responsible enzyme, termed methionine aminopeptidase, has been described in general terms on the basis of the distribution of residues found in mature proteins and in naturally occurring mutants (Burstein & Schechter, 1978; Tsunasawa et al., 1985; Boissel et al., 1985; Sherman et al., 1986). These observations suggest that methionine is readily removed from proteins in which the penultimate amino acid has a small aliphatic side chain and that removal of methionine is inhibited in part or completely by the presence of an adjacent charged residue or one with a bulky side chain. In some instances, the residue adjacent to the penultimate amino acid also appears to affect the activity (Boissel et al., 1985; Prchal et al., 1986).

The methionine as well as the newly exposed penultimate residue can be further modified co- or posttranslationally, usually by the addition of N^{α}-acetyl moieties donated by acetyl coenzyme A (CoA) (Narita, 1958). Although more than one form of N^{α}-acetyltransferase is found in eukaryotic cells, the principal enzyme involved in N^{α}-acetylation is, like methionine aminopeptidase, loosely associated with ribosomes (Yoshida & Lin, 1972; Pestana & Pitot, 1975). The specificity of this enzyme, determined in the same fashion as with the peptidase, appears to favor N-terminal glycine, alanine, threonine, and serine residues as principal substrates (Jornvall, 1975; Bloemendal, 1977; Persson et al., 1985; Driessen et al., 1985). Other residues, including the initiator methionine residue itself, can also be modified. To date, however, no study has systematically determined which specific N-terminal modifications result when all 20 amino acids are substituted into the penultimate position of the same protein.

Thaumatin is a plant protein that has been used as a sweetener in some food products. Amino acid sequences of two closely related forms (I and II) (Iyengar et al., 1979; Edens et al., 1982) and a partial three-dimensional structure (back bone only) (de Vos et al., 1985) have been reported. The mature protein contains 207 residues and 8 disulfide bonds. It is normally exported from the plant cell and is therefore synthesized with an amino-terminal signal peptide, which is excised during translocation into the endoplasmic reticulum (Iyengar et al., 1979) to yield a protein having a free N-terminal alanine. When thaumatin is expressed in yeast from a synthetic gene that lacks the signal peptide, it is found as an insoluble monomer inside the cell. The N-terminus, in this case, is blocked. Expression levels are high (ca. 10–20% of the total yeast protein), and the protein is easily purified to homogeneity. In this paper, we describe the use of this mature thaumatin protein to study cotranslational N-terminal modifications in yeast. All 20 amino acids were placed into the penultimate amino acid position by site-directed mutagenesis of the gene, and the effects on N-terminal modification of the resulting proteins were studied.

Materials and Methods

Strain, Media, and Genetic Manipulation. Yeast strain BB25-1d (*MATa leu2-3, 2-112 gal2 can1*) was the host for expression of the thaumatin gene. BB25-1d is a His^{+} revertant of AH22 (Hinnen et al., 1978). Yeast transformations were

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performed according to the lithium acetate procedure (Ito et al., 1983), and transformants were selected on supplemented SD medium lacking leucine (SD-leu). Recipes for all of the media have been described (Sherman et al., 1979), with the exception that liquid SD-leu medium was brought to 50 mM succinate, pH 5.5, after autoclaving.

Transformant colonies were inoculated into YPD medium and grown to a reading of approximately 200 on a Klett-Summerson colorimeter (red filter). The YPD cultures were then diluted 1/100 into 1.5 L of supplemented SD-leu and grown at 30 °C for approximately 48 h. The cells were harvested by centrifugation (~4500*g*) and washed once with distilled water.

Construction of Thaumatin Expression Vectors with Various Amino Termini. A synthetic gene for thaumatin I, the sequence of which was based primarily on the reported amino acid sequence (Iyengar et al., 1979), was assembled manually by the phosphotriester method (Ito et al., 1982). In this construct, the amino-terminal alanine codon of the mature protein was preceded by that of an initiator methionine. The yeast phosphoglycerate kinase (PGK) promoter and transcription terminator were joined to the 5′ and 3′ ends of the thaumatin gene, respectively (unpublished results). This expression cassette was then cloned between the *Bam*HI and *Hin*dIII sites of pJDB209 (Beggs, 1981), a yeast–*Escherichia coli* shuttle vector, to form pING406. A synthetic DNA fragment containing *Sph*I, *Sst*I, and *Xho*I restriction sites was used to replace the *Sau*3A–*Hpa*I fragment of the thaumatin gene across the ATG codon to generate pSH11 (Figure 1). The approaches used to generate changes in the 5′ penultimate codon of the thaumatin coding sequences are described in Figure 1.

Purification of Thaumatin Analogues from Yeast. The pelleted cells (4–8 g) were resuspended in 20 mM cold sodium phosphate, pH 7.5, containing 20 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 1.0 mM ethylenediaminetetraacetic acid (EDTA) and lysed on a French press (five to six passes at 20 000 psi). Following each lysis pass, the cell debris was cooled on ice. The final lysate was centrifuged at 23000*g* for 20 min at 20 °C to pellet the cell debris and insoluble protein fraction. The supernatant, containing the soluble yeast proteins, was discarded. The cell pellet was resuspended in 4–5 volumes of aqueous 0.5 M NaCl (two washes) and 1.0 M urea (two washes) with a glass homogenizer being used to disperse the pellet. Centrifugation at 23000*g* for 10 min between each step repelleted the insoluble fraction. In each case, the supernatant was discarded.

Thaumatin was finally solubilized by twice extracting the pellet for 30 min at room temperature with 3–5 volumes of 10 mM sodium acetate, pH 4.5, containing 8 M urea (buffer A). Seventy to eighty percent of the thaumatin was solubilized after the first extraction. A second extraction resulted in a quantitative yield of urea-solubilized thaumatin. The pellet was discarded. Generally, all 20 variant constructs produced about 1 g of protein/100 g of cells (wet weight), which was equivalent to about 10–15% of the total protein in the extracts. The derivatives containing the cysteine, phenylalanine, and isoleucine substitutions produced somewhat less thaumatin (~5% of the total protein in the extract). The general uniformity of production indicates that the genetic manipulations of the thaumatin N-terminus did not quantitatively affect the expression of the plasmid gene.

The sample was loaded onto a column of CM-agarose equilibrated with buffer A. The column was washed until the absorbance at 280 nm of the flow-through was zero and then eluted with a linear NaCl gradient (0–0.4 M NaCl in buffer A). The thaumatin-containing fractions [analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis, Figure 2] were pooled and dialyzed against 0.1 M acetic acid. When necessary, the thaumatin pool was concentrated and further purified by chromatography on an HPLC TSK-125 size-exclusion column.

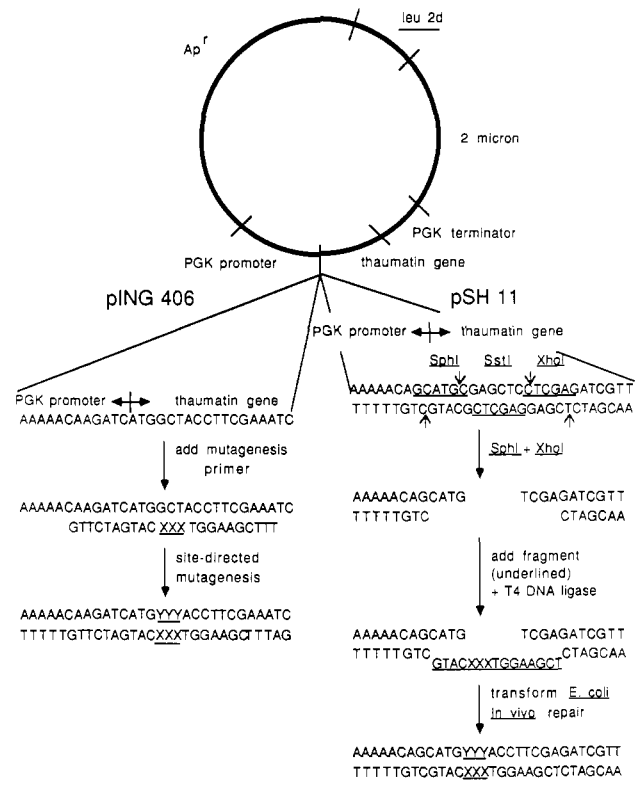


FIGURE 1: Construction of thaumatin expression vectors with various amino termini. Two fragments containing the yeast PGK promoter, the thaumatin gene, and the PGK terminator were cloned between the *Bam*HI and *Hin*dIII sites of pJDB209 (Beggs, 1981), a yeast–*E. coli* shuttle vector, to form pING406 and pSH11. Two approaches were used to generate DNA sequences coding for various amino acids at the penultimate codon of thaumatin. (1) A fragment with the sequence of the PGK promoter–thaumatin gene junction was first cloned into m13mp8. Synthetic 23-mer oligonucleotides (Elesen, Los Angeles, CA) complementary to this junction (except for the codon after the ATG) were used for site-directed mutagenesis to generate DNA sequences coding for proline, lysine, or valine. The wild-type sequence for alanine in pING406 was then replaced with the various DNA fragments containing the change. (2) Sixteen synthetic 15-mer oligonucleotides, varying only at the first codon of thaumatin after the initiator ATG codon, were made. These single-stranded fragments, which complement the cohesive ends of *Xho*I at the 5′-end and *Sph*I at the 3′-end, were ligated to *Sph*I- and *Xho*I-digested pSH11; the ligation mixtures were transformed into *E. coli* strain MC1061 (Casadaban & Cohen, 1980). The single-stranded region on the ligated plasmid apparently was repaired in vivo, and plasmids with correct insert sequences were produced. YYY represents the sequence of the changed codon after the initiator ATG and is complementary to XXX.

Protein Sequencing. Purified thaumatin and its derivatives were sequenced on an Applied Biosystems Model 470 sequencer. Samples were loaded onto a precycled glass fiber frit, allowed to air-dry, and run with the standard phenylthiohydantoin (PTH) cycle program. Individual samples were dried in a Savant Speed-Vac concentrator and resuspended in 10% acetonitrile. A Hewlett-Packard Model 1090 equipped with a 220 × 2.1 mm C-18 column was used for PTH analysis.

Cyanogen Bromide Cleavage. Purified samples of thaumatin that could not be sequenced were subjected to cyanogen bromide cleavage. Any disulfides that may have formed during protein purification were reduced with 100 mM dithiothreitol

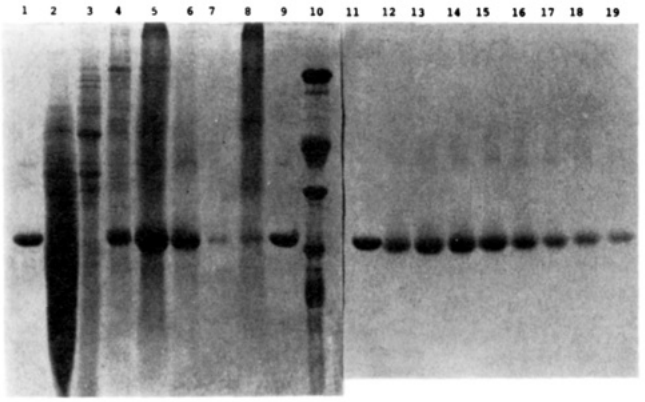


FIGURE 2: Analysis of the progress of thaumatin purification by SDS gel electrophoresis. The gel pattern shows the results of purifying yeast-produced thaumatin by the procedure outlined under Materials and Methods. (Lane 1) Plant thaumatin; (lane 2) cell extract supernatant (-PMSF); (lane 3) NaCl wash of cell pellet; (lane 4) 4 M urea extract of pellet; (lane 5) 6 M urea extract of pellet; (lane 6) second 6 M urea extract of pellet; (lane 7) third 6 M urea extract of pellet; (lane 8) SDS extract of pellet; (lane 9) plant thaumatin; (lane 10) Bio-Rad standards; (lane 11) plant thaumatin; (lanes 12–19) CM-agarose column fractions (salt elution).

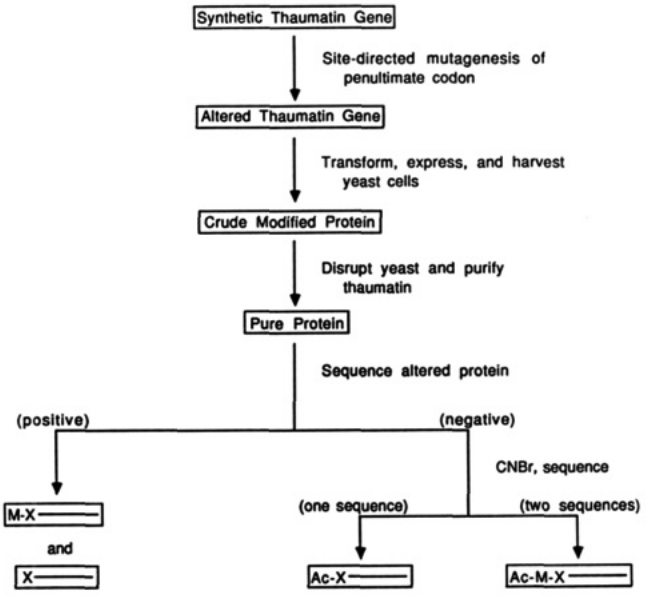


FIGURE 3: Schematic diagram of the protocol used to determine the N-terminal structure of each derivative.

at 37 °C, and the resulting sulfhydryls were blocked with iodoacetic acid (Crestfield et al., 1963). The reaction mixture was dialyzed exhaustively in the dark against water and lyophilized. Carboxymethylated thaumatin was incubated at room temperature in 70% formic acid with a 100-fold excess of CNBr over total protein. After 24 h, the CNBr and formic acid were removed from protein under vacuum and replaced with 0.1 M acetic acid. Thaumatin contains one residue of methionine (in addition to the initiator methionine) resulting in the formation of two fragments. The extent of cleavage was determined by HPLC gel filtration on a TSK-125 column or by SDS gel electrophoresis. These samples were then resuspended and subjected to N-terminal analysis.

Determination of N-Acetyl Groups. The presence of acetylated N-terminal amino groups on some thaumatin derivatives was established by 5-(dimethylamino)naphthalene-1-sulfonyl (dansyl) treatment at pH 3 of the products of hydrazinolysis, essentially according to the method of Schmer and Kreil (1969). Lyophilized samples were redissolved in 95% ethanol and analyzed by thin-layer chromatography on micropolyamide plates (Takagi & Doolittle, 1974). The separation was carried out with 1.5% formic acid in the first dimension and toluene–acetic acid (10:1) in the second dimension. Formic acid hydrazide and acetylhydrazine were used as standards to identify unknown spots.

RESULTS

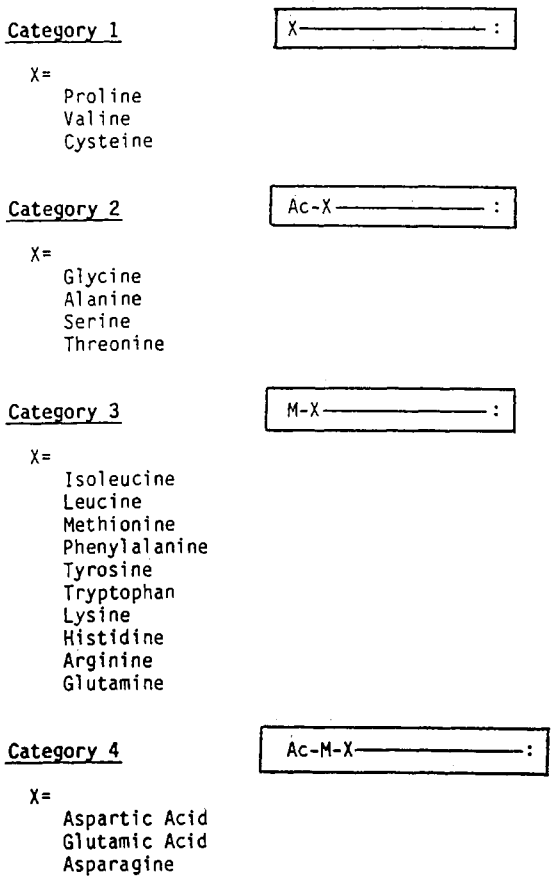
When the synthetic gene for thaumatin, corresponding to the wild-type sequence but lacking the signal peptide, was expressed in yeast, the protein was found to occur in a fully reduced state with a blocked alanine residue in the amino-terminal position. Thus, the yeast host translates this synthetic gene and treats the resulting thaumatin in a fashion similar to other cytoplasmic proteins, i.e., removal of the initiator methionine residue and modification of the newly exposed alanine. The absence of disulfides is also consistent with the cytoplasmic location of this protein.

These observations suggested that this synthetic construct could provide a set of convenient substrates to test the effect of different penultimate amino acids on the catalytic specificities of the yeast methionine aminopeptidase and N^{α}-acetyltransferase following alteration of the appropriate codon by site-directed mutagenesis. Toward this end, synthetic oligonucleotides were used to systematically change the penultimate residue to all 20 genetically directed amino acids. The mutant proteins were in turn isolated and characterized with regard to their N-terminal structures as outlined in Figure 3. Proteins not modified by the N^{α}-acetyltransferase (with or without methionine) were identified by direct analysis in the protein sequencer. Those containing acetyl groups were distinguished by CNBr cleavage followed by a second sequencer analysis. The extent of cleavage was monitored by SDS gel electrophoresis. Since thaumatin contains only a single methionine residue at position 112, this cleavage minimally results in the appearance of the internal sequence Asn-Phe-Ser-Ile (Iyengar et al., 1979). Those derivatives containing blocked initiator methionine residues also yielded an amino-terminal sequence, commencing with the penultimate residue and followed by the sequence Thr-Phe-Glu-. Derivatives that were found to be blocked after the first sequence analysis (suggesting the presence of an N^{α}-acetyl group) were subjected to dansyl analysis following hydrazinolysis to confirm the presence of the acetyl moiety. All such derivatives were found to contain such an entity (data not shown).[1]

Four types of N-terminal derivatives could be expected: (1) proteins containing an unblocked initiator methionine; (2) proteins containing a blocked initiator methionine; (3) proteins containing an unblocked penultimate residue; (4) proteins containing a blocked penultimate residue. In fact, all four kinds of derivatives were observed. Seven of the possible 20 derivatives of thaumatin showed blocked amino-terminal sequences while the remaining 13 had free α-amino termini that were identified by sequence analysis (Chart I). Of these 13, proteins with valine, proline, and cysteine as amino termini did not contain initiator methionine residues and in each case produced the expected amino-terminal sequence (with the alanine of the mature sequence replaced by the appropriate substitution). Some variability in the yield of N-terminal residues from the sequencer was observed with the cysteine derivative, suggesting partial N^{α}-acetylation may have occurred with this amino acid. However, CNBr cleavage did not pro-

[1] pING406 (containing alanine in the penultimate position) showed multiple spots on TLC in addition to the acetyl derivative. These unknown spots, which were absent in the other blocked samples, may represent N-terminal modifications in addition to acetylation.

Chart I: Effect of the Penultimate Amino Acid on Cotranslational Modifications of Plant Thaumatin Expressed in Yeast as Nonsecretory Mutants



duce additional levels of the residues arising from this region, indicating the absence of N^{α}-acetylated methionine. The remaining 10 derivatives that had open N-termini all possessed the initiator methionine followed by the expected replacement. These included the larger aliphatic amino acids (isoleucine and leucine), the aromatic amino acids (tyrosine, phenylalanine, and tryptophan), the basic amino acids (histidine, lysine, and arginine), and methionine itself, arising from a Met-Met sequence. Somewhat surprisingly, the glutamine derivative was included in this group.

Derivatives containing apparently blocked sequences (i.e., those refractory to Edman degradation) were subjected to CNBr cleavage and reevaluated in the sequencer (Figure 3). Four of these derivatives (alanine, glycine, serine, and threonine) showed only the internal sequence following this treatment. In contrast, the remaining three derivatives (aspartic acid, glutamic acid, and asparagine) showed not only the internal sequence but also a second sequence derived from the amino terminus (with the amino-terminal alanine replaced by the appropriate residue), indicating the presence of an Ac-Met structure. This finding is consistent with previous conclusions based on the N-terminal sequences of known proteins (Jornvall, 1975; Bloemendal, 1977). Interestingly, in the case of aspartic acid and glutamic acid, the observed amino-terminal sequence was only 50–75% of that found for the internal sequence, whereas for the asparagine derivative both sequences were present in equivalent amounts. Such a situation could arise for two possible reasons: (1) the CNBr cleavage of the blocked amino-terminal methionine was incomplete, or (2) only a portion of these two derivatives contained the blocked initiator methionine group. If the latter explanation is correct, the remainder of the protein must contain a blocked penultimate derivative, since no free aspartic or glutamic acid sequences were observed on direct analysis of the parental derivative.

Discussion

All eukaryotic proteins synthesized in the cytoplasm are presumably exposed to the ribosomally bound methionine aminopeptidase and N^{α}-acetyltransferase. For most cytoplasmic proteins (as well as some other organelle proteins), these modifications (or lack thereof) are not further altered, and the N-terminal structure of isolated mature proteins can therefore be taken as a reflection of the specificities of these catalysts. Since thaumatin expressed in yeast as a nonsecretory protein possesses all of the characteristics of a cytoplasmic protein, it should be entirely suitable as a model for examining cotranslational N-terminal processing. Noteworthy is the fact that the enzymes in question are apparently stoichiometric for each ribosome and, as they act on nascent chains, are therefore presumably uneffected by the levels of protein ultimately produced by the vector cell.

These results provide the first systematic analysis of the specificity of the methionine aminopeptidase and N^{α}-acetyltransferase in a eukaryote. For the peptidase, they confirm and extend the specificities predicted from isolated mature proteins (from a wide range of eukaryotes) as well as mutant isocytochromes *c* in yeast (Burstein & Schechter, 1978; Sherman et al., 1986; Tsunasawa et al., 1985; Boissel et al., 1985). As suggested from these analyses, the methionine was efficiently removed (quantitatively within the error of the sequence determinations) from seven derivatives in which the penultimate amino acid residue was glycine, alanine, threonine, serine, valine, proline, and cysteine. In all cases in which methionine was retained, no evidence (except as noted above for aspartic and glutamic acids) for partial removal to reveal unblocked penultimate residues was found. The limits of sequencer quantitation are such that we could not have detected (by difference) 10–20% blocked penultimate derivatives in our preparations.

The observed specificity of methionine aminopeptidase is consistent with the view that the presence of a charged residue in the penultimate position is inhibitory (Burstein & Schechter, 1978; Sherman et al., 1986; Tsunasawa et al., 1985), with the exception of cysteine, which is presumably largely protonated under the intracellular conditions in which protein synthesis occurs. Not deduced from previous observations, but predicted on the basis of side-chain size (Sherman et al., 1986), is the finding that aromatic side chains are inhibitory to this enzyme. The early studies of Jackson and Hunter (1970) showed that valine does not inhibit the enzyme in erythrocytes. This was found to be the case in these studies with the yeast enzyme. Since isoleucine and leucine prevent the excision of the methionine, the dimensions of the binding pocket for the penultimate residue in the catalytic site of the peptidase must be tightly restricted.

The acetylation of derivatives bearing glycine, alanine, serine, and threonine at the N-terminus is in agreement with what is found for a wide range of eukaryotic proteins (Jornvall, 1975; Bloemendal, 1977; Driessen et al., 1985) but is in contrast to the observations with mutants of iso-1-cytochrome *c* in yeast (Tsunasawa et al., 1985). The consistency of our results with the broader data base suggests that the conclusions of the cytochrome study may not reflect the general specificity of the transferase. In this regard, it is important to stress that other acetyl transferases that can act *post*translationally clearly exist as well as other enzymes that could give rise to altered N-terminal structures. Since isocytochrome *c* is an intramolecular protein of the mitochondrion, further processing in-

volving this location may well have occurred with those proteins.

These conclusions also suggest that the primary influence is the penultimate residue and that the adjacent residue(s) is (are) less important, if at all, in determining specificity. However, clear examples indicating effects of neighboring residues are known (Boissel et al., 1985; Prchal et al., 1986), and Augen and Wold (1986) have suggested that conformational effects contributed by residues in the first 40 amino acids may also bear on these specificities. Derivatives in which the initiator methionine is the substrate followed by an adjacent acidic residue clearly are N^{α}-acetylated, and asparagine, but not glutamine, also has this effect. Apparently this contribution to the specificity of the N^{α}-acetyltransferase is quite selective.

The interpretation of these data was based on the assumption that only these two enzymes were responsible for the production of the observed derivatives. In cases where the initiator methionine is retained (Chart I, categories 3 and 4), it is unlikely that further modifications would have occurred, although deacylation cannot be rigorously excluded. The des-methionine derivatives of category 1 and/or category 2 may have resulted from additional modifications. Thus, either or both could have been *first* acetylated followed by the removal of N^{α}-acetylmethionine (by an amino acylhydrolase, and then the category 2 proteins produced by a second acetylation) (Wold, 1984). In this case the enzyme(s) responsible might not be associated with ribosomes as such modifications could be post- rather than cotranslational. For this reason, specificity studies using synthetic peptides and homogeneous preparations of enzyme will be valuable correlative studies. However, they cannot test the specificities of the enzymes under their "working" conditions, an approach that synthetic genes and site-directed mutagenesis have now made possible.

ACKNOWLEDGMENTS

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Registry No. Methionine aminopeptidase, 61229-81-0; N^{α}-acetyltransferase, 97002-67-0.

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