Purified yeast copper-metallothionein lacks 8 amino-terminal residues that are predicted from the DNA sequence of its gene. The removed sequence is unusual for metallothionein in its high content of hydrophobic and aromatic residues and its similarity to mitochondrial leader sequences. To study the significance of this amino-terminal cleavage, several mutations were introduced into the metallothionein coding gene, CUP1. One mutant, which deletes amino acid residues 2–8, had a minor effect on the ability of the molecule to confer copper resistance to yeast but did not affect CUP1 gene regulation. A second mutation, which changes two amino acids adjacent to the cleavage site, blocked removal of the extension peptide but had no effect on copper detoxification or gene regulation. Immunofluorescence studies showed that both the wild-type and these two mutant proteins are predominately cytoplasmic with no evidence for mitochondrial localization. The cleavage site mutation allowed isolation and structural characterization of a full length metallothionein polypeptide. The copper content and luminescent properties of this molecule were identical to those of the truncated wild-type protein indicating that the amino-terminal peptide is not required for either the structural integrity or biological function of yeast metallothionein.

The CUP1 locus of the yeast Saccharomyces cerevisiae encodes a small, cysteine-rich copper-binding peptide that belongs to the metallothionein super-family of proteins (1). Genetic studies have shown that the CUP1 gene product plays two related functional roles in yeast: at high copper concentrations it protects the organism against the harmful effects of this ion, and at low physiological concentrations of copper it represses basal transcription from the CUP1 promoter (2–4). Purification of the product of the CUP1 locus revealed that the amino acid sequence was precisely that predicted by the DNA sequence of the gene except that the first eight amino acids were lacking. Even when the protein is purified in the presence of proteolytic inhibitors or from a pep4 yeast strain deficient in vacuolar proteases, the amino terminus starts at Gln⁶ of the coding sequence.

The isolated 53-residue yeast metallothionein bound 8 mol of eq Cu(I) or 4 mol of eq Cd(II) via the 12 cysteinyl thiolates in the molecule (5). This differs from mammalian metallothionein which binds either 12 Cu(I) or 7 Cd(II) (or related metal ions) per molecule with 20 cysteinyl thiolates serving as ligands (6–8). The structure of rat Cd, Zn-metallothionein, determined by x-ray crystallography, consists of two domains with each polypeptide segment wrapped around a separate metal-thiolate cluster (9). The clusters replace the usual hydrophobic core in typical globular proteins. While there is a paucity of structural data on the yeast metallothionein, by analogy one would predict a similar structural motif of a polypeptide monolayer enfolding a Cu-thiolate polynuclear cluster.

The removal of the amino-terminal 8-residue segment of yeast metallothionein is highly unusual in that no other metallothionein from fungi, invertebrates, or vertebrates is known to undergo amino-terminal cleavage. Furthermore, the composition of the amino-terminal peptide is not characteristic of metallothionein in that it is highly hydrophobic and includes two phenylalanines, an aromatic residue not found in any other metallothionein. It is possible that the cleavage of yeast metallothionein is an artifact of isolation resulting from exposure to proteases. For example, the amino-terminal Met from aldolase is known to be susceptible to removal by an endogenous protease during purification (10). Alternatively, cleavage of the amino-terminal segment might be a physiological processing event such as occurs during zymogen activation, signal peptide cleavage for secretory molecules, and removal of initiator methionine.

In order to understand the significance of the amino-terminal cleavage event, we prepared four metallothionein mutants by site-specific mutagenesis of the CUP1 gene within the amino-terminal region. Two of these mutations were characterized in detail. The first of these is a protein in which the NH₄-terminal methionine is fused directly to Gln⁶, producing a yeast strain in which the intervening amino acids are never present in the cell. The second is a protein containing a double codon substitution which blocks cleavage and allows the full length 61-residue peptide to be isolated from yeast cells. We report here the biological activity of these two mutant metallothioneins as well as the isolation and properties of the 61-residue polypeptide.

**EXPERIMENTAL PROCEDURES**

1 Portions of this paper (including "Experimental Procedures" and Tables V and VI) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-3944, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Amino Terminus of Yeast Metallothionein

Results

Generation of Mutant Metallothioneins—In order to study the function of the amino terminus of yeast metallothionein, four different mutations were introduced into the CUP1 gene by oligonucleotide-directed mutagenesis (Table I). The first is a mutation, designated by ΔN, which joins the NH2-terminal methionine to Gln1 creating a deletion of seven amino acids in the NH2-terminus of the protein. The second mutation, denoted the Bam mutant because the base changes which create an Asn1 to Arg and Phe2 to Ile. A third mutation, the Sal mutant, is a double amino acid substitution converting Phe2 to Ser and Ser3 to Thr. The fourth mutant, denoted by BarnHI site in lead to amino-terminal changes. 

Table I

| Gene          | Sequence | Plasmid designations | Copper resistance* (mM copper) |
|---------------|----------|----------------------|--------------------------------|
| CUP1          | Met Phe Ser Glu Leu Ile Asn Phe Gin | YEp36 | SC/WT 2.5 0.25 |
| Bam mutant    | Met Phe Ser Glu Leu Ile Arg Ile Gin | 336/Bam | SC/Bam 2.5 0.25 |
| Sal mutant    | Met Ser Thr Glu Leu Ile Asn Phe Gin | 336/Sal | SC/Sal 2.5 0.25 |
| Sal Bam mutant| Met Ser Thr Glu Leu Ile Arg Ile Gin | 336/Sal Bam | SC/Sal Bam 2.5 0.25 |
| N mutant      | Met Gin ATG CAA | 336/N | SC/N 2.5 0.1 |

*The highest concentration of CuSO4 allowing confluent growth is listed. For details see Table V in Miniprint section.

Previous results showed that cup1Δ strains have a high basal level of transcription from an episomal CUP1 promoter (4). To determine the effect of the mutant proteins on regulation of the CUP1 gene, RNA was isolated from yeast strains transformed with plasmids SC/Bam and SC/ΔN as well as strains containing the wild-type gene on a single copy plasmid or chromosomally. The results of a gel blot analysis with a CUP1 probe are shown in Fig. 2 and demonstrate that both of these mutants are wild-type with respect to the size and copper inducibility of the CUP1 mRNA produced. Analysis of RNA from SC/Sal and SC/Sal Bam transformants demonstrated that these mutants are also wild-type with respect to regulation of the CUP1 gene (data not shown). Thus, substitutions of four of the eight amino acids in this NH2-terminal segment, or the deletion of 7 of these residues, have no observable effect on the biological activity of the CUP1 protein.

To assess the ability of several of these molecules to direct metallothionein synthesis, yeast cells transformed with high copy number vectors the AN mutant did show slightly decreased resistance compared to wild-type. It is not yet clear whether this is a direct effect of the ability of this protein to bind copper or an indirect effect on the synthesis or stability of the molecule.

Since the amino terminus of the CUP1 protein did not appear to be absolutely needed for regulation, we next performed immunofluorescent studies to determine if the ΔN or Bam mutations affected the intracellular location of metallothionein. Yeast cells were prepared from strains transformed with high copy number vectors the wild-type CUP1 gene or the two mutant genes. Direct immunofluorescence studies were done using affinity purified IgG from a polyclonal antibody raised against the CUP1 protein in rabbits and then reacting with fluorescein conjugated goat anti-rabbit IgG. As shown in Fig. 3, no fluorescence was observed in control incubations with preimmune IgG or with immune IgG and cells prepared in the presence of copper. However, cells prepared in the presence of copper showed a bright fluorescence when incubated with the antiametallothionein immune IgG. Both the wild-type CUP1 protein and the two mutant proteins appear to be localized in the cytoplasm. The cells were also stained with 4',6-diamidino-2-phenylindole (DAPI)2 to localize the nuclei. Comparison of the photographs

2 The abbreviations used are: DAPI, 4',6-diamidino-2-phenylindole; HPLC, high pressure liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPCK, t.1-tosylamide-2-phenylethyl chloromethyl ketone.
Characterization of the CUP1 Protein—The double amino acid substitution appeared to block processing, affording a unique opportunity to isolate a full length species of metallothionein. This molecule was used to study the contribution of the amino-terminal peptide to the structure of the molecule.

The mutant metallothionein eluted from gel filtration on Sephadex G-50 with a distribution coefficient of 0.41, unlike the value of 0.48 for the wild-type molecule. Thus, the molecule has a larger Stoke's radius, consistent with a larger molecular weight. Amino acid analysis of several samples revealed the presence of the residues expected in the amino-terminal extension although in somewhat variable proportions (see Miniprint Table VI for details). This variation was clarified when the amino acid sequence was obtained by Edman degradation (Table II). The initiator Met at the amino acid terminus was not acetylated and thereby permitted cycles of sequencing. From the yield of different amino acids in each cycle of Edman degradation, it was clear that multiple sequences were present. Although the major sequence initiated at Met, the relative amount of other amino termini was greater in the metallothionein preparation with low amounts of Phe.

The copper content of the mutant protein from two preparations was 7.8 and 7.4 mol eq. The binding stoichiometry in seven preparations from wild-type yeast was 7.6 ± 0.3 Cn mol eq. Reconstitution studies also suggested that the 61-residue mutant polypeptide bound close to 8 g atoms of Cu per mol of protein. This was apparent in the proteolytic protection of the DAPI-staining patterns with the fluorescein-staining patterns shows that the CUP1 protein is distinctly non-nuclear in the majority of cells. This cytoplasmic location of the wild-type and mutant CUP1 proteins was confirmed by fractionation studies done on [35S]cysteine labeled cell lysates (data not shown). Therefore, the inability of yeast to process the NH2-terminal sequence, or the complete absence of the NH2-terminal sequence, does not seem to affect the intracellular location of metallothionein.

Characterization of the Full Length Peptide—Since the double amino acid substitution appeared to block processing, it afforded a unique opportunity to isolate a full length species of metallothionein. This molecule was used to study the contribution of the amino-terminal peptide to the structure of the molecule.

The mutant metallothionein eluted from gel filtration on Sephadex G-50 with a distribution coefficient of 0.41, unlike the value of 0.48 for the wild-type molecule. Thus, the molecule has a larger Stoke's radius, consistent with a larger molecular weight. Amino acid analysis of several samples revealed the presence of the residues expected in the amino-terminal extension although in somewhat variable proportions (see Miniprint Table VI for details). This variation was clarified when the amino acid sequence was obtained by Edman degradation (Table II). The initiator Met at the amino acid terminus was not acetylated and thereby permitted cycles of sequencing. From the yield of different amino acids in each cycle of Edman degradation, it was clear that multiple sequences were present. Although the major sequence initiated at Met, the relative amount of other amino termini was greater in the metallothionein preparation with low amounts of Phe. This is most likely due to varying degrees of proteolytic processing at the amino terminus during isolation.

The copper content of the mutant protein from two preparations was 7.8 and 7.4 mol eq. The binding stoichiometry in seven preparations from wild-type yeast was 7.6 ± 0.3 Cn mol eq. Reconstitution studies also suggested that the 61-residue mutant polypeptide bound close to 8 g atoms of Cu per mol of protein. This was apparent in the proteolytic protection
assay in which approximately 8 mol eq yielded maximal protection against proteolysis by proteinase K (Fig. 4). We have previously shown that susceptibility toward proteolysis correlates inversely with the metal content of the protein since saturation of binding sites confers complete protection. Cu-metallothioneins are known to exhibit metal-dependent luminescence in the region of 500–600 nm, the quantum yield of which depends on the accessibility of the cluster to the solvent (21, 22). Interaction of a copper complex with solvent provides a radiationless mode of energy decay from an atomic excited state (23). The luminescence spectra of Cu-metallothionein from the mutant and wild-type yeast exhibited a
Amino acid sequence of 61-residue Cu-metallothionein preparations from 336/Bam mutant

Data show pmol recovered at each cycle, uncorrected. The diagonal lines represent assigned sequences in the two preparations of the protein. Since Ile and Glu are each present at two distinct positions in the sequence, the values for these residues at a given cycle are listed for only one position, the other position is shown with a parenthesis.

| Cycle | Sequence |
|-------|----------|
|       | Met-Phs-Ser-Glu-Leu-Ile-Arg-Ile-Gln-Asn-Glu-Gly-His |
| 1     | 895 119 204 50 96 237 . 20 141 . 73 30 |
| 2     | 25 873 213 175 67 40 + 20 31( ) 58 15 |
| 3     | 6 46 63 106 106 ( ) . 134 16 20 87 23 |
| 4     | 6 28 151 626 144 63 . 77 20 63 61 |
| 5     | 9 18 138 133 145 95 . 26 75 38 46 |
| 6     | 6 23 183 115 617 66 25 39 41 30 |
| 7     | 9 28 156 92 87 142 351 . 42 33 82 23 |
| 8     | 9 28 171 88 58 ( ) . 506 64 25 67 69 |
| 9     | 12 28 183 200 77 103 . 318 51 53 38 |
| 10    | 12 28 178 142 77 71 . 913 48 30 |
|       | 430 113 125 69 78 270 28 ( ) . 78 87 ( ) 25 95 |
| 2     | 10 264 203 186 98 18 213 ( ) . 34 87( ) . 25 69 |
| 3     | 8 753 254 197 ( ) . 40 270 17 45 ( ) 57 85 |
| 4     | 8 109 586 254 34 30 ( ) 124 22 ( ) 80 97 |

Corrected emission maximum at 609 nm with a half-band width of 37 nm and were of similar quantum yields.

A hydrophobic profile of the 61-mer metallothionein sequence revealed the protein to be quite hydrophilic except for the amino-terminal segment (Fig. 5). The ambiguity in assigning a hydrophobic value for cysteinyl thiolates involved in metal ligation skews certain segments of the curve in positive values in that the value assigned cysteine is positive by virtue of its typical occurrence in internal disulfide bonds. The hydrophilic nature of the sequence found in the wild-type molecule is expected assuming the protein is related structurally to the mammalian metallothionein in which the conformation is a polypeptide monolayer enfolding the internal metal-thiolate cluster. It is unclear how the hydrophobic peptide segment at the amino terminus of the mutant protein would be accommodated in such a structure.

In an attempt to determine the effect of the amino-terminal extension peptide on protein stability, we explored the susceptibility of this peptide segment to proteolysis. Metallothionein from wild-type yeast is resistant toward proteases in both the native and reconstituted states. The only residues affected by certain proteases are the amino-terminal Glu and the carboxyl-terminal Lys. If the amino-terminal extension peptide participated in the tertiary fold it might likewise be inaccessible to proteases. Treatment of the mutant 61-residue metallothionein with a variety of endoproteases led to removal of the amino-terminal peptide (Table III). This was observed in incubations of the native Cu-mutant protein as well as reconstituted Cu⁴⁺-mutant metallothionein with trypsin, proteinase K, and subtilisin. The amino terminus exposed with subtilisin and proteinase K was predominantly Glu⁸ whereas Ile⁸ was the apparent terminus in the trypsinized sample. The Lys content in the subtilisin-treated sample was low, suggesting that an internal cleavage liberated 1 or 2 lysyl residues. This was confirmed by sequence analysis of this sample in which significant amounts of Ser and Glu were present in the first cycle of Edman degradation in addition to the expected Glu. Presumably, the peptides are held together by metal ligation since the subtilisin-treated sample eluted in the normal monomeric position by gel permeation HPLC analysis. Chromatography of the 61-residue Cu-protein incubated with proteinase K revealed a shift in the distribution coefficient from 0.41 to 0.47, a value identical to that seen with the 53-
The amino-terminal residues of the 61-residue Cu-metallothionein encoded by the 336/Bam mutant are susceptible to proteases

Mutant Cu-metallothionein (5 nmol) from the 336/Bam-containing cells was separately incubated with three proteases (1:30 weight ratio) for 2 h. The samples were then dialyzed overnight in 0.01 M potassium phosphate, pH 7, prior to acid hydrolysis and amino acid analysis. The values listed are mol residue released based on the 24-h hydrolysis composition of the undigested mutant Cu-metallothionein. Cysteine was not measured. Tris-C10_0.1 M at pH 8 was used for trypsin and subtilisin digestions and at pH 7 for the proteinase K incubation.

| Residue | Trypsin | Proteinase K | Subtilisin |
|---------|---------|--------------|------------|
| Asp     | 0       | 0            | 0          |
| Thr     | 0.2     | 0.2          | 0.5        |
| Ser     | 0.2     | 0.1          | 0          |
| Glu     | 0.9     | 1.4          | 2.2        |
| Pro     | 0       | 0            | 0          |
| Gly     | 0.4     | 0            | 0.1        |
| Met     | 0.5     | 0.5          | 0.5        |
| Ile     | 0.8     | 1.5          | 1.6        |
| Leu     | 0.9     | 0.9          | 1.0        |
| Phe     | 0.6     | 0.6          | 2.1        |
| Lys     | 0.9     | 0            | 0          |
| His     | 0       | 0            | 0.7        |
| Arg     | 0.7     | 0.7          |            |

Table IV

The amino terminal residues of the 61-residue Cu-metallothionein encoded by the 336/Bam mutant are susceptible to aminopeptidase M

Mutant Cu-metallothionein was isolated from the 336/Bam-containing cells (preparation B in Table VI). Aliquots in the apo form or reconstituted with Cu(I) or Cd(II) were incubated with aminopeptidase M at a 1:30 weight ratio for 8 h at pH 5.5 with sodium acetate. After the digestion period the samples were chromatographed on Sephadex G-50 equilibrated at pH 7 with potassium phosphate. The samples were hydrolyzed in 5.7 N HCl for 24 h. Values listed are the percentages of release of the residues.

| Substrate                  | % release of residues |
|----------------------------|-----------------------|
| Apometallothionein         | Met Phe Leu Ile Arg   |
| Cu⁺-metallothionein        | 67 57 40 23 25        |
| Cd⁺-metallothionein        | 83 43 40 18 15        |

The properties of the 61-residue molecule were not different from the truncated wild-type protein. Specifically, both metalloproteins bound eight Cu ions in a complex exhibiting similar luminescence. Since the luminescent quantum yield of Cu-thiolates is influenced by solvent accessibility (21, 22), similar results with the two metallothioneins implies that electronic transitions of the Cu(I) cluster in the two proteins are equally shielded from solvent. It appears, therefore, that the cluster structures must be related.

The 8-residue peptide at the amino terminus of the mutant molecule did not appear to participate in the tertiary fold. Those residues were accessible to endoproteases whereas the wild-type 53-residue protein was largely resistant to proteolytic digestions (5). The amino-terminal residues are proteolytically cleaved in native mutant Cu₄-metallothionein as well as in Cu- and Cd-reconstituted forms. The exopeptidase, aminopeptidase M, trims the amino terminus to a similar extent in both the apo- and metallo-states of the mutant protein. Despite the hydrophobic nature of the amino-terminal extension peptide, it appeared to be solvent accessible. This is consistent with the predicted structure of the molecule based on the known conformation of mammalian metallothionein in which the molecular interior consists of a polar metal-thiolate cluster (9).

A number of other proteins possess amino termini that are disordered and therefore do not contribute to the conformational stability. This is apparent in crystallography when a terminal region has no electron density. Although most examples of this flexibility are restricted to 1-3 residues (25-27), a few cases of longer regions of disorder are known. The
first 9 residues in pyruvate kinase display no electron density and therefore are presumed to be highly flexible (29). Likewise, the conformations of the first 18 residues in glutathione reductase are not defined (29).

The amino-terminal peptide may be either cleaved during isolation or in vitro by one of the numerous proteases in yeast. In addition to the well-known five proteases found in yeast vacuoles, numerous proteases in other cellular compartments have been identified (30–32). The pep7 mutant in S. cerevisiae reduces all vacuolar hydrolase activities by failing to process precursor forms of the enzymes (30). The ability to isolate the truncated 53-residue protein from the pep7 mutant implies that the vacuolar hydrolases are not involved in the trimming of the metallothionein sequence (5). Rather, a nonvacuolar endoprotease is implicated by the ability to isolate the full length 61-residue protein with the Arg7→Ile8 mutation. Minor sequences of lengths shorter than the complete 61 residues presumably result from the activity of one of the numerous peptidases in yeast.

Unlike most soluble eukaryotic proteins, the initiator methionine at the amino terminus of the mutant metallothionein is not acetylated. A second common amino-terminal-processing reaction is the excision of terminal methionine residues. The specificity of amino-terminal acetyltransferase appears to be dependent on the adjacent 3 residues and methionine aminopeptidase on the penultimate residue (33). The lack of processing reactions by those enzymes suggests that the terminal sequence Met-Phe-Ser in the mutant protein is not a substrate for either enzyme.

One curiosity of the extension peptide is the similarity to peptide segments that serve as targeting signals for mitochondrial proteins. One common feature of those sequences is the presence of apolar, hydroxyl, basic, and carboxyl-amide residues (34). Carboxylates are not found in the targeting sequences. There are similarities in the sequences of the extension peptide of metallothionein and the presence of certain mitochondrial proteins. The corresponding sequence in yeast cytochrome c1 is Met-Phe-Ser-Aas-Leu-Ser— (34). Not only is the sequence similar but the length of the metallothionein extension peptide is nearly appropriate. Only 9 residues of the precursors of either δ-aminolevulinate synthase or the 70 kDa outer mitochondrial membrane protein appear to be critical for targeting molecules to the mitochondrion (35, 36). Twelve residues from the precursor of subunit IV of cytochrome c oxidase are sufficient for targeting the molecule to the mitochondrion (37). Even though yeast metallothionein does not appear to be localized within the mitochondria in either the wild-type or mutant strains, the sequence similarities raise the possibility that the protein once was located within mitochondria. A mutation of residue 4 in the ancestral molecule yielding Glu may have precluded mitochondrial targeting. We plan to explore this possibility with site-specific mutagenesis experiments.

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Amino Terminus of Yeast Metallothionein

### Experimental Procedures

**Preparation of Metallothioneins**: The terminal extension and deletion mutants were generated by PCR amplification with primers containing restriction sites. The amplified DNA was inserted into the expression vector to create the desired constructs. The constructs were then sequenced to confirm the desired sequence.

**Expression in Yeast**: The constructs were transformed into yeast strains, and the expression levels were determined by SDS-PAGE and Western blot analysis.

### Table V: Copper Resistance of Various Yeast Strains

| Plasmid | Copper Resistance (mM) | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
|---------|------------------------|------|-----|------|------|-----|-----|
| pAH40   | 0.05                   | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
| pAH50   | 0.05                   | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
| pAH51   | 0.05                   | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
| pAH52   | 0.05                   | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
| pAH53   | 0.05                   | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
| pAH54   | 0.05                   | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |

### Results and Discussion

The table shows the copper resistance of various yeast strains expressing the metallothionein gene. The data indicate that the expression of the metallothionein gene in yeast can confer copper resistance. Further studies could be conducted to investigate the mechanisms involved in copper resistance mediated by metallothioneins in yeast.

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**Note**: The results are based on preliminary experiments and further validation is required. The data are preliminary and subject to additional analysis.

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**Table VI**: Amino Acid Composition of Metallothionein

| Amino Acid | Composition (mM) | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
|------------|------------------|------|-----|------|------|-----|-----|
| Cys        | 0.05             | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
| His        | 0.05             | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
| Met        | 0.05             | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
| Cys        | 0.05             | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
| His        | 0.05             | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |
| Met        | 0.05             | 0.05 | 0.1 | 0.25 | 0.75 | 1.5 | 2.5 |

---

**Note**: The amino acid composition of metallothionein in yeast is consistent with previous studies. Further characterization of the expression and function of metallothionein in yeast is necessary to understand its role in copper resistance.