Armitage, I. M., Otvos, J. D., Briggs, R. W., & Boulanger, Y. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 2974–2980.
Boulanger, Y., Armitage, I. M., Miklossy, K.-A., & Winge, D. R. (1982) *J. Biol. Chem. 257*, 13717–13719.
Bremner, I., & Young, B. W. (1976) *Biochem. J. 157*, 517–520.
Briggs, R. W., & Armitage, I. M. (1982) *J. Biol. Chem. 257*, 1259–1262.
Bühler, R. H. V., & Kägi, J. H. R. (1979) in *Metallothionein* (Kägi, J. H. R., & Nordberg, M., Eds.) pp 211–220, Birkhäuser, Basel.
Cousins, R. J. (1979) *Curr. Concepts Nutr. 37*, 97–103.
Day, F. A., Funk, A. E., & Brady, F. O. (1984) *Chem.-Biol. Interact. 50*, 159–174.
Durnam, D. M., & Palmiter, R. D. (1981) *J. Biol. Chem. 256*, 5712–5716.
Harris, R. K., & Mann, B. E. (1978) *NMR and the Periodic Table*, Academic Press, New York.
Kägi, J. H. R., & Vallee, B. L. (1961) *J. Biol. Chem. 236*, 2435–2442.
Kägi, J. H. R., Kojima, Y., Kissling, M. M., & Lerch, K. (1980) *Ciba Found. Symp. 72*, 223–237.
Karin, M., & Herschman, H. R. (1980) *Eur. J. Biochem. 107*, 395–401.
Kennedy, J. D., & McFarlane, W. (1977) *J. Chem. Soc., Perkin Trans. 2*, 1187.
Li, T.-Y., Kraker, A. J., Shaw, C. F., & Petering, D. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6334–6338.
Nielson, K. B., & Winge, D. R. (1983) *J. Biol. Chem. 258*, 13063–13069.
Nordberg, M., & Kojima, Y. (1979) in *Metallothionein* (Kägi, J. H. R., & Nordberg, M., Eds.) pp 41–117, Birkhäuser, Basel.
Ohi, S., Cardenosa, G., Pine, R., & Huang, P. C. (1981) *J. Biol. Chem. 256*, 2180–2184.
Otvos, J. D., & Armitage, I. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7094–7098.
Otvos, J. D., & Armitage, I. M. (1982) in *Biochemical Structure Determination by NMR* (Sykes, B. D., Glickson, J., & Bothner-By, A. A., Eds.) pp 65–96, Marcel Dekker, New York.
Piscator, M. (1964) *Nord. Hyg. Tidskr. 45*, 76–82.
Squibb, K. S., Cousins, R. J., & Feldman, S. L. (1977) *Biochem. J. 164*, 223–228.
Udom, A., & Brady, F. O. (1980) *Biochem. J. 187*, 329–335.
Vasak, M., & Kägi, J. H. R. (1983) *Metal Ions Biol. Syst. 15*, 213–273.
Vasak, M., Kägi, J. H. R., & Hill, H. A. O. (1981) *Biochemistry 20*, 2852–2856.
Vasak, M., Hawkes, G. E., Nicholson, J. K., & Sadler, P. J. (1985) *Biochemistry 24*, 740–747.
Winge, D. R., & Miklossy, K.-A. (1982) *J. Biol. Chem. 257*, 3471–3476.
Winge, D. R., Premakumar, R., & Rajagopalan, K. V. (1975) *Arch. Biochem. Biophys. 170*, 242–252.
Winge, D. R., Premakumar, R., & Rajagopalan, K. V. (1978) *Arch. Biochem. Biophys. 188*, 466–475.

Copper Metallothionein from the Fungus *Agaricus bisporus*: Chemical and Spectroscopic Properties†

Karl Münger and Konrad Lerch*
Biochemisches Institut der Universität Zürich, CH-8057 Zürich, Switzerland
Received April 19, 1985

ABSTRACT: The isolation and chemical characterization of the copper metallothionein from the common mushroom *Agaricus bisporus* are presented together with the complete amino acid sequence of the protein. It consists of 25 amino acids with a characteristically high cysteine content (28%) and binds 6 mol of copper per molecule. The protein reveals a high degree of sequence homology to both vertebrate metallothioneins (38.4% identity with the amino-terminal part of human metallothionein 2) and *Neurospora crassa* copper metallothionein (76.9% identity). The spectroscopic properties of *Agaricus* copper metallothionein are compared to those reported for *N. crassa* copper metallothionein [Beltramini, M., & Lerch, K. (1983) *Biochemistry 22*, 2043–2048] and suggest a very similar structure of the metal thiolate chromophore.

Metallothioneins (MTs)[1] are an ubiquitous class of low molecular weight proteins sequestering high amounts of heavy metals such as Zn, Cd, Hg, and Cu (Kägi & Nordberg, 1979). They were first isolated from equine kidney (Kägi & Vallee, 1960) and were shown to be responsible for the natural accumulation of Cd in this organ (Margoshes & Vallee, 1957). By now the amino acid sequences of MTs from a wide variety of sources including mammals, lower vertebrates, and invertebrates have been elucidated (Kägi et al., 1984).

Mammalian MTs have a typical chain length of 61 amino acid residues with the sequence positions of the 20 cysteinyl

† This work was supported by Swiss National Science Foundation Grant 3.285-0.82 and by the Kanton of Zürich.
* Author to whom correspondence should be addressed.

[1] Abbreviations: MT(s), metallothionein(s); PTH, phenylthiohydantoin; HPLC, high-pressure liquid chromatography; CD, circular dichrosism; T, trypsin; Quadrol, *N,N,N',N'*-tetrakis(2-hydroxypropyl)-ethylenediamine; R, regeneration of PTH derivative; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-6751$01.50/0

residues strongly conserved (Kägi et al., 1984). All the cysteines are involved in metal binding, and recent spectroscopic investigations have given strong evidence for the metals being bound in distinct metal thiolate clusters (Otvos & Armitage, 1980; Vašák & Kägi, 1981).

In contrast to vertebrate MTs, which bind different metal ions, the fungal MTs are reported to contain copper exclusively (Lerch, 1981). From the ascomycete *Neurospora crassa* a copper MT has been isolated and sequenced (Lerch, 1980). It consists of 25 amino acid residues and binds 6 mol of copper. Despite its rudimentary amino acid composition, sequence analysis revealed complete agreement in the positions of the seven cysteinyl residues with the amino-terminal part of the mammalian Cd/Zn MTs.

From the yeast *Saccharomyces cerevisiae* a low molecular weight copper-binding protein has been isolated (Weser et al., 1977). It shares with other MTs some spectroscopic properties (Bordas et al., 1983). The gene coding for this protein has recently been cloned and sequenced (Butt et al., 1984a,b; Karin et al., 1984). It codes for a polypeptide of 61 amino acid residues in length and exhibits only a remote sequence homology to vertebrate MTs.

The biological functions of copper MTs are still not fully understood, but they are assumed to play an important role in both metal storage and detoxification (Lerch, 1981; Lerch & Beltramini, 1983). In vitro experiments moreover suggest this protein acts as a metal donor during the biosynthesis of a number of metalloproteins (Beltramini & Lerch, 1982).

In the ascomycete *N. crassa*, MT occurs only in vegetatively growing cultures and is degraded during fructification (Lerch, 1980). We therefore studied the accumulation of Cu and Cd in the liquid-cultured mycelium of *Agaricus bisporus*. Here we report the isolation and chemical characterization as well as the amino acid sequence of a low molecular weight copper-binding protein from the liquid-cultured, copper-supplemented mycelium of the common mushroom *A. bisporus*. The data presented unequivocally demonstrate that this protein is a member of the superfamily of MT (Kägi & Nordberg, 1979).

Experimental Procedures

Materials. Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone was purchased from Worthington. Carboxypeptidase Y was a generous gift from J. T. Johansen, Carlsberg Laboratories, Copenhagen. Sephadex G-50 and Sephadex G-25 were purchased from Pharmacia. DE-32 cellulose (Whatman) was treated according to the direction of the manufacturer. The cation exchanger M-72 was obtained from Beckman. Chemicals employed for manual or automated Edman degradation were from Pierce or Fluka. HPLC-grade acetonitrile was purchased from Baker. All other chemicals were of the highest grade of purity commercially available and where not otherwise stated were used without further purification.

Isolation of the Protein. Mycelium of *A. bisporus* strain A-32 (Hauser Champignonkulturen, CH-8625 Gossau, Switzerland) was grown on a chemically defined medium (Hänseler et al., 1983) in liquid shaking cultures supplemented with $CuSO_4$ (final concentration, 250 μM). Cultures were harvested 72 h after inoculation by suction through G-4 glass filters (Schott), washed with distilled H_2O extensively, and lyophilized immediately. MT was purified by a combination of gel filtration and ion-exchange chromatography at 4 °C. Special care was taken to avoid air oxidation during the purification procedure (Minkel et al., 1980). Therefore all buffers were kept under a constant stream of nitrogen or argon. In a typical preparation, 20 g of lyophilized mycelium was pulverized in a Waring blender and extracted with 200 mL of 0.1 M sodium phosphate, pH 7.2. The suspension was centrifuged for 15 min at 48000*g* at 4 °C. The supernatant was applied on a Sephadex G-50 column (5 × 160 cm) in 10 mM Tris-HCl, pH 8.0. The low molecular weight copper-containing protein fraction was immediately applied on a DE-32 cellulose column (2 × 6 cm) in 10 mM Tris-HCl, pH 8.0. The column was developed with a linear gradient by using 250 mL each of 10 mM Tris-HCl, pH 8.0, and 10 mM Tris-HCl, pH 8.0, plus 0.1 M NaCl. Further purification was achieved by passage over Sephadex G-25 (2 × 80 cm) in 2 mM potassium phosphate, pH 7.5. The purified protein was either used immediately or kept at −80 °C under argon.

Modification of MT. MT was rendered metal free by exposure to 0.1 M HCl for 4 h at room temperature, followed by gel filtration on Sephadex G-25 (2 × 45 cm) in 7% formic acid. Thionein was reacted either with 4-vinylpyridine (redistilled) at pH 7.5 (Friedman et al., 1970) or with ethylenimine at pH 8.0 (Cole, 1976). The modified proteins were recovered by gel filtration on Sephadex G-25 (1 × 45 cm) in 7% formic acid. The S-aminoethylated thionein was submitted to tryptic digestion essentially as described elsewhere (Lerch et al., 1982).

Purification of Peptides. Tryptic peptides of S-aminoethylated thionein were fractionated on Beckman M-72 resin (0.9 × 20 cm) in 0.05 M pyridine/acetate, pH 2.5 at 55 °C. The column was developed with a four-chamber gradient containing 200 mL each of 0.05 M pyridine/acetate, pH 2.5, 0.2 M pyridine/acetate, pH 3.1, 0.5 M pyridine/acetate, pH 3.75, and 2.0 M pyridine/acetate, pH 5.0. The separation of the peptides was monitored with a Technicon autoanalyzer (Hill & Delaney, 1967). The resulting peptides were desalted with Sephadex G-25 (1 × 140 cm) in 50% acetic acid.

Amino Acid Analysis. Peptides were hydrolyzed for 22 h at 110 °C in vacuo in 6 M HCl and applied on a Durrum D-500 amino acid analyzer. The values given in the tables are corrected for the partial loss of serine and threonine during the hydrolytic procedure. For the determination of cysteine, samples were oxidized with performic acid (Hirs, 1967) before acid hydrolysis.

Metal Analysis. Metal analyses were performed on an IL 157 atomic absorption spectrophotometer (Instrumentation Laboratory). Triply distilled water and acid-washed glassware were used.

HPLC Analysis. Samples containing 2 μg of copper were subjected to HPLC analysis on a Lichrosorb RP-18 column (0.46 × 25 cm). The buffer system used was buffer A, 0.01 M sodium perchlorate in 0.1% phosphoric acid, pH 2.1, and buffer B, as in (A) except 60% (v/v) in acetonitrile. Chromatography was carried out with a linear gradient of buffer B (0.4%/min) at room temperature with a flow rate of 1.0 mL/min. Absorbance was recorded at 220 nm.

Amino Acid Sequence Analysis. Peptides were sequenced by the manual Edman degradation method previously described (Lerch & Fischer, 1975). Automated Edman degradation of the S-pyridylethylated thionein was performed on a Beckman 890 B (updated) sequenator with the Beckman Quadrol program 060275 by using the Edman & Begg (1967) procedure as modified by Hermodson et al. (1972). The protein (0.5 μmol) was dissolved in 50% acetic acid and the solvent removed by using the Beckman application program 031872. Thiazolinone derivatives were converted to phenylthiohydantoins (PTH) by treatment with 1 M HCl at 80 °C for 10 min. The PTH derivatives were identified by HPLC

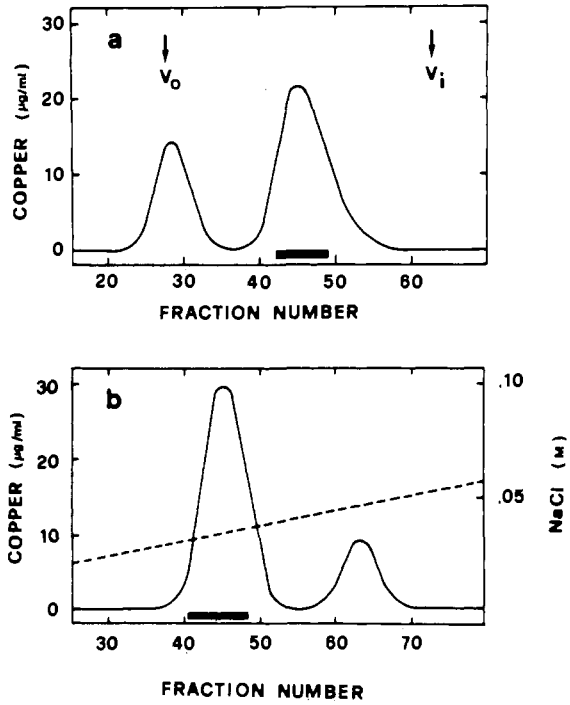

FIGURE 1: Isolation of *A. bisporus* copper MT by Sephadex G-50 gel filtration (a) and DE-32 cellulose ion-exchange chromatography (b). Fractions indicated by bars were pooled and subjected to further purification. Experimental details are given under Experimental Procedures.

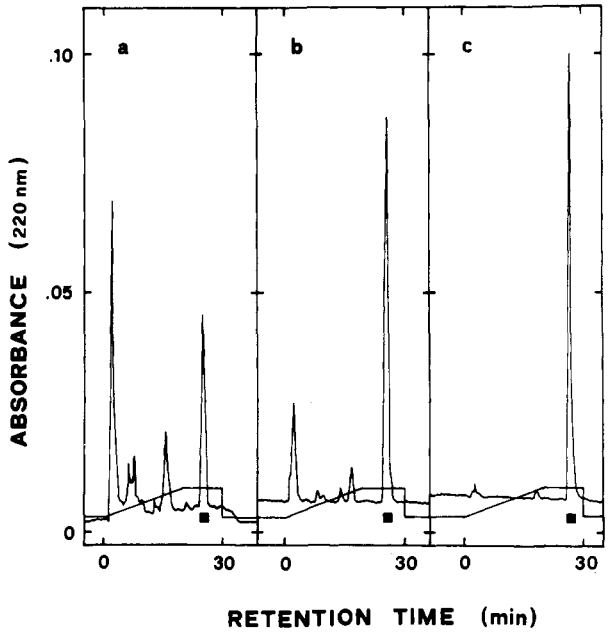

FIGURE 2: HPLC profiles of MT-containing fractions at different stages of the purification procedure: after Sephadex G-50 gel filtration (a), DE-32 cellulose ion-exchange chromatography (b) and Sephadex G-25 gel filtration (c). Samples containing 2 μg of copper were applied as described under Experimental Procedures. MT-containing peaks (identified by comparison with a sample of purified MT) are denoted by bars.

Table I: Amino Acid Composition of *Agaricus* Copper MT

residue	analysis	sequence
Cys	6.8	7
Asx	1.1	
Asp		1
Asn		–
Ser	5.0	5
Glx	1.1	
Glu		–
Gln		1
Gly	6.0	6
Ala	2.0	2
Lys	1.0	1
Thr	2.0	2
total residues	25	
chain weight	2230	
copper content (mol)	5.8	
M_r including copper	2600	

(Frank & Strubert, 1973) combined with back-conversion with 6 M HCl containing 0.1% $SnCl_2$ (Mendez & Lai, 1975) followed by amino acid analysis. Carboxypeptidase Y digests were performed according to the method of Hayashi (1977a,b).

Spectroscopic Measurements. Absorption spectra were recorded with a Hitachi Perkin-Elmer Model 340 recording spectrophotometer. Circular dichroism measurements were performed in a Cary 61 spectropolarimeter. The ϵ and $[\theta]$ values were calculated on a per mole MT basis. For fluorescence measurements a Perkin-Elmer MPF-2A spectrofluorimeter was used, operating in the "ratio" mode. Fluorescence spectra are given in arbitrary units (a.u.). Spectra were corrected for the inner filter effect according to the method of Chignell (1972). The excitation spectrum was corrected for the photon output of the lamp (Parker & Rees, 1960) by using a concentrated alcoholic solution of Rhodamine 6G. The quantum yield was calculated by comparing the areas under the emission spectra of the protein sample and a solution of Rhodamine 6G in ethanol excited at the same wavelength and normalized for the same absorbance (Kuiper et al., 1980). All spectroscopic measurements were carried out at 10 °C.

Results

Isolation. Figure 1 shows the elution profiles of the Sephadex G-50 and DE-32 cellulose columns. The low molecular weight copper-containing protein fraction is separated in two peaks on DE-32 cellulose. The first major fraction yielded a copper-binding protein essentially homogeneous as judged by HPLC analysis (Figure 2) and was used throughout this study. The minor peak eluting at higher ionic strength was inhomogeneous; however, when further purified by HPLC, the fraction showed the same amino acid composition as the protein in the first DE-32 peak (data not shown).

Chemical Properties. The amino acid composition of *A. bisporus* copper MT is presented in Table I. The protein consists of only eight different amino acids. Besides the high cysteine content (28%), it contains also high amounts of glycine (24%) and serine (20%). The protein is devoid of aromatic amino acids as well as histidine. The copper content was

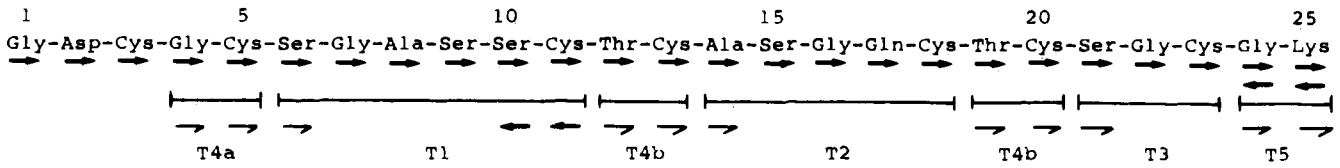

FIGURE 3: Amino acid sequence of *A. bisporus* copper MT and a schematic outline of the peptides used to establish the primary structure. The following symbols were used to indicate the methods of sequential degradation: →, automated Edman degradation; →, manual Edman degradation; ←, carboxypeptidase Y digestion.

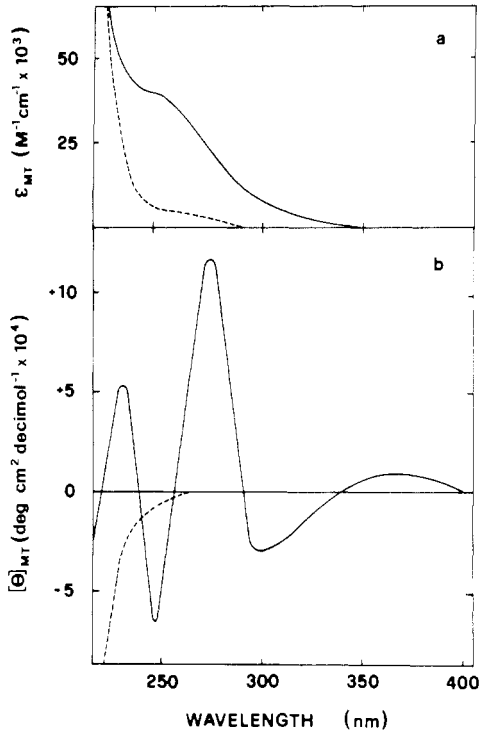

FIGURE 4: Electronic absorption (a) and circular dichroism (b) spectra of *Agaricus* copper MT (—) and apothionein (---).

calculated to be 5.8 mol per molecule. No other metal ions were detected by atomic absorption spectroscopy.

Amino Acid Sequence. The primary structure of *A. bisporus* MT is presented in Figure 3 together with the peptides used to establish the amino acid sequence. Automated Edman degradation of the S-pyridylethylated thionein allowed the unambiguous identification of all 25 amino acids (Table III of the supplementary material). The amino acid sequence was further corroborated by amino acid and partial sequence analyses of the tryptic peptides of the S-aminoethylated derivative (Tables IV–VI of the supplementary material). All peptides with the exception of the amino-terminal tripeptide were obtained in good yields by ion-exchange chromatography and gel filtration (Figure 7 of the supplementary material). An increased yield was observed for the dipeptide Thr-Cys, which occurs twice in the sequence of *Agaricus* MT. The amino acid composition of the performic acid oxidized protein is also in good agreement with the composition calculated from the sequence data (Table I).

Spectral Properties. The absorption spectrum of freshly isolated *Agaricus* copper MT is characterized by a broad band with a distinct shoulder at 250 nm (Figure 4a, solid line). No features were detected at higher wavelengths. Upon removal of the metal ions by lowering the pH to 0.5 with HCl, nearly complete bleaching of this band was observed (Figure 4a, dashed line). The CD spectrum of the native protein (Figure 4b, solid line) displays a number of well-resolved Cotton extrema located at 230 (+), 245 (−), 275 (+), 300 (−), and 365 (+) nm. Again these spectral properties disappeared almost completely when copper was removed by acid treatment (Figure 4b, dashed line). Excitation and emission spectra of native *A. bisporus* copper MT are presented in Figure 5. Upon excitation at 310 nm the protein exhibits a broad emission band (half-bandwidth, 70 nm) centered at 565 nm. The quantum yield was calculated to be 0.014. Again the luminescence properties proved to be strictly metal dependent, vanishing completely when 1 M HCl was added to the holoprotein.

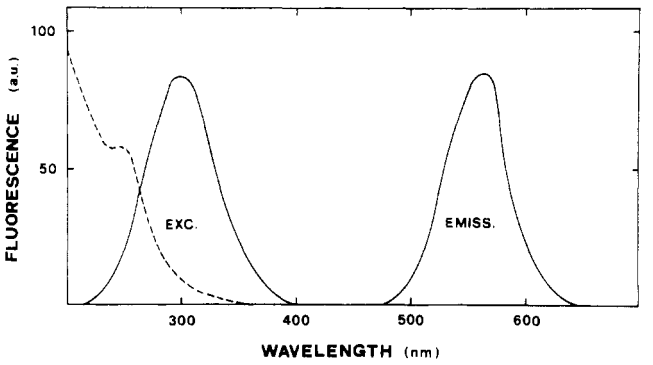

FIGURE 5: Observed excitation (EXC) and emission (EMISS) spectra of *Agaricus* copper MT (—) and corrected excitation spectrum (---). Fluorescence is given in arbitrary units (a.u.).

Discussion

The accumulation of heavy metals in higher mushrooms is a well-known phenomenon and has been the subject of numerous investigations (Meisch et al., 1977; Seger, 1978; Stijve & Besson, 1976; Tyler, 1980). The consumption of mushrooms harvested close to heavy metal polluted areas (e.g., motorways, industrial plants, etc.) may be a potential health hazard due to their high content of heavy metals. We recently studied the uptake of Cu and Cd in commercial cultures of *A. bisporus* (Münger et al., 1982). Both metals were accumulated to about the same extent, but surprisingly no low molecular weight metal-binding protein could be detected in the fruiting bodies. In this study we have investigated the uptake of different metal ions by mycelia using liquid cultures of *A. bisporus*. Additions of $CuSO_4$ to a chemically defined growth medium (Hänseler et al., 1983) led to the induction of a low molecular weight copper-binding protein. The elution properties both on Sephadex G-50 and on DE-32 cellulose columns are very similar to those reported for *N. crassa* copper MT (Lerch, 1980).

Additions of Cd and Zn, which are known to be potent inducers of MT synthesis in vertebrates (Kägi & Nordberg, 1979), did not show any effect on MT synthesis in this organism as judged by Sephadex G-50 and G-75 gel filtration experiments (data not shown). Similar observations were made earlier with *N. crassa* (Lerch, 1980), where the induction of MT synthesis is also strictly copper-dependent. Other metals like Cd, Zn, or Ni, however, led to the formation of chemically and spectroscopically well-defined, stable derivatives of the protein in vitro (Beltramini et al., 1984).

A. bisporus copper MT consists of 25 amino acids with a characteristically high value for cysteine (28%) (Table I). In agreement with the fact that *A. bisporus* is a highly evolved fungus, the amino acid composition of *Agaricus* copper MT is less rudimentary than the one of *Neurospora*. In particular, the serine and glycine contents are lower in *A. bisporus* MT with the new appearance of glutamic acid and threonine (Table I). The overall sequence homology between *A. bisporus* and *N. crassa* copper MTs is close to 80%. A sequence comparison of the *A. bisporus* copper MT with the amino-terminal part of mammalian MTs reveals almost 40% identity (Figure 6). This value clearly allows the classification of this protein as a member of the superfamily of MTs. Comparison of the primary structures of *Agaricus* MT with that of *Neurospora* showed the first 11 residues to be identical (Figure 6). In some respects, however, the sequence differs markedly from that of *Neurospora* MT. A threonine residue at position 14 and an alanine residue at position 16 are common to many vertebrate MTs (Figure 4). The threonine residue at position 20,

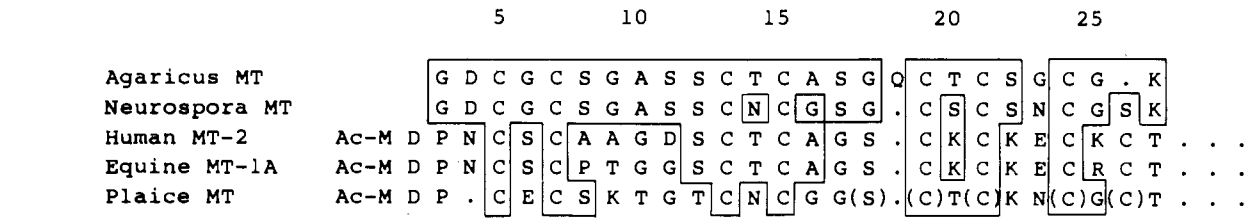

FIGURE 6: Amino acid sequence comparison of *A. bisporus* MT with *N. crassa* MT (Lerch, 1980) and the amino-terminal parts of human MT-2 (Kissling & Kägi, 1977), equine MT-1A (Kojima et al., 1979), and plaice MT (Overnell et al., 1981). Identical residues are indicated by boxes. The different amino acid sequences are aligned so as to obtain maximal sequence homology. The residues enclosed within parentheses require further identification. For the one-letter notation for amino acids see IUPAC–IUB Commission on Biochemical Nomenclature: A One-Letter Notation for Amino Acid Sequences (1968).

Table II: Position of Cotton Extrema in Circular Dichroism Spectra of Different Copper-Containing MTs

source							
Agaricus MT	365 (+)		300 (−)	275 (+)	245 (−)	230 (+)	
Neurospora MT[a]	355 (+)		295 (−)	270 (+)	245 (−)	230 (+)	
bovine fetal liver MT-1[b]			285 (−)		250 (+)		207 (−)
bovine fetal liver MT-2[b]	325 (−)		295 (+)		255 (+)		205 (−)
yeast MT[c]	360 (+)	330 (+)	300 (−)	280 (−)	250 (+)	215 (−)	

[a] Beltramini & Lerch (1983). [b] Münger et al. (1985). [c] Weser & Rupp (1979).

however, was only found in plaice MT (Overnell et al., 1981). Moreover, there is an insertion of one amino acid between positions 18 and 19. Interestingly enough a deletion was found to occur in the same region in the amino acid sequence of crab MT (Lerch et al., 1982). In a three-dimensional model of rabbit MT proposed by Boulanger et al. (1983), this very region forms a loose loop in which an insertion or deletion would not influence the metal-binding properties of the protein. Moreover, the MT from *Drosophila melanogaster*, which has been cloned and sequenced recently (Lastowsky-Perry et al., 1985), shows an insertion of five amino acids at the same position.

Six of the seven cysteines are arranged in the form of -Cys-X-Cys- clusters, a unique feature of all class I MTs sequenced so far (Kägi et al., 1984). Because of their strong conservation in evolution, they are supposed to be essential as primary metal chelating sites and are probably determining the formation of metal thiolate clusters in these systems.

Like *N. crassa* copper MT, the protein from *A. bisporus* contains an unusually high amount of copper (5.8 mol/mol, Table I). The absorption, chiroptical, and luminescence properties of *Agaricus* copper MT can be attributed to metal thiolate complexation as they are absent in the apoprotein (Figures 4 and 5). The absorption spectrum is rather featureless with a characteristic shoulder at 250 nm, attributable to metal sulfur charge-transfer transitions (Beltramini & Lerch, 1983). The CD measurements document a high degree of asymmetry in the copper thiolate chromophore. Although the positions of the Cotton extrema are similar to those of *Neurospora* MT (Beltramini & Lerch, 1983), the rotational strengths of corresponding transitions were found to be different. Vast differences are also observed when the chiroptical data are compared to those of bovine fetal liver MT (Münger et al., 1985) and the copper binding protein from *S. cerevisiae* (Weser & Rupp, 1979) (Table II). This may be related to the different metal thiolate stoichiometry and in the case of the structurally different yeast protein also to a different metal-binding mode. All the spectroscopic features taken together suggest a structure of *A. bisporus* copper MT very similar to that of the *Neurospora* protein, for which a polymeric copper(I) μ-thiolate structure has been proposed (Lerch, 1980; Beltramini & Lerch, 1983).

ACKNOWLEDGMENTS

We thank Dr. H. J. Tschierpe (Hauser Champignonkulturen, CH-8625 Gossau, Switzerland) for *A. bisporus* strains, C. Kasper for expert technical assistance, and Dr. D. M. Rast and Dr. M. Beltramini for helpful suggestions.

SUPPLEMENTARY MATERIAL AVAILABLE

Tables III–VI containing amino acid composition and amino acid sequence data and Figure 7 showing a chromatogram of the separation of the tryptic peptides of S-aminoethylated *A. bisporus* MT (5 pages). Ordering information is given on any current masthead page.

Registry No. Metallothionein (*Agaricus bisporus* protein moiety reduced), 98526-74-0.

REFERENCES

Beltramini, M., & Lerch, K. (1982) *FEBS Lett. 142*, 219–222.
Beltramini, M., & Lerch, K. (1983) *Biochemistry 22*, 2043–2048.
Beltramini, M., Vašák, M., & Lerch, K. (1984) *Biochemistry 23*, 3422–3427.
Bordas, J., Koch, M. H. J., Hartmann, H.-J., & Weser, U. (1983) *Inorg. Chim. Acta 78*, 113–120.
Boulanger, Y., Goodman, C. M., Forte, C. P., Fesik, S. W., & Armitage, I. M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1501–1505.
Butt, T. R., Sternberg, E. J., Herd, J., & Crooke, S. T. (1984a) *Gene 27*, 23–33.
Butt, T. R., Sternberg, E. J., Gorman, J. A., Clark, D., Hamer, D. H., Rosenberg, M., & Crooke, S. T. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3332–3336.
Chignell, C. F. (1972) *Methods Pharmacol. 2*, 33–61.
Cole, D. R. (1976) *Methods Enzymol. 11*, 315–317.
Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80–91.
Frank, G., & Strubert, W. (1973) *Chromatographia 6*, 522–524.
Friedman, M., Krull, L. H., & Cavins, J. F. (1970) *J. Biol. Chem. 245*, 3868–3871.
Hänseler, E., Nyhlén, L. E., & Rast, D. M. (1983) *Exp. Mycol. 7*, 17–30.
Hayashi, R. (1977a) *Methods Enzymol. 47*, 84–93.

Hayashi, R. (1977b) *Methods Enzymol. 47*, 339-351.
Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., & Walsh, K. A. (1972) *Biochemistry 11*, 4493-4502.
Hill, R. L., & Delaney, R. (1967) *Methods Enzymol. 11*, 339-351.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 59-62.
IUPAC-IUB Commission on Biochemical Nomenclature: A One-Letter Notation for Amino Acid Sequences (1968) *J. Biol. Chem. 243*, 3557-3559.
Kägi, J. H. R., & Vallee, B. L. (1960) *J. Biol. Chem. 235*, 3460-3465.
Kägi, J. H. R., & Nordberg, M., Eds. (1979) *Metallothionein*, Birkhäuser, Basel.
Kägi, J. H. R., Vašák, M., Lerch, K., Gilg, D. E. O., Hunziker, P., Bernhard, W. R., & Good, M. (1984) *EHP, Environ. Health Perspect. 54*, 93-103.
Karin, M., Najarian, R., Haslinger, A., Valenzuela, P., Welch, J., & Fogel, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 337-341.
Kissling, M. M., & Kägi, J. H. R. (1977) *FEBS Lett. 82*, 247-250.
Kojima, Y., Berger, C., & Kägi, J. H. R. (1979) in *Metallothionein* (Kägi, J. H. R., & Nordberg, M., Eds.) Birkhäuser, Basel.
Kuiper, H. A., Finazzi-Agro, A., Antonini, E., & Brunori, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2387-2389.
Lastowsky-Perry, D., Otto, E., & Maroni, G. (1985) *J. Biol. Chem. 260*, 1527-1530.
Lerch, K. (1980) *Nature (London) 284*, 368-370.
Lerch, K. (1981) *Met. Ions Biol. Syst. 13*, 299-316.
Lerch, K., & Fischer, E. (1975) *Biochemistry 14*, 2009-2014.
Lerch, K., & Beltramini, M. (1983) *Chem. Scr. 21*, 109-115.
Lerch, K., Ammer, D., & Olafson, R. W. (1982) *J. Biol. Chem. 257*, 2420-2426.
Margoshes, M., & Vallee, B. L. (1957) *J. Am. Chem. Soc. 79*, 4813-4814.
Meisch, H.-U., Schmitt, J. A., & Reinle, W. (1977) *Z. Naturforsch., C: Biosci. 32C*, 172-181.
Mendez, E., & Lai, C. Y. (1975) *Anal. Biochem. 68*, 47-53.
Minkel, D. T., Poulson, K., Wielgus, S., Shaw, C. F., III, & Petering, D. H. (1980) *Biochem. J. 191*, 475-485.
Münger, K., Lerch, K., & Tschierpe, H. J. (1982) *Experientia 38*, 1039-1041.
Münger, K., Germann, U. A., Beltramini, M., Niedermann, D., Baitella-Eberle, G., Kägi, J. H. R., & Lerch, K. (1985) *J. Biol. Chem. 260*, 10032-10038.
Otvos, J. D., & Armitage, I. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7094-7098.
Overnell, J., Berger, C., & Wilson, K. J. (1981) *Biochem. Soc. Trans. 9*, 217-218.
Parker, C. A., & Rees, W. T. (1960) *Analyst (London) 85*, 587-600.
Seger, R. (1978) *Z. Lebensm.-Unters. -Forsch. 166*, 23-34.
Stijve, T., & Besson, R. (1976) *Chemosphere 2*, 151-158.
Tyler, G. (1980) *Trans. Br. Mycol. Soc. 74*, 41-49.
Vašák, M., & Kägi, J. H. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6709-6713.
Weser, U., & Rupp, H. (1979) in *Metallothionein* (Kägi, J. H. R., & Nordberg, M., Eds.) pp 221-230, Birkhäuser, Basel.
Weser, U., Hartmann, H.-J., Fretzdorff, A., & Strobel, G.-J. (1977) *Biochim. Biophys. Acta 493*, 465-477.

Mechanism of Poly(ethylene glycol) Interaction with Proteins†

Tsutomu Arakawa‡ and Serge N. Timasheff*
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254
Received April 30, 1985

ABSTRACT: Poly(ethylene glycol) (PEG) is one of the most useful protein salting-out agents. In this study, it has been shown that the salting-out effectiveness of PEG can be explained by the large unfavorable free energy of its interaction with proteins. Preferential interaction measurements of β-lactoglobulin with poly(ethylene glycols) with molecular weights between 200 and 1000 showed preferential hydration of the protein for those with $M_r \geq 400$, the degree of hydration increasing with the increase in poly(ethylene glycol) molecular weight. The preferential interaction parameter had a strong cosolvent concentration dependence, with poly(ethylene glycol) 1000 having the sharpest decrease with an increase in concentration. The preferential hydration extrapolated to zero cosolvent concentration increased almost linearly with increasing size of the additive, suggesting steric exclusion as the major factor responsible for the preferential hydration. The poly(ethylene glycol) concentration dependence of the preferential interactions could be explained in terms of the nonideality of poly(ethylene glycol) solutions. All the poly(ethylene glycols) studied, when used at levels of 10-30%, decreased the thermal stability of β-lactoglobulin, suggesting that caution must be exercised in the use of this additive at extreme conditions such as high temperature.

A large number of compounds with a variety of chemical structures are known to be preferentially excluded from the immediate domain of proteins in aqueous solution; i.e., when they are present at high concentration proteins are preferentially hydrated. These include amino acids (Arakawa & Timasheff, 1983), sugars (Lee & Timasheff, 1981; Lee et al., 1975; Arakawa & Timasheff, 1982a), salts (Arakawa & Timasheff, 1982b; Aune & Timasheff, 1970; Timasheff et al., 1976), glycerol (Gekko & Timasheff, 1981a; Na & Timasheff, 1981), 2-methyl-2,4-pentanediol (MPD)[1] (Pittz & Timasheff,

† This is Publication No. 1566 from the Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254. This work was supported by grants from the National Institutes of Health (GM 14603 and CA 16707).
* Author to whom correspondence should be addressed.
‡ Present address: AMGen, Thousand Oaks, CA 91320.

[1] Abbreviations: PEG, poly(ethylene glycol); MPD, 2-methyl-2,4-pentanediol; β-LG, β-lactoglobulin; Gdn·HCl, guanidine hydrochloride; RNase A, ribonuclease A.

0006-2960/85/0424-6756$01.50/0