Effect of Copper and Manganese on the de Novo Generation of Protease-Resistant Prion Protein in Yeast Cells†

Carina Treiber, Andreas Simons, and Gerd Multhaup*

Institut fuer Chemie und Biochemie, Freie Universitaet Berlin, Thielallee 63, D-14195 Berlin, Germany

Received February 6, 2006; Revised Manuscript Received March 20, 2006

ABSTRACT: The prion protein (PrP) is the key protein implicated in diseases known as transmissible spongiform encephalopathies. PrP has been shown to bind manganese and copper, the latter being involved in the normal function of the protein. Indeed, upon expression in yeast we noted a major increase in intracellular copper and a decrease in manganese. Interestingly, protease-resistant PrP^{Sc}-like protein (PrP^{res}) formation was induced when PrP-expressing yeast cells were grown in copper- and/or manganese-supplemented media. The pattern of PrP banding in SDS−PAGE was dominantly determined by manganese. This conformational transition was stable against EDTA treatment but not in the presence of the copper chelators bathocuproinedisulfonic acid or clioquinol. Conclusively, PrP itself influences manganese and copper metabolism, and a replacement of copper in PrP complexes with manganese is highly likely under the condition of copper depletion or if excess amounts of copper and manganese are present. Taken together, our present study demonstrates the involvement of PrP in the regulation of intracellular metal ion homeostasis and uncovers copper and, more severely, manganese ions as in vivo risk factors for the conversion into PrP^{Sc}.

A fundamental event in the transmissible spongiform encephalopathies (TSEs)[1] is the conversion of the normal isoform of the prion protein (PrP^{C}) to a scrapie-associated, proteinase K-resistant isoform (PrP^{Sc}). The accumulation of PrP^{Sc} in the central nervous system of infected animals is a pathological hallmark (*1*−*3*). PrP^{Sc} is considered to be the principal or sole component of the prion, a transmissible agent consisting of proteinaceous infectious particles (*4*). PrP^{Sc} features a template-assisted and nucleation-dependent conversion in the interaction with PrP^{C} (*5*, *6*). This implies that prions when regarded as genetic elements can induce conformational changes when acting as adaptive conduits of memory and inheritance as shown for neuronal members of the CPEB family (cytoplasmic polyadenylation element binding proteins) upon adaptation of a prion-like state (*7*−*9*).

To distinguish between PrP^{Sc} and structurally altered isoforms of PrP which have been converted into a newly misfolded proteinase K-resistant (PrP^{res}) form in a cell-free system, the term PrP^{res} has been invented for this potentially infectious form (*10*). Nevertheless, as yet, no in vitro generated full-length PrP^{res} has been shown to be infectious although experimental evidence has been provided that infectious prions can be generated from recombinantly expressed mouse prion protein (residues 89−230) in vitro (*11*).

PrP^{C} is mainly localized on the cell membrane, and accordingly, its physiological role has been associated with ligand uptake, cell signaling, or cell adhesion (*12*). Exposure to Cu and Zn but not to Mn stimulated PrP^{C} endocytosis (*13*). Recently, extracellular Cu has been identified as a possible factor that induces shedding of PrP^{C} into the medium of cultured neurons (*14*), and thus Cu reduces the amount of PrP^{C} bound to neuronal cell membranes (*15*). PrP^{C} itself binds Cu (*16*, *17*), Zn, Mn, and Ni (*18*−*21*). Cu binding to PrP was found to be highly cooperative with approximately five Cu ions binding to the N-terminal domain (*22*, 23). A second Cu binding site exists outside the repeat region between residues 90 and 115 of PrP with two Cu(II) binding to His96, His111, and His187 independently of each other (*19*, *20*, *24*). Crude membranes and synaptosomal fractions of the brains of mice lacking the expression of PrP^{C} have a major deficit in Cu (*25*). A C4 mouse line expressing the prion protein that lacks the octameric repeat region exhibited reduced Cu levels in the brain, almost no Cu binding to PrP^{C}, and a loss of antioxidant activity.

Taken together, PrP could cause an early change in the intracellular level of Cu and Mn, and the PrP structure itself might be controlled by Cu, Mn, and other divalent ions that directly bind to PrP. Although Mn has been suggested to participate in the PrP conversion mechanism in vivo and in the pathogenesis of the disease (*26*, *27*), it is not known if factors such as Mn, Cu, or other divalent metal ions may participate in the PrP conversion mechanism in vivo.

To test the involvement of PrP in the regulation of intracellular metal ion homeostasis, the methylotrophic yeast

† This work was supported in part by the International Copper Association (ICA), the EC (QLK4-CT-2002-02723), and the MWK Baden-Wuerttemberg and the Free University of Berlin.

* To whom correspondence should be addressed. E-mail: multhaup@chemie.fu-berlin.de. Phone: +49-30-838-555-33. Fax: +49-30-838-565-09.

[1] Abbreviations: TSE, transmissible spongiform encephalopathy; PrP^{C}, cellular prion protein; wtPrP, wild-type PrP; PrP^{res}, protease-resistant protein; PrP^{Sc}, scrapie-associated PrP; PrPΔN, PrP lacking the octapeptide repeat region; ICP-MS, inductively coupled plasma mass spectrometry; BCS, bathocuproinedisulfonic acid; EDTA, ethylenediaminetetraacetic acid; CQ, clioquinol; PK, proteinase K.

10.1021/bi060244h CCC: $33.50
Published on Web 05/04/2006

Pichia pastoris was used. In this report we found that the expression of PrP increased the intracellular Cu level by a factor of 1.6. Even when the four octarepeats that specifically bind Cu within the N-terminal half of PrP^C were deleted, the intracellular Cu level increased by a factor of 1.4. This result indicates that PrP had a regulatory or buffering function for the cellular Cu content not only through the octarepeat region. Supplementation of the growth medium with Cu and/or Mn led to the conversion of PrP^C into protease-resistant PrP^{res}, which was stable even in the presence of EDTA but not in the presence of bathocuproine or clioquinol. When cells were grown with clioquinol and Cu in the medium, clioquinol inhibited the formation of PrP^{res} inside the cells.

MATERIALS AND METHODS

PrP Expression in P. pastoris. 3F4 epitope tagged PrP(23−230) with residues 109 and 112 of murine PrP replaced by methionine (*28*) was expressed in the methylotrophic yeast *P. pastoris* with (PrP) and without the octapeptide region encompassing residues 27−89 (PrPΔN). Both forms lack the N-terminal signal peptide of 22 amino acids and the C-terminal signal peptide of 23 amino acids that is required for attachment of the GPI anchor.

To target PrP to the secretory pathway, cloning was done by fusing the prepro-α-factor signal sequence of *Saccharomyces cerevisiae* to the PrP encoding sequence 23−230 using the *Eco*RI and *Not*I cloning sites of the vector pPICZαA (Invitrogen). In agreement with mass spectrometry data it is expected that the resulting hybrid molecule is cleaved at the site -EKR*EAEAEFMKKRPK- by the yeast protease Kex2 that is highly specific for cleavage of their peptide substrates C-terminal to paired basic sites.

Cell Culture. Yeast cells were grown for 48 h in 75 mL of BMMY medium [BMGY with 2% (v/v) methanol instead of glycerol], harvested in the late exponential phase of growth and centrifuged at 3500 rpm for 10 min, resuspended in 1 mL of H_2O, washed once with 0.5 M EDTA, pH 8.0, and then resuspended again in 1 mL of H_2O and further analyzed by ICP-MS for metal ions and by Western blotting to detect PrP. For inhibition of PrP glycosylation, tunicamycin was solubilized in DMSO and was added to the induction medium to a final concentration of 30 μg/mL. To investigate the effect of copper and manganese on the de novo generation of PrP^{res} and intracellular metal content, induction medium was adjusted to 1 mM $Cu^{II}Cl_2$ or 1 mM $Mn^{II}Cl_2$, respectively, by using 1 M stock solutions.

Spheroblasting. Washed yeast pellets were carefully resuspended in 500 μL of 2 M sorbitol, 20 mM potassium phosphate, pH 7.6, and 10 mM DTT, incubated for 30 min at 30 °C, washed once with 500 μL of 1.2 M sorbitol followed by 500 μL of 1.2 M sorbitol, 20 mM potassium phosphate, pH 7.5, and 1000 units of lyticase per 1 g wet weight of cells, and incubated for 1 h at 30 °C. Samples were washed again twice with 500 μL of 1.2 M sorbitol. Supernatants were collected for further analysis by Western blotting. Microscopic observation confirmed that the cells were fully converted to spheroplasts.

Cell Lysis and Proteinase K Treatment. Pellets of 1×10^9 cells were resuspended in 100 μL of lysis buffer [50 mM potassium phosphate buffer, pH 7.4, 5% (v/v) glycerol] and an equal volume of acid-washed glass beads (0.5 mm, Sigma) followed by eight cycles of vortexing for 30 s and alternating cooling for 30 s on ice. Samples were then treated with 50 μg/mL proteinase K for 1 h at 37 °C for 24 h with end-over-end rotation unless specified otherwise. Proteolytic digestion was terminated by addition of 2 mM PefablocSC (Roche). The digested samples were boiled in SDS sample loading buffer for 10 min prior to electrophoresis. Depending on the experiment 20 mM CQ, BCS, or EDTA was added to the lysis buffer.

Western Blotting. Culture supernatant and lysed cell pellets were analyzed for protein expression. After centrifugation the cell lysate as well as the supernatant of the cell culture was mixed with SDS−PAGE gel loading buffer [4% (w/v) SDS, 40% (v/v) glycerol, 0.04% (w/v) bromphenol blue, 20% (v/v) mercaptoethanol, 250 mM Tris-HCl, pH 6.8], boiled for 7 min, and loaded on a 10−20% SDS gel (ANAMED). After electrophoresis proteins were transferred to nitrocellulose (Schleicher & Schuell) by tank blotting (380 mA, 4 °C for 3 h). Unspecific binding sites were blocked with 10% (w/v) milk powder in 1 × PBS, incubated with primary antibody (3F4, 1:10000 in 1 × PBS), and incubated with the secondary antibody (anti-mouse IgG HRP, 1:10000; Promega). The detection of immunoreactivity was performed by using the Enhanced-Chemi-Luminescene system (Amersham Biosciences) according to the manufacturer's instructions.

Inductively Coupled Mass Spectrometry (ICP-MS). Washed cell pellets were analyzed by ICP-MS. ICP-MS was performed by using a HP4500 Series 300 ShieldTorch system instrument (Agilent, Waldbronn, Germany) in peak-hopping mode with spacing at 0.05 atomic mass unit, three points per peak, three scans per replicate, and an integration time of 300 ms per point as described (*29, 30*). The rate of plasma flow was 15 L/min with an auxiliary flow of 0.9 L/min and a blend gas flow rate of 0.1 L/min. The radio-frequency power was 1.2 kW. The sample was introduced by using a cross-flow nebulizer at a flow rate of 1.02 L/min. The apparatus was calibrated by using a 6.5% HNO_3 solution containing Cu and Zn at 1, 5, 10, 25, 50, 100, 200, and 400 ppb with Rh-103 as internal standard for all isotopes of Cu and Zn. Obtained data were normalized by the amount of yeast cells to compare the determined intracellular amount of metal ion per cell; samples were measured three times. Statistical analysis was performed using three independent measurements by calculating the standard error of the mean (SEM). Statistical significance was determined by the Student's *t* test.

RESULTS

The analysis of PrP expression revealed the fusion proteins within the yeast plasma membrane fraction but not in the media. When cell walls were digested with lyticase followed by Western blot analysis, formerly insoluble PrP showed bands of 38, 33, and 23 kDa that were released into the supernatant without disruption of the plasma membrane (Figure 1A). Upon deglycosylation the 38 kDa band shifted to 33 kDa, and the minor band of 23 kDa remained unaffected (Figure 1B).

Since we had previously shown that yeast is an appropriate system to examine the influence of recombinant metalloproteins on ion homeostasis when overexpression of secreted

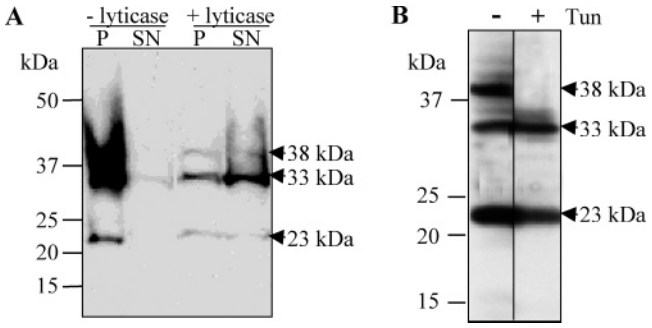

FIGURE 1: Expression and biochemical characterization of PrP in *P. pastoris*. (A) Yeast cells were treated with lyticase which specifically disrupts yeast cell walls. Cell lysates were subjected to immunoblotting with the anti-PrP antibody 3F4 without (lane P, −lyticase) and with lyticase treatment and were released into the supernatant (lane SN, +lyticase). (B) Yeast cells were grown with the N-glycosylation inhibitor tunicamycin (30 μg/mL) for 24 h (lane +) and without (lane −). The protein loaded was adjusted according to the cell number; samples were separated on 10−20% SDS gels. Arrows indicate PrP specific bands at 38, 33, and 23 kDa.

forms of the human amyloid precursor protein (APP) decreased intracellular Cu levels (*31*), we tested whether similar biochemical properties were also present in PrP-transfected yeast cells. Whereas APP is suggested to be involved in Cu efflux (*31*−*33*), there is evidence from several groups that Cu is taken up by PrP^C into cells (*13*, *34*). In agreement with previous studies showing substantially lower Cu content in PrP knockout mice relative to wild-type (*25*) and increased cellular Cu binding associated with increased levels of PrP^C expression (*35*), PrP increased the cellular Cu level in yeast cells 1.6-fold and PrPΔN 1.4-fold compared with mock control cells as determined by ICP-MS analysis (Figure 2A). Whereas Zn levels remained unchanged, PrP expression reduced intracellular levels of Co to 54% (data not shown). When the cells were grown in the presence of 1 or 5 mM Cu, the increase of intracellular Cu was limited by PrP expression to 14-fold for both concentrations tested (Figure 2B, white bars). In contrast, mock control cells showed a 52-fold increased intracellular Cu level upon treatment with 1 mM Cu, which further increased to 94-fold when cells were grown in medium supplemented with 5 mM Cu (Figure 2B, black bars). This suggests that PrP expression has a regulatory or buffering function for the cellular Cu content of yeast cells, most likely due to its multiple Cu binding sites. On the basis of the finding that Mn was found incorporated into native PrP from cells cultured with Mn in the medium, it had been suggested that PrP^C would also influence Mn metabolism (*18*). Upon expression of PrP, intracellular levels of Mn were significantly reduced by 50% for nonsupplemented medium and when 1 or 5 mM Cu was added to the culture medium (Figure 2C, white bars). To obtain information regarding the cellular Cu level influenced by Mn, the medium was supplemented with 1 or 5 mM Mn. The analysis showed that the intracellular Cu level remained constant upon Mn supplementation in mock- and PrP-transfected cells (Figure 2D). An increase of intracellular Mn levels was observed in PrP-expressing cells and in mock-transfected cells (Figure 2E). Taken together, PrP expression increased the cellular Cu level and had no effect on intracellular Zn concentrations but lowered cellular Co (data not shown), and Mn levels decreased by ∼50%. PrP limited the increase of intracellular Cu levels only in the presence of additional Cu but did not limit the increase of Mn in the presence of additional Mn. Since only Mn could substitute for Cu binding to PrP (*18*), we tested if the combined supplementation of the growth medium with 1 mM Mn and varying Cu concentrations from 1 to 10 mM had an effect on intracellular Cu levels. Surprisingly, the Cu levels of PrP-expressing cells remained constant even when the Cu concentration in the medium was further elevated (Figure 2F, white bars). In contrast, in mock-transfected cells the cellular Cu level increased with increased Cu concentrations in the medium tested for concentrations of up to 10 mM Cu (Figure 2F, black bars). The varying concentrations of Mn and Cu in the medium let the ratio of intracellular Mn/Cu raise from 1:1 under normal conditions to 150:1 in PrP-transfected cells and from 2:1 to 20:1 in mock-transfected cells (Figure 2F). Cell viability assessed by counting was not impaired by any of the treatments. These results indicate that PrP expression downregulates intracellular Cu levels in yeast cells even in the presence of excess Mn.

Analysis of Protease Resistance. Prion-infected brains contain both protease-sensitive and protease-resistant forms of PrP^{Sc}-like molecules (*36*). Therefore, it is important to identify possible mechanisms involved in the conversion of PrP^C into PrP^{Sc} molecules through the formation of PrP^{res} molecules in vitro. Fractions enriched for scrapie infectivity primarily contain molecules relatively resistant to protease digestion (PrP^{res}), which possibly might be equivalent to PrP^{Sc} (*37*). There is increasing evidence that PrP^{Sc} exists in variable structures and that these structures are controlled by Cu or other divalent ions.

To investigate the effect of those metal ions on PrP protease resistance, yeast cells were grown either in normal or in Cu-, Co-, or Mn-supplemented medium. When we performed protease digestion assays using proteinase K in crude yeast cell homogenates, we discovered that PrP molecules were protease-sensitive under normal conditions (lanes +/0 in Figure 3) in the absence of transition metals. When tested at concentrations of 1 mM (lanes +/1) and 5 mM (lanes +/5), Cu ions were most effective at inducing PrP^{res} formation (Figure 3A), whereas Mn was less effective at 1 mM but highly effective at the higher ionic strength (Figure 3B). Co was ineffective even at a concentration of 5 mM (data not shown).

Control experiments indicated that Cu or Mn effects on protease resistance of PrP molecules could not be attributed to inhibition of proteinase K activity. Qualitatively, bands recognized by the monoclonal antibody 3F4 after PK treatment differed between Mn- and Cu-treated cells (Figure 3A,B). PrP from Mn-treated cells was more resistant to PK treatment (Figure 3B) as revealed by the higher size of the bands generated (33, 27, and 24 kDa). Cu treatment alone revealed two specific bands at 23 kDa (double band) and 10 kDa that were not observed upon Mn treatment (Figure 3A,B). When PrP metal ion binding was analyzed, it was found that only Mn could substitute for Cu (*18*). Therefore, we tested in medium supplemented with Cu plus Mn if one of the metal ions can dominantly determine the pattern of banding in SDS−PAGE. Unexpectedly, at equimolar concentrations (1 mM Cu and 1 mM Mn; Figure 3C) and even in the presence of a 5-fold molar excess of Cu (5 mM Cu and 1 mM Mn; Figure 3C) bands of higher electrophoretic

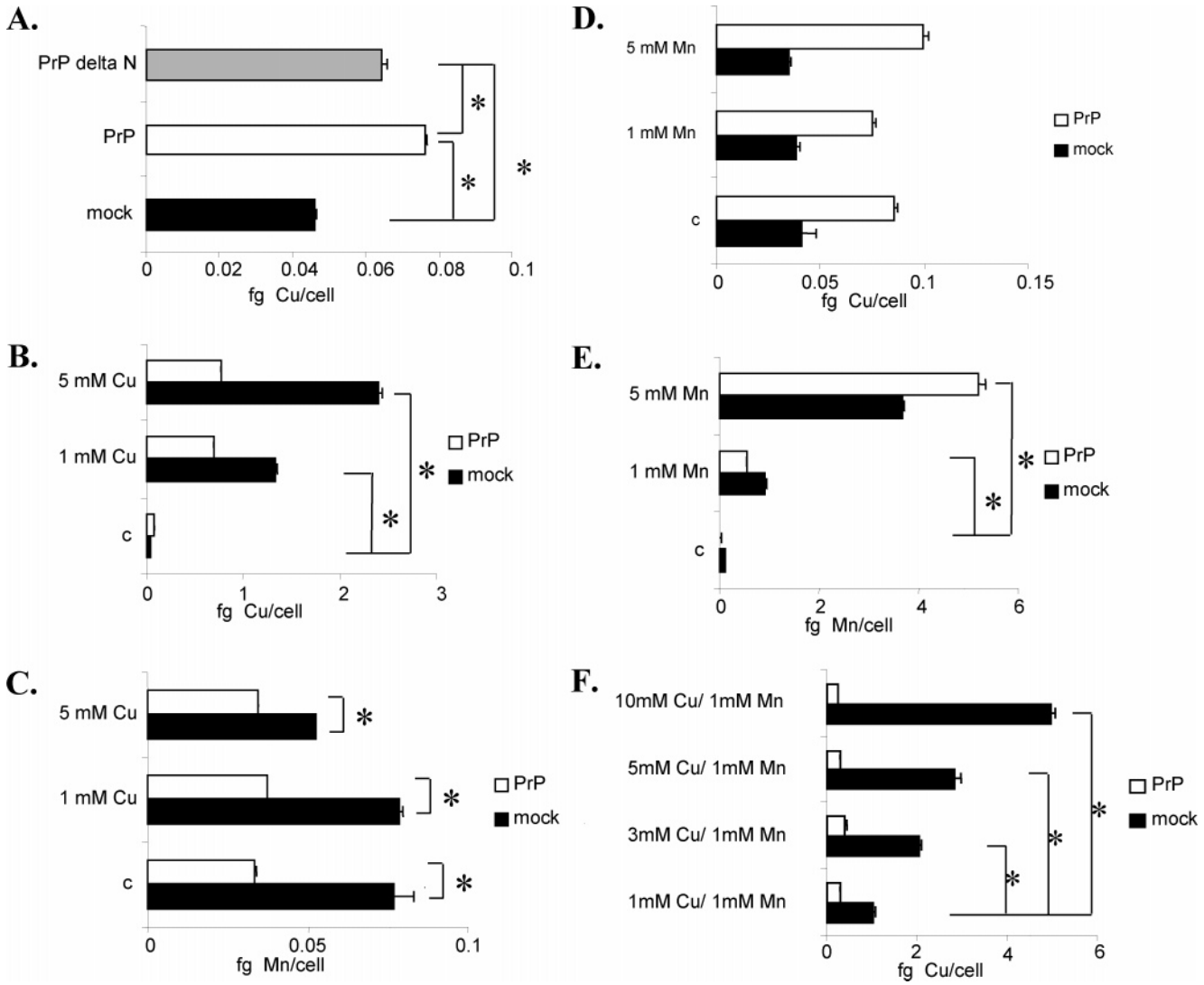

FIGURE 2: Effect of PrP expression on intracellular Cu and Mn levels. PrP, PrPΔN, and mock-transfected yeast cells were grown for 48 h in BMMY medium to the same density, and cell pellets were analyzed by ICP-MS for Cu or Mn. The normal copper concentration in the medium was 0.5 μM. (A) The increase of intracellular Cu is significant for PrP versus mock-transfected ($p < 0.00001$), PrPΔN versus mock-transfected ($p < 0.0001$), and PrP versus PrPΔN-transfected cells ($p < 0.00046$). (B) A statistically significant difference of intracellular copper concentration was found among mock-transfected cells at 1 mM ($p < 0.0001$) or 5 mM ($p < 0.0001$) supplemented copper, respectively. (C) A statistically relevant reduction of intracellular Mn was found for normal medium ($p < 0.0003$) and for Cu-supplemented growth medium ($p < 0.0001$). (D) In the presence of 0, 1, or 5 mM $Mn^{II}Cl_2$, a statistically significant difference was found among PrP-transfected cells and mock-transfected cells ($p < 0.0001$). (E) Total Mn content was determined of vector and PrP-transfected cells. A significant increase of intracellular Mn for PrP- and mock-transfected cells was found with a statistical significance of $p < 0.0001$. (F) Yeast cells grown in medium supplemented with 1 mM $Mn^{II}Cl_2$ and 1, 3, 5, or 10 mM $Cu^{II}Cl_2$, respectively, showed a statistically significant difference only among mock-transfected cells with increased supplemented Cu ($p < 0.0001$) whereas PrP expression limited the increase up to the maximum concentration of 10 mM Cu tested. Values were obtained from two to four independent experiments using different yeast clones. Values need to be multiplied by 10^{-3}. The mean ± SD was calculated from three measurements each.

mobility (33, 27, and 24 kDa) resembled the characteristic pattern as obtained for Mn treatment alone (Figure 3B).

To investigate if the effects observed were reversible in the presence of a divalent metal ion chelator, cells were lysed in the presence of 0.5 M EDTA and subsequently treated as described with proteinase K with 20 mM EDTA in the digestion buffer. The banding pattern of the samples obtained after SDS−PAGE was identical to the one generated without EDTA (data not shown). But when clioquinol (CQ) (20 mM) was added during the lysis of yeast cells, PK resistance was lost in cells grown in Cu-supplemented medium (Figure 4A) but not when cells were grown in the presence of Mn and/or Mn/Cu (Figure 4A). This further confirms the specific effects of Mn or Cu on the conversion of PrP, which was evident by the different pattern of bands upon Cu or Mn treatment (Figure 3A,B). To assess if the specific effect of CQ on PrP^{res} from cells treated with Cu was possibly due to the formation of CQ−Cu complexes, the experiment was performed with BCS, which is a specific chelator for Cu(I) (*38*). Indeed, BCS was able to convert PrP^{res} back into the proteinase-sensitive form (Figure 4B). We followed persistent PrP^{res} formation upon exposure of cells to the membrane-permeable CQ. When cells were grown in the presence of Cu and CQ, CQ inhibited the formation of PrP^{res} in living cells (Figure 5). This was either due to the inhibition of Cu uptake, which is less likely because CQ was shown to enable the cellular uptake of CQ−Cu complexes, or due to a competition between CQ and PrP for Cu(I), which is the intracellular transport form of Cu. This explanation is supported by the results obtained for the divalent metal ion

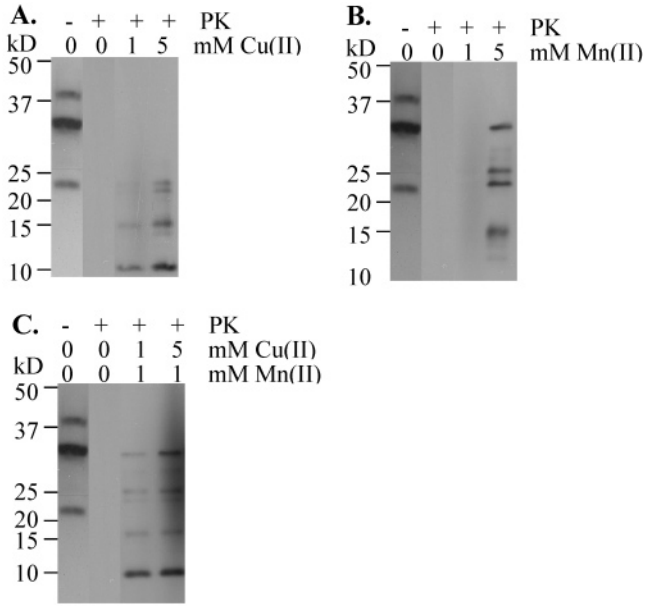

FIGURE 3: Proteinase K resistance of yeast cells treated with metal ions. PrP-transfected yeast cells treated with proteinase K (50 μg/mL) showed PK-resistant core proteins after Cu (A), or Mn (B), Cu and Mn supplementation (C) of the growth medium, respectively. Protein amounts loaded were standardized according to the cell number, samples were separated on 10−20% SDS gels, and PrP was detected with the monoclonal antibody 3F4 by using the ECL technique.

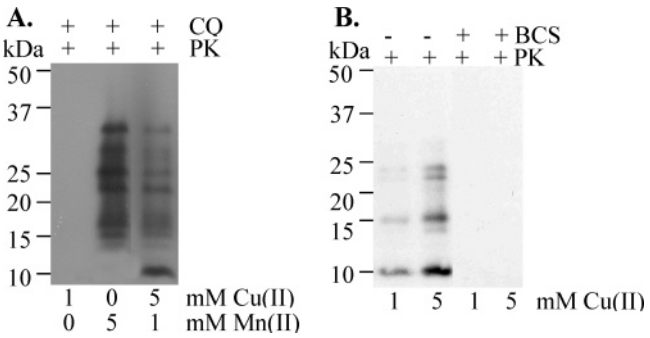

FIGURE 4: Proteinase K resistance of metal-treated yeast cells lysed in the presence of CQ or BCS. PK-resistant core proteins disappeared after CQ (20 mM) or BCS (20 mM) treatment, respectively, during cell lysis and PK digestion. Protein amounts loaded were standardized according to the cell number, samples were separated on 10−20% SDS gels, and PrP was detected with the monoclonal antibody 3F4 by using the ECL technique.

chelator EDTA that did not have an effect on PrP^{res} stability (see above).

Thus, our results suggest that the events leading to the initial formation of PrP^{res} in living cells may differ from those reported for PrP^{res} formation from recombinant protein in several in vitro studies. Taken together, Mn and Cu(I) rather than Cu(II) may represent risk factors for the formation of different forms of PrP^{res} inside the cell and primarily during cellular synthesis of the protein. This could, at least in part, explain the very rare reports of PrP^{res} formation in PrP expressing cells, which may essentially occur under the conditions of cellular metal ion imbalances.

DISCUSSION

Recently, the gene encoding for the prion protein has been identified in a gene expression profiling approach in chronic copper overload to be responsible for a copper-specific homeostatic response (*39*). Current theories link the normal activity of PrP^{C} to Cu binding and suggest that (i) PrP is a transmembrane Cu transporter which is involved in Cu uptake (*34*) and (ii) PrP is a Cu buffer that sequesters excess metal ion at the plasma membrane (*35*). In the present work we demonstrate that the methylotrophic yeast *P. pastoris* is useful to examine these hypotheses at the cellular level and to study the influence of PrP expression on intracellular concentrations of Cu or Mn. Yeast is an appropriate system as most of what we know about Cu transport in eukaryotes is derived from yeast studies (*40*−*42*). Moreover, the yeast genome does not encode a direct structural homologue of PrP, making it an attractive model system for analyzing the role of PrP in Cu homeostasis. We have derived *P. pastoris* lines that produce murine PrP, which was trapped in the cell wall from where it could only be released after lyticase digestion as full-length PrP23−230. Therefore, we were able to investigate how PrP influences metal ion utilization during cellular synthesis and transport before it is withdrawn from the cellular turnover without being secreted or internalized.

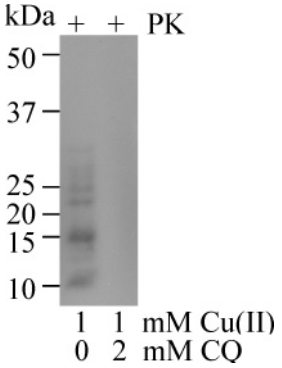

FIGURE 5: Proteinase K resistance of yeast cells grown in the presence of Cu and CQ. PrP-transfected yeast cells treated with proteinase K (50 μg/mL) showed PK-sensitive PrP after supplementation of the growth medium with Cu and CQ. Protein amounts loaded were standardized according to the cell number, samples were separated on 10−20% SDS gels, and PrP was detected with the monoclonal antibody 3F4 by using the ECL technique.

Previous experiments with overexpression of PrP^{C} did not have a dramatic effect on Cu metabolism. The lack of increased brain Cu in transgenic mice suggested that although PrP^{C} is involved in Cu metabolism, it would not have a major role in Cu uptake, transport, or removal (*43*). Mn was earlier speculated to be incorporated into native PrP from cells cultured with Mn in the medium (*18*). We found that PrP expression increased the cellular Cu level 1.6-fold and decreased the Mn concentration by 57%. PrPΔN showed a similar increase of the cellular Cu level (1.4-fold). This is in accordance with data indicating that the affinity for the fifth copper binding site of PrP is increased when the octarepeat region is lacking (*44*). Due to the small differences of wtPrP and PrPΔN on the metal ion homeostasis the following experiments were only performed with wtPrP. Under the condition of Cu excess PrP expression limited the increase of the total number of Cu atoms per cell to a maximum of 14-fold and independently from the presence of excess Mn. In contrast, in Mn-treated cells PrP did not limit the intracellular increase of Mn. Moreover, cosupplementation of the medium with a defined concentration of Mn and varying concentrations of Cu revealed that PrP

expression facilitated the intracellular increase of Mn up to a ratio of 150:1 in PrP-expressing cells compared with controls reaching a ratio of 25:1. When Mn binding to PrP^C was observed to lead to its conversion into an abnormal form of PrP (*18*), it was suggested that an imbalance in brain trace elements, such as the loss of Cu and an excess of Mn, might have led to the formation of this misfolded form. However, we found that PrP itself influences Mn and Cu metabolism, and a replacement of Cu in PrP−Cu complexes with Mn is highly likely when excess amounts of Cu and Mn are present. Cu depletion might obviously not be a prerequisite in the presence of excess Mn.

It is known that in CJD brain homogenates the electrophoretic mobility is controlled by Cu and Zn ions (*45*), the level of PrP^{res} detected in scrapie-infected mouse brain is increased upon addition of Cu to the homogenate (*46*), and Cu enhances the reversal of scrapie inactivation by guanidine hydrochloride (*47*). Also, the method of cyclic amplification applied to normal hamster brain homogenates upon addition of a number of transition metals (Mn, Cu, Fe) led to the conversion of PrP^C into protease-resistant PrP^{res} (*48*). However, there is no consistency in transition metals tested to increase the protease resistance of PrP^{Sc}. Co and Ni caused only small increases, and Mn was mostly reported to be ineffective. The effects caused on PrP^{Sc} conformation reported so far were always reversible when EDTA was used to chelate divalent metal ions in a stoichiometric manner. This indicates that metal ion induced conformational changes were only partially preserved in PrP^{res}. Most interestingly, we have shown that specific PrP^{res} formation was induced when PrP-expressing yeast cells were grown in Cu- and/or Mn-supplemented media. Mn- and Cu-generated PrP^{res}-derived bands qualitatively differed in their size. Although Cu treatment specifically led to the formation of a 10 kDa band, cotreatment of Mn and Cu revealed that Mn mainly determined the physical characteristics of PrP^{res} even in the presence of excess Cu. Thus, PrP^{res} had been generated most likely during the de novo synthesis and adopted a structure that was not interconvertible by EDTA but by BCS and CQ when cells were grown in Cu-supplemented medium. Several trials to charcterize the PrP-specific bands by mass spectrometry were unsuccessful due to the limited amount of protein enriched from yeast cell homogenates. Although the individual roles for the two metal ions Cu and Mn for the conformational change into PrP^{res} are evident, their mechanism of action is unknown. It is possible that the Cu(I)-specific chelator BCS and CQ which can also be assumed to bind Cu(I) could successfully compete for the Cu(I) ion bound to PrP^{res}. Alternatively, metal(chelator)$_2$ complexes could have formed and functioned as fibril inhibitors as it was earlier shown for phthalocyanine compounds which are structurally very similar to the metal(chelator)$_2$ complexes used in these experiments (*49*).

Therefore, it is possible that the methods used so far to study Cu or Mn effects on PrP in cell-free systems were not adequate to transform the molecule into PrP^{res} or PrP^{Sc}, respectively. We showed that not a loss in the amount of Cu is required but an excess of Mn over Cu is sufficient. This implies that imbalances in environmental Mn entering the food chain might favor the formation of proteinase-resistant PrP as formerly predicted for the cause of scrapie, CJD, and chronic wasting disease occurring in disease clusters in Iceland, Slovakia, and Colorado where the soil is high in Mn in these specific regions (*50*). Mn is found in all body tissues as it is essential for many enzymatic reactions, including synthesis of amino acids, lipids, proteins, and carbohydrates (*51*). In laboratory animals Mn deficiency was observed to cause impaired growth, skeletal defects, reduced fertility, birth defects, abnormal glucose tolerance, and altered lipid and carbohydrate metabolism (*52*).

Taken together, we conclude that Cu stabilizes the native conformation of PrP^C. This is further supported by the finding that PrP downregulates the Mn(II) level even in cells treated with Mn(II). Only an excess of Mn revealed specific forms of PrP^{res} that could not be folded back again into PrP^C in the presence of chelators. PrP might belong to protective systems that operate in cells heavily exposed to Cu and/or Mn. In prion disorders there is now an established link to Mn metabolism which increases the need to better understand its involvement in the pathogenesis.

ACKNOWLEDGMENT

We thank Jörg Tatzelt for PrP-expressing clones and G. Buchlow, T. Wons, and M. Nündel for valuable assistance.

REFERENCES

1. Hope, J., Morton, L. J., Farquhar, C. F., Multhaup, G., Beyreuther, K., and Kimberlin, R. H. (1986) The major polypeptide of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP), *EMBO J. 5*, 2591−2597.
2. Meyer, R. K., McKinley, M. P., Bowman, K. A., Braunfeld, M. B., Barry, R. A., and Prusiner, S. B. (1986) Separation and properties of cellular and scrapie prion proteins, *Proc. Natl. Acad. Sci. U.S.A. 83*, 2310−2314.
3. Oesch, B., Westaway, D., Walchli, M., McKinley, M. P., Kent, S. B., Aebersold, R., Barry, R. A., Tempst, P., Teplow, D. B., Hood, L. E., et al. (1985) A cellular gene encodes scrapie PrP 27−30 protein, *Cell 40*, 735−746.
4. Prusiner, S. B. (1982) Research on scrapie, *Lancet 2*, 494−495.
5. Jarrett, J. T., and Lansbury, P. T., Jr. (1993) Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie?, *Cell 73*, 1055−1058.
6. Prusiner, S. B. (1991) Molecular biology of prions causing infectious and genetic encephalopathies of humans as well as scrapie of sheep and BSE of cattle, *Dev. Biol. Stand. 75*, 55−74.
7. Bussard, A. E. (2005) A scientific revolution? The prion anomaly may challenge the central dogma of molecular biology, *EMBO Rep. 6*, 691−694.
8. Si, K., Lindquist, S., and Kandel, E. R. (2003) A neuronal isoform of the aplysia CPEB has prion-like properties, *Cell 115*, 879−891.
9. Shorter, J., and Lindquist, S. (2005) Prions as adaptive conduits of memory and inheritance, *Nat. Rev. Genet. 6*, 435−450.
10. Hill, A. F., Antoniou, M., and Collinge, J. (1999) Protease-resistant prion protein produced in vitro lacks detectable infectivity, *J. Gen. Virol. 80* (Part 1), 11−14.
11. Legname, G., Baskakov, I. V., Nguyen, H. O., Riesner, D., Cohen, F. E., DeArmond, S. J., and Prusiner, S. B. (2004) Synthetic mammalian prions, *Science 305*, 673−676.
12. Mouillet-Richard, S., Ermonval, M., Chebassier, C., Laplanche, J. L., Lehmann, S., Launay, J. M., and Kellermann, O. (2000) Signal transduction through prion protein, *Science 289*, 1925−1928.
13. Perera, W. S., and Hooper, N. M. (2001) Ablation of the metal ion-induced endocytosis of the prion protein by disease-associated mutation of the octarepeat region, *Curr. Biol. 11*, 519−523.
14. Parkin, E. T., Watt, N. T., Turner, A. J., and Hooper, N. M. (2004) Dual mechanisms for shedding of the cellular prion protein, *J. Biol. Chem. 279*, 11170−11178.
15. Toni, M., Massimino, M. L., Griffoni, C., Salvato, B., Tomasi, V., and Spisni, E. (2005) Extracellular copper ions regulate cellular

prion protein (PrP^C) expression and metabolism in neuronal cells, *FEBS Lett. 579*, 741−744.
16. Hornshaw, M. P., McDermott, J. R., Candy, J. M., and Lakey, J. H. (1995) Copper binding to the N-terminal tandem repeat region of mammalian and avian prion protein: structural studies using synthetic peptides, *Biochem. Biophys. Res. Commun. 214*, 993−999.
17. Hornshaw, M. P., McDermott, J. R., and Candy, J. M. (1995) Copper binding to the N-terminal tandem repeat regions of mammalian and avian prion protein, *Biochem. Biophys. Res. Commun. 207*, 621−629.
18. Brown, D. R., Hafiz, F., Glasssmith, L. L., Wong, B. S., Jones, I. M., Clive, C., and Haswell, S. J. (2000) Consequences of manganese replacement of copper for prion protein function and proteinase resistance, *EMBO J. 19*, 1180−1186.
19. Jackson, G. S., Murray, I., Hosszu, L. L., Gibbs, N., Waltho, J. P., Clarke, A. R., and Collinge, J. (2001) Location and properties of metal-binding sites on the human prion protein, *Proc. Natl. Acad. Sci. U.S.A. 98*, 8531−8535.
20. Jones, C. E., Klewpatinond, M., Abdelraheim, S. R., Brown, D. R., and Viles, J. H. (2005) Probing $copper^{2+}$ binding to the prion protein using diamagnetic $nickel^{2+}$ and ^1H NMR: the unstructured N terminus facilitates the coordination of six $copper^{2+}$ ions at physiological concentrations, *J. Mol. Biol. 346*, 1393−1407.
21. Pan, K. M., Stahl, N., and Prusiner, S. B. (1992) Purification and properties of the cellular prion protein from Syrian hamster brain, *Protein Sci. 1*, 1343−1352.
22. Stockel, J., Safar, J., Wallace, A. C., Cohen, F. E., and Prusiner, S. B. (1998) Prion protein selectively binds copper(II) ions, *Biochemistry 37*, 7185−7193.
23. Whittal, R. M., Ball, H. L., Cohen, F. E., Burlingame, A. L., Prusiner, S. B., and Baldwin, M. A. (2000) Copper binding to octarepeat peptides of the prion protein monitored by mass spectrometry, *Protein Sci. 9*, 332−343.
24. Brown, D. R., Guantieri, V., Grasso, G., Impellizzeri, G., Pappalardo, G., and Rizzarelli, E. (2004) Copper(II) complexes of peptide fragments of the prion protein. Conformation changes induced by copper(II) and the binding motif in C-terminal protein region, *J. Inorg. Biochem. 98*, 133−143.
25. Brown, D. R., Qin, K., Herms, J. W., Madlung, A., Manson, J., Strome, R., Fraser, P. E., Kruck, T., von Bohlen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D., and Kretzschmar, H. (1997) The cellular prion protein binds copper in vivo, *Nature 390*, 684−687.
26. Wong, B. S., Brown, D. R., Pan, T., Whiteman, M., Liu, T., Bu, X., Li, R., Gambetti, P., Olesik, J., Rubenstein, R., and Sy, M. S. (2001) Oxidative impairment in scrapie-infected mice is associated with brain metals perturbations and altered antioxidant activities, *J. Neurochem. 79*, 689−698.
27. Wong, B. S., Chen, S. G., Colucci, M., Xie, Z., Pan, T., Liu, T., Li, R., Gambetti, P., Sy, M. S., and Brown, D. R. (2001) Aberrant metal binding by prion protein in human prion disease, *J. Neurochem. 78*, 1400−1408.
28. Gilch, S., Winklhofer, K. F., Groschup, M. H., Nunziante, M., Lucassen, R., Spielhaupter, C., Muranyi, W., Riesner, D., Tatzelt, J., and Schatzl, H. M. (2001) Intracellular re-routing of prion protein prevents propagation of PrP(Sc) and delays onset of prion disease, *EMBO J. 20*, 3957−3966.
29. Bayer, T. A., Schafer, S., Simons, A., Kemmling, A., Kamer, T., Tepest, R., Eckert, A., Schussel, K., Eikenberg, O., Sturchler-Pierrat, C., Abramowski, D., Staufenbiel, M., and Multhaup, G. (2003) Dietary Cu stabilizes brain superoxide dismutase 1 activity and reduces amyloid Abeta production in APP23 transgenic mice, *Proc. Natl. Acad. Sci. U.S.A. 100*, 14187−14192.
30. Simons, A., Ruppert, T., Schmidt, C., Schlicksupp, A., Pipkorn, R., Reed, J., Masters, C. L., White, A. R., Cappai, R., Beyreuther, K., Bayer, T. A., and Multhaup, G. (2002) Evidence for a copper-binding superfamily of the amyloid precursor protein, *Biochemistry 41*, 9310−9320.
31. Treiber, C., Simons, A., Strauss, M., Hafner, M., Cappai, R., Bayer, T. A., and Multhaup, G. (2004) Clioquinol mediates copper uptake and counteracts copper efflux activities of the amyloid precursor protein of Alzheimer's disease, *J. Biol. Chem. 279*, 51958−51964.
32. Bellingham, S. A., Lahiri, D. K., Maloney, B., La Fontaine, S., Multhaup, G., and Camakaris, J. (2004) Copper depletion down-regulates expression of the Alzheimer's disease amyloid-beta precursor protein gene, *J. Biol. Chem. 279*, 20378−20386.
33. White, A. R., Reyes, R., Mercer, J. F., Camakaris, J., Zheng, H., Bush, A. I., Multhaup, G., Beyreuther, K., Masters, C. L., and Cappai, R. (1999) Copper levels are increased in the cerebral cortex and liver of APP and APLP2 knockout mice, *Brain Res. 842*, 439−444.
34. Pauly, P. C., and Harris, D. A. (1998) Copper stimulates endocytosis of the prion protein, *J. Biol. Chem. 273*, 33107−33110.
35. Rachidi, W., Vilette, D., Guiraud, P., Arlotto, M., Riondel, J., Laude, H., Lehmann, S., and Favier, A. (2003) Expression of prion protein increases cellular copper binding and antioxidant enzyme activities but not copper delivery, *J. Biol. Chem. 278*, 9064−9072.
36. Safar, J., Wille, H., Itri, V., Groth, D., Serban, H., Torchia, M., Cohen, F. E., and Prusiner, S. B. (1998) Eight prion strains have PrP(Sc) molecules with different conformations, *Nat. Med. 4*, 1157−1165.
37. Bolton, D. C., McKinley, M. P., and Prusiner, S. B. (1982) Identification of a protein that purifies with the scrapie prion, *Science 218*, 1309−1311.
38. Multhaup, G., Schlicksupp, A., Hesse, L., Beher, D., Ruppert, T., Masters, C. L., and Beyreuther, K. (1996) The amyloid precursor protein of Alzheimer's disease in the reduction of copper(II) to copper(I), *Science 271*, 1406−1409.
39. Armendariz, A. D., Gonzalez, M., Loguinov, A. V., and Vulpe, C. D. (2004) Gene expression profiling in chronic copper overload reveals upregulation of Prnp and App, *Physiol. Genomics 20*, 45−54.
40. Huffman, D. L., and O'Halloran, T. V. (2001) Function, structure, and mechanism of intracellular copper trafficking proteins, *Annu. Rev. Biochem. 70*, 677−701.
41. Labbe, S., and Thiele, D. J. (1999) Pipes and wiring: the regulation of copper uptake and distribution in yeast, *Trends Microbiol. 7*, 500−505.
42. Thiele, D. J. (2003) Integrating trace element metabolism from the cell to the whole organism, *J. Nutr. 133*, 1579S−1580S.
43. Brown, D. R. (2003) Prion protein expression modulates neuronal copper content, *J. Neurochem. 87*, 377−385.
44. Thompsett, A. R., Abdelraheim, S. R., Daniels, M., and Brown, D. R. (2005) High affinity binding between copper and full-length prion protein identified by two different techniques, *J. Biol. Chem. 280*, 42750−42758.
45. Wadsworth, J. D., Hill, A. F., Joiner, S., Jackson, G. S., Clarke, A. R., and Collinge, J. (1999) Strain-specific prion-protein conformation determined by metal ions, *Nat. Cell Biol. 1*, 55−59.
46. Sigurdsson, E. M., Brown, D. R., Alim, M. A., Scholtzova, H., Carp, R., Meeker, H. C., Prelli, F., Frangione, B., and Wisniewski, T. (2003) Copper chelation delays the onset of prion disease, *J. Biol. Chem. 278*, 46199−46202.
47. McKenzie, D., Bartz, J., Mirwald, J., Olander, D., Marsh, R., and Aiken, J. (1998) Reversibility of scrapie inactivation is enhanced by copper, *J. Biol. Chem. 273*, 25545−25547.
48. Kim, N. H., Choi, J. K., Jeong, B. H., Kim, J. I., Kwon, M. S., Carp, R. I., and Kim, Y. S. (2005) Effect of transition metals (Mn, Cu, Fe) and deoxycholic acid (DA) on the conversion of PrP^C to PrPres, *FASEB J. 19*, 783−785.
49. Caughey, W. S., Raymond, L. D., Horiuchi, M., and Caughey, B. (1998) Inhibition of protease-resistant prion protein formation by porphyrins and phthalocyanines, *Proc. Natl. Acad. Sci. U.S.A. 95*, 12117−12122.
50. Purdey, M. (2000) Ecosystems supporting clusters of sporadic TSEs demonstrate excesses of the radical-generating divalent cation manganese and deficiencies of antioxidant co-factors Cu, Se, Fe, Zn. Does a foreign cation substitution at prion protein's Cu domain initiate TSE?, *Med. Hypotheses 54*, 278−306.
51. Finley, J. W., and Davis, C. D. (1999) Manganese deficiency and toxicity: are high or low dietary amounts of manganese cause for concern?, *Biofactors 10*, 15−24.
52. Keen, C. L., Ensunsa, J. L., Watson, M. H., Baly, D. L., Donovan, S. M., Monaco, M. H., and Clegg, M. S. (1999) Nutritional aspects of manganese from experimental studies, *Neurotoxicology 20*, 213−223.

BI060244H