Role of Protein in Fungal Biomineralization of Copper Carbonate Nanoparticles

Highlights

- Proteins are involved in the formation of mycogenic CuCO₃ nanoparticles (CuNPs)
- Triosephosphate isomerase (TPI) exhibited a strong affinity to the CuNPs
- TPI played an important role in controlling nanomaterial morphology and structure
- Proteomics identified key fungal proteins in biomass-free spent culture media

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In Brief
Liu et al. show that triosephosphate isomerase plays an important role in controlling morphology and structure of mycogenic CuCO₃ nanoparticles. The results extend understanding of how microbial systems can influence biomineral formation through protein secretion, which is relevant to the synthesis of inorganic protein-based nanomaterials.

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Role of Protein in Fungal Biomineralization of Copper Carbonate Nanoparticles

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SUMMARY

Biomineralization processes are of key importance in the biogeochemical cycling of metals and other elements by microorganisms, and several studies have highlighted the potential applications of nanoparticle synthesis via biomineralization. The roles played by proteins in the transformation and biologically induced biomineralization of metals by microorganisms is not well understood, despite the interactions of protein and nanoparticles at mineral interfaces attracting much interest in various emerging fields for novel biomaterial synthesis. Here, we have elucidated the association and involvement of fungal proteins in the formation of biogenic copper carbonate nanoparticles (CuNPs) using a carbonate-enriched biomass-free ureolytic fungal culture supernatant. Proteomic analysis was conducted that identified the major proteins present in the culture supernatant. Of the proteins identified, triosephosphate isomerase (TPI) exhibited a strong affinity to the CuNPs, and the impact of purified TPI on CuNP formation was studied in detail. The combined use of scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) confirmed that TPI played an important role in controlling the morphology and structure of the nanomaterials. Fourier transform infrared spectroscopy (FTIR) was applied to examine conformational changes of the proteins to further clarify the interaction mechanisms with CuNPs during biomineralization. Such analyses revealed unfolding of proteins on the mineral surface and an increase in β sheets within the protein structure. These results extend understanding of how microbial systems can influence biomineral formation through protein secretion, the mechanisms involved in formation of complex protein/inorganic systems, and provide useful guidelines for the synthesis of inorganic-protein based nanomaterials.

INTRODUCTION

Many microorganisms, including bacteria, yeasts, and filamentous fungi, are able to produce a variety of nanoscale materials with promising industrial applications.1–4 Among these microbial groups, fungi are well known for biomineralization of metals, i.e., conversion of free metal ions into insoluble mineral forms, through, e.g., redox transformations and/or metabolite excretion. Such biomineralization can result in the formation of elemental or mineral nanoparticles (NPs) of differing compositions and morphologies.5,6 For example, Fusarium oxysporum can reduce silver ions and generate silver NPs extracellularly.7 Neurospora crassa produces mono- and bimetallic gold/silver NPs intracellularly8 and cadmium carbonate NPs extracellularly.9 Metal-containing NP production by biological systems presents some important advantages over traditional chemical syntheses and could help promote clean, non-toxic, and environmentally friendly manufacturing.

The actual mechanisms of formation of many kinds of NPs by microorganisms is still an open question, and the limited knowledge about how to control the properties of final products so far precludes industrial scale bioproduction of NPs. Many researchers have attempted different means of studying the possible mechanisms involved in the biological process and how organic molecules can influence the nucleation and crystal growth of nanoscale biominerals.1,3,10,11 Several studies have provided evidence that proteins play an important role in controlling NP formation. An NADH-dependent reductase was found to be responsible for reduction of silver ions and subsequent formation of silver NPs by Fusarium oxysporum.12–14 The reduction of platinum salts to platinum NPs by F. oxysporum was achieved in two reduction processes aided by a hydrogenase enzyme.15 Proteins can act as both reducing and stabilizing agents, and it was reported that gold NPs produced on the surface of Rhizopus oryzae biomass remained stable without agglomeration for up to 6 months.16 Proteins identified from an iterative gene library were shown to influence the morphology of the Au NPs by controlling the gold crystal growth rate.17 Recently, Li et al.11 and Liu et al.10 have reported that Neurospora crassa was able to mediate production of nanoscale metal carbonate NPs through the reaction of soluble metal ions and carbonate ions that were released from urea hydrolysis. It was
proposed that a change in the conformation of fungal extracellular proteins might provide more NP nucleation sites and promote the crystallization of metal carbonate NPs. In order to clarify the role of extracellular proteins in controlling NP formation, the precipitation of copper carbonate NPs by N. crassa was investigated. Cu is an element of interest for electronic and catalytic applications, and the biomineralization of copper-bearing NPs could supply an efficient and affordable method for production of monodispersed NPs. In addition, Cu species show a high affinity to bind to a variety of specific or non-specific proteins. Copper can shift easily between Cu⁺ and Cu²⁺ states, with affinities for thiol and thioether groups of cysteine/methionine, and oxygen or imidazole groups of aspartic/glutamic acids or histidine, respectively. In this research, a proteomic study was conducted with microscopy and traditional surface chemistry analysis to investigate the association of protein with biogenic CuNPs produced by N. crassa. Proteomic analysis provided the first detailed information on protein excretion by N. crassa, and the combined use of scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) revealed the spatial relationship of metal ions to proteins at the micrometer scale. The long-term goal of this work was to identify proteins that play an important role in the biomineralization of Cu and other metals, to unravel the interaction mechanisms of proteins and metal NPs, and to inform potential applied protocols for NP surface modification and optimization.

RESULTS AND DISCUSSION

Identification of N. crassa Proteins Produced during Growth

In previous work, metal carbonate minerals were found to precipitate by simply mixing soluble metal ion solutions with biomass-free Neurospora crassa spent culture media, which was enriched with carbonate ions released from urea hydrolysis by the enzyme amidohydrolase (urease). The reaction equations are as follows:

$$\text{NH}_2\text{CONH}_2 + 2\text{H}_2\text{O} \rightarrow 2\text{NH}_4^+ + \text{CO}_3^{2-}$$  \hspace{0.5cm} (Equation 1)
$$\text{H}_2\text{O} + \text{Cu}^{2+} + \text{CO}_3^{2-} \rightarrow \text{CuCO}_3(\text{OH})_2(s) \downarrow$$  \hspace{0.5cm} (Equation 2)

After mixing 20 mM CuCl₂ with a biomass-free growth supernatant of N. crassa, fine green particles were precipitated, and protein measurements showed that more than 90% of protein was removed from solution by the formation of these copper carbonate minerals. The protein distribution was examined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and it was found that the protein pattern of the mineral was strongly consistent with the growth supernatant of N. crassa. This indicated that enrichment of the excreted fungal proteins occurred in the produced biominerals (Figure S1). After the biomineralization, there was a dramatic decrease in the protein content of the reactive supernatant, which showed only a few remaining bands for proteins of molecular weights around 15, 20, and 70 kDa. The proteins in the fungal growth supernatant were distributed throughout the entire molecular weight range and representative of the whole proteome. Therefore, the goal of the following proteomic study was to identify the proteins that were potentially involved in the biomineralization of copper carbonate NPs.

Proteomic analysis of the growth supernatant of N. crassa was performed in three biological repeats. The results shown in Table 1 are the proteins that were detected in all three tests, with the confidence level >95% and an ionic score (IS) >90 to ensure accurate protein identification. The mass spectrometry results confirmed that 42 proteins were found in the N. crassa growth medium, which were related to a variety of metabolic processes, including carbohydrate metabolism, stress responses, and hydrogen peroxide removal. As glucose was used as the sole carbon and energy source for growth, large amounts of proteins involved in glycolysis were detected in the medium. After 12 days of growth, autolysis of aged hyphal structures, resulting in disruption of cell walls, membranes, and organelles, would lead to the release of such proteins originally produced in the cytoplasm, cytosol, and mitochondria to the extracellular environment. Only 12 proteins were detected in the reactive supernatant after copper carbonate NP precipitation (Table S1), and an obvious explanation for such protein subtraction was the association of proteins within the NP structure. It is relevant that many detected proteins require metal cofactors, including Cu, Zn, Mg, Mn, Fe, Co, and others, suggesting that the affinity of these proteins to bind metals could explain their strong association with CuNPs. Another possibility for the binding of proteins and copper NPs is electrostatic attraction. The calculated isoelectric point (IP) of proteins detected in the fungal growth supernatant generally ranged around pH 5–7, lower than the reaction pH = 8.5, which suggested that most of these proteins were negatively charged in the reaction of copper carbonate formation and therefore would tend to combine with positive Cu ions on the biominerals.

Proteins Associated with CuNPs

In order to further study specific proteins and examine their strength of binding to NPs, CuNPs produced from the biomineralization step were washed with increasingly strong solutions of desorption of the proteins. The proteins that were still bound to the minerals after washing with the denaturing solutions were detected in the reactive supernatant after copper carbonate NP precipitation (Table S1), and an obvious explanation for such protein subtraction was the association of proteins within the NP structure. It is relevant that many detected proteins require metal cofactors, including Cu, Zn, Mg, Mn, Fe, Co, and others, suggesting that the affinity of these proteins to bind metals could explain their strong association with CuNPs. Another possibility for the binding of proteins and copper NPs is electrostatic attraction. The calculated isoelectric point (IP) of proteins detected in the fungal growth supernatant generally ranged around pH 5–7, lower than the reaction pH = 8.5, which suggested that most of these proteins were negatively charged in the reaction of copper carbonate formation and therefore would tend to combine with positive Cu ions on the biominerals.

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Table 1. Proteins Detected in Biomass-free Spent Culture Medium after Growth of N. crassa

| Name               | Size (kDa) | IP  | Cofactor       | Function                                             |
|--------------------|------------|-----|----------------|------------------------------------------------------|
| ENO enolase         | 48         | 5.21| Mg<sup>2+</sup> | glycolysis                                           |
| ALF fructose-bisphosphate aldolase | 40         | 5.42| Zn<sup>2+</sup> | glycolysis                                           |
| G6PI glucose-6-phosphate isomerase | 61         | 6.03|                 | gluconeogenesis, glycolysis                          |
| TPI triosephosphate isomerase | 27         | 5.40|                 | gluconeogenesis, glycolysis                          |
| KPYK pyruvate kinase | 58         | 6.36| Mg<sup>2+</sup>, K<sup>+</sup> | glycolysis                                           |
| ASD1 putative rhamnogalacturonase | 58         | 8.77|                 | carbohydrate metabolism, cell wall biogenesis/degradation |
| KATG catalase-peroxidase | 83         | 5.97|                 | hydrogen peroxide catabolic process                  |
| AMYG glucoamylase   | 67         | 5.47|                 | carbohydrate metabolism, polysaccharide degradation  |
| SODC superoxide dismutase [Cu-Zn] | 16         | 5.74| Cu<sup>2+</sup>, Zn<sup>2+</sup> | cell redox homeostasis                              |
| HSP88 heat shock protein hsp88 | 79         | 5.09|                 | stress response                                     |
| CAT3 catalase-3     | 79         | 5.69| iron (heme axial ligand) | hydrogen peroxide removal                           |
| PGK phosphoglycerate kinase | 45         | 6.17|                 | glycolysis                                           |
| LAC1 laccase        | 69         | 6.70| Cu<sup>2+</sup> | lignin degradation                                   |
| CYM1 mitochondrial presequence protease | 113        | 5.84| Zn<sup>2+</sup> | mitochondrial transit peptides degradation           |
| NDK nucleoside diphosphate kinase | 17         | 7.82| Mg<sup>2+</sup> | nucleotide metabolism                               |
| SPEE spermidine synthase | 33         | 5.53|                 | spermidine biosynthesis                             |
| CVNH cyanovirin-N homolog | 13         | 4.73|                 | mannose-binding lectin                               |
| GSHR glutathione reductase | 51         | 5.99|                 | cell redox homeostasis                              |
| PDC pyruvate decarboxylase | 63         | 5.71| metal cation | pyruvate metabolism                                 |
| CAT1 catalase-1     | 82         | 6.21| iron (heme axial ligand) | hydrogen peroxide catabolic process                  |
| GRP78 78 kDa glucose-regulated protein homolog | 72         | 4.93|                 | secretory polypeptide translocation                 |
| FDH formate dehydrogenase | 41         | 5.93|                 | formate catabolic process                           |
| AMPP1 probable Xaa-Pro aminopeptidase P | 76         | 5.61| Mn<sup>2+</sup> | removal of a penultimate prolyl residue from the N termini of peptides |
| GBLP guanine nucleotide-binding protein subunit beta-like protein | 36         | 6.79|                 | positive regulation of protein phosphorylation      |
| MET3 sulfate adenyltransferase | 65         | 6.20|                 | hydrogen sulfide biosynthesis                        |
| NNRD ATP-dependent (S)-NAD(P)H-hydrate dehydratase | 38         | 6.41| Mg<sup>2+</sup> | nicotinamide nucleotide metabolic process           |
| HIS2 histidine biosynthesis trifunctional protein | 94         | 5.52| Zn<sup>2+</sup> | L-histidine biosynthesis                            |
| IPYR inorganic pyrophosphatase | 33         | 5.28| Mg<sup>2+</sup> | phosphate-containing compound metabolic process     |
| EIF3B eukaryotic translation initiation factor 3 subunit B | 86         | 5.00|                 | formation of cytoplasmic translation initiation complex |
| CARP vacuolar protease a | 43         | 4.69|                 | protein catabolic process, proteolysis              |
| MASY malate synthase, glyoxysomal | 61         | 7.27| (S)-malate synthesis from isocitrate                |
| MAP2 methionine aminopeptidase 2 | 48         | 5.78| Co<sup>2+</sup>, Zn<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>2+</sup> | protein initiator methionine removal                |
| MTAP S-methyl-5′-thioadenosine phosphorylase | 34         | 5.58|                 | L-methionine biosynthesis via salvage pathway        |
| APTH1 acyl-protein thioesterase 1 | 27         | 5.99|                 | fatty acid metabolic process, protein depalmitoylation |
| CISY citrate synthase, mitochondrial | 52         | 6.73|                 | synthesis of isocitrate from oxaloacetate           |
| LYS1 saccharopine dehydrogenase | 41         | 5.27|                 | L-lysine biosynthesis                               |
| NTF2 nuclear transport factor 2 | 14         | 4.71|                 | nucleocytoplasmic transport                         |
| PDX1 probable pyridoxal 5′-phosphate synthase subunit pdx-1 | 33         | 5.85|                 | pyridoxal 5¢-phosphate biosynthesis                 |

(Continued on next page)
confirmed by the SDS-PAGE gel results (Figure S2), as lanes corres-
ding to purified TPI and the Cu minerals produced in the presence of TPI both showed strong and clear protein bands of the correct size, whereas protein bands were not detected in the supernatant after mineral precipitation. This showed conclusively that TPI was strongly binding to the minerals, and therefore most of the protein was removed by mineral precipitation from the reaction system.

The potential impact of purified TPI protein on copper carbonate formation was further investigated, and the biogenic minerals produced in the presence of TPI were compared to chemically synthesized Cu carbonate. Chemically synthesized Cu carbonate minerals, used as a control, were obtained by mixing 20 mM (NH₄)₂CO₃ and 2 mM/20 mM CuCl₂. Two Cu concentrations were used to investigate the effect of different metal concentrations on the formation of metal-bearing NPs. In order to examine the impact of TPI, TPI was added to the chemical reaction mixture to final concentrations of 0.02 and 0.1 mg/mL, the reaction pH being around pH 8.0 to 8.1. Precipitated minerals were collected and examined by SEM and CLSM to reveal the impact of the associated protein on mineral morphology.

After a 24 h reaction period, few crystallization nuclei were found in the mixture of 20 mM (NH₄)₂CO₃ and 2 mM CuCl₂, as nuclei in the solution were undersaturated (Figure 3A). No obvious crystalline structures were observed in the samples with 0.02 mg/mL TPI, and sample surfaces were covered by a thin layer of dried organic materials (Figure 3B). NPs with an average diameter of ~60 nm were precipitated when the concentration of TPI was increased to 0.1 mg/mL. Nucleation and growth of NPs can be critically affected by the microenvironment of the particle system. The solution chemistry, pH, and temperature and the addition of biological molecules are believed to be crucial factors. The nitrogen atoms of the amide groups in the protein backbone and some amino acid residues can form complexes with Cu²⁺. It was observed that proteins were tightly bound within the mineral structure (Figure 1C). At early stages of crystal growth, such biomolecules are bound to mineral surfaces, resulting in the blocking of growth sites on the surface of copper carbonate particles after “burst nucleation,” resulting in the formation of NPs. In the reaction of 20 mM (NH₄)₂CO₃ and 20 mM CuCl₂ (Figures 3D–3F), micrometer-scale spherical particle agglomerations made of large numbers of nanocrystals were formed. Compared with the morphology of samples without TPI or with 0.02 g/L TPI (Figures 3D and 3E), the addition of a high concentration of protein (0.1 mg/mL TPI) enabled the formation of more dispersible particles and retarded particle aggregation. The formation of NPs with diameters

| Name                  | Size (kDa) | IP     | Cofactor | Function                        |
|-----------------------|-----------|--------|----------|---------------------------------|
| GLYC                  | 53        | 6.93   |          | interconversion of serine and glycine |
| EIF3L                 | 55        | 5.09   |          | protein synthesis of a specialized repertoire of mRNAs |
| CAPZA                 | 30        | 5.91   |          | actin cytoskeleton organization |
| ARGI                  | 39        | 5.71   | Mn²⁺     | synthesis L-ornithine and urea from L-arginine |

Data show proteins detected after 12-day growth of N. crassa in AP1 liquid medium, with a confidence level >95% and an ion score (IS) >90 in three tests to ensure accurate protein identification. The proteins are ranked from a high to low IS. IP refers to the isoelectric point for each protein. See also Table S1.
around 100 nm was observed with 2 mM CuCl$_2$ and 0.1 g/L TPI after 14 days (Figure 3I), compared with the inorganic control sample that showed spherical structures with diameters in micrometers (Figure 3G). The NP-protein complexes formed at early reaction stages could supply more nucleation sites for crystallization and further promote the development of the NPs. Continuous attachment of CuNPs to the NP-protein surface can also be the result of strong electrostatic forces. The addition of proteins to excess can therefore inhibit or promote nucleation rate and crystal growth, determine the morphology of the final product, and may also enhance the stability of NPs, and prevent particle aggregation.

These results therefore show that TPI can play an important role in the biomineralization of nanoscale CuNPs. We used a commercial protein, bovine serum albumin (BSA), and another purified *N. crassa* protein [Mn] superoxide oxidase (SOD), the latter having a similar molecular weight (27,804 Da) as TPI, as positive controls in order to compare the impact of various proteins on the morphology of copper carbonate NPs. As shown in Figure S3, after a 14-day reaction between 2 mM CuCl$_2$, 20 mM...
(NH₄)₂CO₃, and 0.1mg/mL protein, the formation of spherical particle agglomerations was extensive, and single NPs were not observed. As mentioned previously, TPI was chosen as a target protein as it showed a strong affinity to combine with the copper carbonate minerals, and, specifically, the CuNPs produced in the presence of TPI were all in the nanoscale range. Products synthesized with other proteins were micro-scale and formed by aggregation. Different protein sizes and structures therefore resulted in the formation of different Cu-protein complexes, which subsequently influenced crystal development and morphologies of the final minerals. However, stable NPs were not produced from the reaction of TPI with Cu, when [Cu²⁺] was 20 mM. This suggested that the formation of NPs using the fungal growth supernatant may be the combined effect of several proteins, or even with an impact of other biomolecules such as amino acids and polysaccharides, rather than the sole impact of TPI.

In order to investigate the spatial relationships of minerals and proteins, the minerals produced from the reaction of 20 mM (NH₄)₂CO₃, 2 mM CuCl₂, and 0.1 mg/mL TPI were examined by CLSM. CLSM is able to reveal the distribution of metals and biomacromolecules in biological samples but has been rarely applied in geomicrobiology. Here, we generated 3D images of protein-mineral aggregates by scanning the specimen at 5 μm intervals and stacking those images. The use of Fura 2AM visualized the distribution of Cu and FilmTracer Sypro stained the protein, enabling the visualization of Cu association with protein as well as spatial information. Figure 4 shows the 3D datasets from CLSM illustrating the interaction of proteins (red) and copper (green). The CLSM images showed strong signals for protein covering the mineral surface and inside the mineral clusters, which might indicate multilayer protein adsorption onto the mineral surface (Video S1). Colocalization analysis was applied to assess the amount of colocalization between the signals for protein and Cu in the images. The Mander’s split colocalization coefficient of each channel can reveal the proportion of signal in that channel that colocalizes with the other channel, with zero meaning no colocalization and one meaning perfect colocalization. The Mander’s coefficient for the channel of Cu (MCu = 0.948) was much higher than for the protein channel (Mprotein = 0.788) and very close to 1, indicating that Cu therefore strongly colocalized with protein in our samples. This also confirms that the proteins have a major role in formation of the Cu minerals. Following initial nucleation of Cu as a NP-protein complex, the mineral precursor can subsequently interact with more proteins, which results in the formation of a matrix of protein and CuNPs.

### Fourier-Transform Infrared Spectroscopy Analysis of Conformational Changes in Proteins

Fourier-transform infrared spectroscopy (FTIR) was conducted to further identify the interactions between the copper minerals and proteins and to provide additional information on chemical composition. Minerals produced in the presence of 0.1 g/L TPI were compared with standard malachite (Cu₂(OH)₂CO₃) (Figure 5A). Malachite showed characteristic peaks for CO₃²⁻ at around 1,490 and 1,378 (asymmetric stretch), 1,041 (symmetric stretch), and 816 cm⁻¹ (bending mode) in the FTIR spectrum. Broad bands were observed at around 3,400 cm⁻¹, which represented the stretching vibration of intermolecular bonded O-H groups. The biominerals precipitated from the 14-day reaction of 2 mM CuCl₂ and 0.1 g/L TPI, which showed a similar pattern to the standard, therefore confirmed as malachite. For the sample produced from the reaction of 2 mM CuCl₂ and 0.1 g/L TPI, a strong band appeared around 1,600–1,700 cm⁻¹, which is the characteristic amide I band of proteins describing C = O stretching in protein structures. The Amide II peak of protein occurred around 1,452 cm⁻¹, and it is possible that the
interaction of amide and carbonyl groups through hydrogen bonding shifted the carbonate peak at ~1,490 cm\(^{-1}\) to a higher wavenumber (~1,520 cm\(^{-1}\)), when compared with standard malachite. The sample that was produced in a protein-enriched environment also showed other protein peaks, such as the Amide III peak (CN stretching, NH bending) at 1,240 cm\(^{-1}\), the Amide IV peak (OCN bending), and additional fluctuations around 3,000–3,400 cm\(^{-1}\), which represent NH stretching.\(^{36}\) In addition, the occurrence of the peak around 2,960 cm\(^{-1}\) is attributable to C–H stretching, which also provides evidence for the association of organic materials with the mineral structure.\(^{37}\)

FTIR spectroscopy is a useful tool to determine protein secondary structure, and conformational changes were therefore studied by carrying out deconvolution and curve fitting of the amide I peak (Figures 5B and 5C).\(^{36,38}\) The Amide I peak can be resolved into peaks attributable to α helices, β sheets, β-turns, and random coils: the percentage content of the different secondary structures in the minerals are shown in Figure 5. Homology modeling of TPI showed that TPI is a helix-
rich protein with a β-barrel structure, with the percentage of α-helices in the original TPI protein being 48.8%, β-sheets being 15.3%, and β-turns 9.7%. The TPI protein possesses hydrophobic residues (e.g., alanine, valine) orientated into the interior of the barrel, while the polar residues are orientated toward the outside, which makes most β-barrel proteins highly water soluble and hydrophilic. After protein incorporation into the mineral structure, the content of α-helices decreased to varying extents: the α-helices percentage was 38.4% for minerals synthesized from the reaction of 2 mM CuCl₂ and 0.1 g/L TPI, but this value decreased for the sample produced with a higher Cu concentration (20 mM), to 25.7%. A significant increase in β-sheet content to 33.7% occurred for the sample produced from reaction of 2 mM CuCl₂ and 0.1 g/L TPI, which was an increase of 13.4% compared with original TPI. For the sample produced from 20 mM CuCl₂ and 0.1 g/L TPI, the β sheet content increased by 8.1% to 23.4% (Figure 5).

Several mineral-protein interactions, including electrostatic attraction, van-der-Waals interactions, hydrogen bonding, and the entropy gain of solvent molecules or counterion release, can induce significant conformational changes in the protein. For a hydrophilic protein with a rich α-helical
structure interacting with NPs, the NP surface replaces water molecules on the protein surface. The rearrangement of hydrogen bonds involved in this process results in a decrease in the content of helical structures and other conformational changes. Helices with polar and hydrophilic properties are more prone to attach to NP surfaces, which can explain the strong affinity of TPI with CuNPs. The decrease in the percentage of α helices and an increase in β sheets indicated unfolding of TPI during such association with CuNPs. Similar phenomena have been reported previously for different proteins and various NP surfaces. The decrease in α helices was expected to be balanced by an increase in β structures in most cases. The extra increase in β structures for the sample synthesized from 2 mM CuCl₂ and 0.1 g/L TPI can be attributed to a protein-protein reaction, which refers to multiplayer adsorption of the protein molecules or the attachment of more TPI molecules onto the pre-adsorbed TPI. It has been reported that the assembly of protein molecules can result in an increased level of β structures on a mineral surface. The different initial concentrations of Cu²⁺ showed different impacts on the secondary structure of adsorbed TPI, and the higher [Cu²⁺] induced more conformational change. This suggests that, when minerals were produced in the presence of a higher metal concentration, the larger surface area of the minerals would supply more sites.
for protein adsorption, leading to a higher level of conformational change.

This work has identified the spectrum of proteins produced by \(N.\ crassa\) during growth and provided insights into the role of such proteins in the biomineralization of CuNPs. Proteins with a high affinity to bind with CuNPs were screened using proteomic analyses, and further detailed studies on the impact of purified TPI on mineral formation demonstrated the extensive formation of NPs in the presence of TPI. Application of SEM and CLSM revealed the spatial relationship of metal ions to proteins at the micrometer scale. Examination of conformational changes in the protein using FTIR spectroscopy confirmed the association of proteins with the CuNPs and provided further understanding of the NP-protein interactions including conformational changes. As there is no need to maintain living organisms for NP production, a protein-based NP biosynthesis process may offer advantages for potential industrial application. Understanding the interaction mechanisms between proteins and metal NPs may provide protocols for industrial NP surface modification and optimization of production.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.10.044.

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AUTHOR CONTRIBUTIONS

F.L. and G.M.G. conceived the study and performed the experimental design. F.L. performed biomimetical synthesis, proteomic analysis, SEM, and FTIR analysis. D.S.S. performed the CLSM image collection. F.L. and D.S.S. interpreted the obtained data and results. F.L., D.S.S., and G.M.G. wrote the manuscript, and all authors edited and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and Virus Strains** |
| _Escherichia coli_ DH5α competent cells | Invitrogen | Cat#18265017 |
| _E. coli_ BL21(DE3)pLysS competent cells | From Prof Daan van Aalten’s lab | 46 |
| **Chemicals, Peptides, and Recombinant Proteins** |
| Fura 2-AM stain | Abcam | ab120873 |
| FilmTracer SYPRO Biofilm Matrix Stain | Thermal Fisher | F10318 |
| IAA | Abcam | ab146212 |
| DTT | Melford | Cat#3483-12-3 |
| Pierce Trypsin Protease | Thermal Fisher | 90057 |
| **Critical Commercial Assays** |
| Coomassie (Bradford) Protein Assay Kit | Thermal Fisher | 23200 |
| **Deposited Data** |
| Raw and analyzed datasets | This paper | https://dx.doi.org/10.17632/r2b5g763jj.1 |
| **Experimental Models: Organisms/Strains** |
| Neospora crassa | FGSC | FGSC: 2489 |
| **Oligonucleotides** |
| TPIS forward oligo: 5'-CGTGGA TCCATGGCTCGCAAGTCTTC3' | This paper | N/A |
| TPIS reverse oligo: 5'-TATGCGGCC GCTTACAGGGTGCTTTGAT-3' | This paper | N/A |
| **Recombinant DNA** |
| TPIS cloned in modified version of pGEX6P1 plasmid | This paper | N/A |
| **Software and Algorithms** |
| ImageJ | NIH | https://imagej.nih.gov/ij/ |
| Excel | Microsoft | https://www.microsoft.com/en-gb/microsoft-365/excel |
| Origin9.1 | OriginLab | https://www.originlab.com/ |
| Mascot Search Engine | Matrix Science | https://www.matrixscience.com/ |
| Swiss-model | SIB | https://swissmodel.expasy.org/ |
| Pymol 2.3.2 | Schrödinger | https://pymol.org/2/ |
| Zen | Zeiss | https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html |

RESOURCE AVAILABILITY

**Lead Contact**
Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Geoffrey Michael Gadd (g.m.gadd@dundee.ac.uk).

**Materials Availability**
This study did not generate new unique reagents.

**Data and Code Availability**
The datasets generated or analyzed during this study are available at Mendeley Data: https://dx.doi.org/10.17632/r2b5g763jj.1.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

The experimental fungus used in this study was *Neurospora crassa* (FGSC: 2489, Fungal Genetics Stock Centre (FGSC), Manhattan, Kansas, USA). After 3d growth on malt extract agar (MEA, Lab M limited, UK) in 90-mm diameter Petri dishes at 25°C in the dark, *N. crassa* was inoculated to a urea-modified AP1 liquid medium (pH = 5.5) consisting of: 2% (w/v) glucose (Merck, USA), 40 mM urea (Sigma-Aldrich, USA), 4 mM K$_2$HPO$_4$, 3H$_2$O (Sigma-Aldrich, USA), 0.8 mM MgSO$_4$, 7H$_2$O (Sigma-Aldrich, USA), 0.2 mM CaCl$_2$, 6H$_2$O (Sigma-Aldrich, USA), 1.7 mM NaCl (Sigma-Aldrich, USA), 9 x 10^{-3} mM FeCl$_3$, 6H$_2$O (Sigma-Aldrich, USA) and trace metals.0.014 mM ZnSO$_4$, 7H$_2$O (VWR, USA), 0.018 mM MnSO$_4$, 4H$_2$O (Sigma-Aldrich, USA) and 1.6 x 10^{-3} mM CuSO$_4$, 5H$_2$O (VWR, USA). 3. After 3d growth in complete AP1 medium, fungal biomass was collected and washed twice in sterile Milli-Q water by centrifugation (×4,000 g, 30 min), and then incubated in sterile phosphate-free AP1 medium for 12 days at 125 rpm, 25°C in the dark.

METHOD DETAILS

Biomineralization of copper carbonate nanoparticles (CuNP)

CuNP were produced following previous protocols. Briefly, *N. crassa* spent culture media was collected by centrifugation (×4,000 g, 30 min) after 12d growth, filtered using a 0.2 μm pore diameter syringe filter, and mixed with CuCl$_2$ solution at a 20 mM final concentration. The samples were placed on a roller shaker (60 rpm) overnight, and precipitated products were collected and washed twice with Milli-Q water by centrifugation (×10,000 g, 30 min).

Protein identification

The original fungal culture medium and reacted supernatant after mineral precipitation were collected, and proteins in solution were precipitated by mixing with a 20% (v/v)trichloroacetic acid (TCA) (Sigma-Aldrich, USA) overnight at 4°C and then washed twice with acetone. To investigate the association of proteins within the NP structures, pelleted CuNP were washed with (1) 100 mM Tris-Cl (VWR, USA) pH 7.4, (2) 2% Triton X-100 (Sigma-Aldrich, USA), (3) 2% SDS (Melford, UK), (4) 10% SDS and boiling for 10 min and (5) 10% SDS and boiling for 30 min respectively. Some CuNP samples were mixed with the respective denaturing solution, and mixed on a roller shaker at 60 rpm at room temperature for 20 min. Subsequently, all CuNP samples were centrifuged and washed twice with Milli-Q water (10,000 x g, 4°C, 30 min). The carbonate NP dissolved easily in 20% (v/v) TCA, and the proteins that were bonded to the NP were precipitated and purified using the TCA/acetone method.

Protein samples were quantified using the Bradford assay (Thermal Fisher, USA) and mixed with Laemmli Buffer, boiled for 5 min to denature the protein, and then loaded on a 12% (w/v) SDS-polyacrylamide gel. After running at 200 V for 40 min, the gel was stained with Quick Coomassie Stain (Geroner, UK) following the manufacturer’s protocol. The relative densities of protein bands were calculated using ImageJ (NIH, https://imagej.nih.gov/ij/). In order to obtain proteomic information, the bands on the gel were cut out and sliced into 1 mm pieces, and examined by mass spectroscopy. Briefly, samples were reduced and alkylated with 10 mM dithiothreitol (DTT) (Melford, UK) and 50 mM iodoacetic acid (IAA) (Abcam, UK) respectively to reduce disulfide bonds. Samples were dried and in-gel digested with 12.5 μg/mL trypsin (Thermal Fisher, USA) overnight at 30°C. The peptides were extracted from the gel slices and concentrated using a Speedvac (Eppendorf, Hamburg, Germany). Samples were finally analyzed on an Ultimate 3000 RS Lucnano system (Thermo Fisher, USA) coupled to a LTQ Orbitrap Velos Pro (Thermo Fisher, USA). RAW data files were extracted and converted to mascot generic files (.mgf) and searched against Sprot and the Local peptide database (Mascot Search Engine, Version 2.3.2 https://www.matrixscience.com/) to perform identification.

TPI cloning and expression

Biomass of *N. crassa* grown in AP1 medium for 3d was collected, flash frozen and homogenized in liquid nitrogen by grinding with a mortar and pestle and stored at -80°C until use. Total RNA was extracted using a Monarch Total RNA Miniprep Kit (New England Biolabs, USA) according to the manufacturer’s instructions. Single-strand cDNA was synthesized from total RNA using oligo (dt) primer for reverse transcription (qScript cDNA SuperMix, Quantabio, USA). In accordance with the amino acid sequence of TPI from *N. crassa*, two oligonucleotide primers were designed and synthesized (forward oligo: 5'-CGTGGATCCATGGCTCG-3'; reverse oligo: 5'-TTATGCGGCCGCTTACAGGTTGGCGTTGAT-3'). TPI DNA was amplified by the polymerase chain reaction (PCR) using Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA) following the manufacturer’s protocol. The final PCR products were isolated from 0.8% agarose/TBE gel and purified using a Monarch DNA Gel Extraction Kit (New England Biolabs, USA). DNA of TPI was cloned as a BamHI-NotI fragment into the pGEX6P1 plasmid (modified version of pGEX which contains a His6 tag instead of GST). The recombinant plasmid was transformed into competent *Escherichia coli* DH5α cells (Invitrogen, Thermo Fisher Scientific, USA) and grown in Luria-Bertani (LB) broth with 100 μg/mL ampicillin overnight. The correct cloning was confirmed by sequencing analysis.

TPI was expressed in *E. coli* BL21(DE3)pLysS by inoculating 1 L of LB medium with 10 mL of an overnight culture grown in the presence of 100 μg/mL ampicillin and allowing it to grow at 37°C until the OD$_{600}$ reached 0.6. The culture was induced with 250 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, USA) and incubated at 18°C, 160 rpm at dark for 20 h. Cells were harvested by centrifugation (3,000 x g, 4°C, 30 min) and the pellet resuspended in lysis buffer (25 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM TCEP (tris(2-carboxyethyl)phosphine), 0.1 g/L DNase I, 1g/L lysozyme, 1 vial/50 mL protease inhibitor cocktail) and lysed.
using a French press. The lysate was centrifuged (20,000 x g, 4 °C, 1 h) and the supernatant filtered through 0.45 μm pore size cellulose acetate filters and incubated with Ni-NTA resin (GE Healthcare, USA) for 2 h at 4 °C on a rotating shaker (60 rpm). The Ni beads were thoroughly washed using washing buffer (25 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM TCEP, 30 mM Imidazole pH = 8), and His-tag fusion proteins were eluted using eluent buffer (25 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM TCEP, 100 mM Imidazole pH = 8). The protein samples were incubated with PreScission protease at 4 °C overnight to cleave the His-tag, followed by concentration using a Sartorius Vivaspin ultrafiltration concentrator 10,000 MWCO (Sartorius, Germany) and passing through a HiLoad 26/600 Superdex 75 pg (GE Healthcare, USA) pre-equilibrated with base buffer (25 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM TCEP). The peak fractions were concentrated to 10 mg/mL and stored at −80 °C until use. The protein 3D structure model was calculated in the SWISS-MODEL workspace (https://swissmodel.expasy.org/) based on homology modeling.

Impact of TPI on biomineral formation
Chemically synthesized copper carbonate was used as a control and obtained by mixing 20 mM (NH4)2CO3 and 2mM / 20 mM CuCl2 and collecting and washing the precipitate. In order to identify the impact of TPI on biomineralization, TPI was added into the chemical reaction mixture to final concentrations of 0.02 and 0.1mg/mL. The samples were mixed on a roller shaker (60 rpm), and collected by centrifugation (x10,000 g, 30 min) after 1d and 14d reaction times. The precipitated samples were examined by SEM and CLSM to investigate the influence of TPI on their structure and morphology.

Scanning electron microscopy (SEM)
After washing in Milli-Q water twice, a drop of sample suspension was mounted on carbon adhesive tape on aluminum electron microscopy stubs. Samples were air-dried at room temperature overnight in a desiccator, followed by coating with 7 nm gold and platinum using a Cressington 208HR sputter coater (Ted Pella, Redding, CA, USA). SEM images were obtained by using a field emission scanning electron microscope (FESEM) (Jeol JSM7400F) at 5 kV.

Confocal Laser Scanning Microscopy (CLSM)
Biominerals generated during a 14d reaction where 2 mM CuCl2, 20 mM (NH4)2CO3 and 0.1 mg/mL TPI were combined were examined by CLSM. Prior to staining, the sample was washed twice and suspended in Milli-Q water. Copper was stained for 30 min at room temperature using 70 μM Fura-2AM (Abcam, UK) in the dark. Fura-2AM stained samples were pelleted and subsequently resuspended in the protein stain FilmTracer Sypro (Thermal Fisher, USA) at room temperature for 20 min in the dark. The protein and copper stained samples were washed twice and resuspended using Milli-Q water. Approximately 3 μL of resuspended samples were encased between a glass slide and a coverslip and sealed using nail varnish. Protein-mineral aggregates were imaged in real time using a Zeiss 710 confocal microscope with a 60x oil-immersion objective at room temperature with excitation (nm) /emission (nm) set at 380/510 for Fura-2AM and 450/610 for FilmTracer Sypro. Zen software was used to process images and to generate 3-dimensional (3D) images (https://www.zenlabs.com/microscopy/int/products/microscope-software/zen.html) whereby multiple (up to 20) 2D images taken at 5 μm intervals were combined. Colocalization analysis was applied to quantify colocalization between the signals for protein and Cu in the images by using ImageJ.

Attenuated total reflectance–Fourier-transform infrared (ATR-FTIR) spectroscopy
ATR-FTIR spectra of samples in the form of powders were obtained using a Bruker Vertex 70 FTIR spectrometer (Billerica, Massachusetts, USA). All spectra were measured over the wavelength range from 400 to 4000 cm⁻¹, with a 4 cm⁻¹ spectral resolution. Second-derivative spectral analysis was applied to the amide I peak at 1600-1700cm⁻¹ after background subtraction, in order to locate the position of the overlapping components of the band. The amide I band was then fitted to a Gaussian curve by Origin 9.1 (https://www.originlab.com/), and the proportion of each component was calculated as the fractional area of the corresponding peak divided by the sum of the area of the amide I band.50

QUANTIFICATION AND STATISTICAL ANALYSIS
The density of protein bands was calculated by ImageJ from triplicate tests, and the error bars are shown as one standard error. The typical SEM and CLSM images and spectra are shown from several separate determinations.