Fungal ferritins: The ferritin from mycelia of *Absidia spinosa* is a bacterioferritin

Carl J. Carrano*,**, Rudi Böhnke, Berthold F. Matzanke*,***

Microbiology and Biotechnology, University of Tübingen, Auf der Morgenstelle 1, 72076 Tübingen, Germany

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Abstract Two distinct ferritin like iron containing proteins have been identified and isolated from the fungus *Absidia spinosa*; one from the spores and another from the mycelia. The mycelial protein has been purified and consists of two subunits of approx. 20 kDa. The N-terminal sequences of both subunits have been determined. The holoprotein as isolated contains approx. 750 iron atoms/molecule and exhibits a heme-like UV-Vis spectrum. Based on the heme spectrum and the high degree of sequence homology found, it has been established that the mycelial protein is a bacterioferritin. This is the first example demonstrating the presence of a bacterioferritin in a eukaryotic organism.

Key words: Iron metabolism; Ferritin; Bacterioferritin; Fungi; *Absidia spinosa*

1. Introduction

The ferritins constitute a broad superfamily of iron storage proteins found in most aerobic organisms [1]. One of the major branches of this family is the so-called mammalian-type ferritins, which have been widely studied, and are found in mammals, fish, invertebrates, and other eukaryotes [2–6]. The structure of horse spleen ferritin has been determined by single-crystal X-ray diffraction analysis and consists of 24 subunits of approx. 20 000 Da arranged in 432 point symmetry to form a hollow spherical shell into which up to 4000 iron atoms can be deposited [3]. The mineral core is primarily made up of hydrated ferric oxide or ferrihydrate [7]. It is presumed that the other members of this branch share the same structure as they show a degree of sequence homology. The mammalian-type ferritins also display subunit heterogeneity with two clearly different types, designated H and L, having been found in the horse spleen protein [3,4]. The phytoferritins found in many plant species also belong to this group although there is an extension peptide of approx. 3 kDa and no subunit heterogeneity has been reported [8].

The second branch of the superfamily consists of the bacterioferritins (Bfr) found in many prokaryotes. These proteins have similar molecular masses in both their holo form (400 000–500 000 Da) and subunits (20 000 Da) to those of the mammalian type but are otherwise quite different. They do not cross-react immunologically and show very little (approx. 20%) sequence homology with the mammalian ferritins [1,5]. In addition, they contain from 0.5 to 0.2 hemes (protoporphyrin IX) per subunit and their mineral cores are primarily hydrated ferric phosphate rather than oxide [4,5]. The role of the hemes and the significance of the different core composition remain unknown.

The fungi are an important class of eukaryotic microorganisms, however, almost nothing is known about their ferritins, or even whether they contain such molecules at all. As eukaryotes they can be viewed as evolutionarily distant from, but still related to, other members of this phylum in which only mammalian ferritins have thus far been found. In fact, ferritin-like molecules have been identified in only two species of fungi, *Phycomyces blankesleeanus* and *Mortierella alpina*, both of which belong to the Zygomycotina [9–11]. Unfortunately, neither of these ferritins has been well characterized and hence it is difficult to determine their relationship, if any, with the other members of the ferritin superfamily.

The function(s) of the ferritins is probably multi-faceted and stems from the need to provide a stable source of the not very bioavailable yet essential metal, iron, while at the same time protecting the organism from the toxic effects of free ferrous ion [12]. These toxic effects derive from the interaction of iron with dioxygen which leads to Haber-Weiss-Fenton chemistry producing highly deleterious hydroxyl radicals. There exists some speculation that the roles of the mammalian and bacterio-type ferritins may in fact be somewhat different. This view is supported by the recent isolation of a mammalian-type ferritin from *E. coli*, an organism which also contains Bfr [13,14] as well as from *Helicobacter pylori* [15].

It is widely recognized that Mössbauer spectroscopy is a technique well suited for the in vitro study of iron-containing biomolecules such as the ferritins. What is less appreciated is its use in nondestructive investigations of iron metabolism in vivo. In several studies one of us (B.F.M.) has demonstrated that Mössbauer spectroscopy of whole cells yields valuable information about the major components of iron metabolism as well as their time-dependent changes [16–18]. All of this has been done without the need for interference from any isolation procedure. In particular, we have used in vivo Mössbauer spectroscopy to probe iron metabolism in two fungal species of the class Zygomycotina, *Absidia spinosa* and *Rhizopus arrhizus* [19,20]. The results indicated that *A. spinosa* did indeed contain ferritins and that two distinct types were observable. One of these ferritins had Mössbauer parameters similar to those of Bfr and was found primarily in the growing mycelial haplophase of the fungus, while a second ferritin was concentrated in the asexual spores. This ferritin displayed Mössbauer parameters much closer to those of mammalian ferritin than

*Corresponding authors. ***Permanent address:* Department of Chemistry, Southwest Texas State University, San Marcos, TX 78666, USA; Fax: (1) (512) 245-2374.
E-mail: cc05@swt.edu

****Permanent address:* Isotopenlaboratorium der Technisch-Naturwissenschaftlichen Institute, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany.

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the mycelial one, indicating clear differences in the mineral core structure. In this report, we follow up these observations with detailed biochemical studies and show for the first time that the mycelial haplophase of *A. spinosa* contains a bacteriotype ferritin which differs substantially from the spore-type ferritin found in the same organism.

2. Materials and methods

A. spinosa Tü268 was an isolate from the stock of the Botany Department of our university (collection G. Kost, G. Weber, F. Spaaij). Asexual spores for the inoculation of liquid cultures of *A. spinosa* were grown in 500 ml Erlenmeyer flasks for 3 weeks at 23°C on 150 ml of agar containing (per l): yeast extract, 4 g; malt extract, 10 g; glucose, 4 g; and agar, 20 g. Spores were harvested by shaking with 50 ml saline plus 2 drops Tween 80 and scraping the surface with a sterile spatula. The resulting suspension was filtered through glass wool to remove hyphal fragments and the spores were washed twice with saline. Spores used to inoculate mineral salt media were allowed to sediment overnight at 4°C yielding a titer between 3 and 70×10^8 spores/ml. For ferritin isolation the agar was supplemented with 100 μM ferric citrate (Fe/citrate 1:20). Isolation of ferritins from *A. spinosa* followed a modification of the method of La-Bombardi et al. [11]. Mycelia were grown via shaker culture in a medium containing (per l): L-asparagine, 2.5 g; K_2HPO_4, 1.0 g; $MgSO_4\cdot7H_2O$, 1.0 g;$CaCl_2\cdot2H_2O$, 0.5 g; $ZnSO_4\cdot7H_2O$, 0.1 g. Glucose (5 g/l) and ferric citrate (final [Fe] = 100 μM) were autoclaved separately while biotin (0.01 mg/l) and thiamin (0.1 mM) were filter sterilized. After shaking for 72 h at 27°C, the mycelia were harvested by filtration and washed with water. A portion of the mycelial mass was then dispersed in 0.5 M phosphate buffer, pH 6.4, frozen at −20°C and disrupted by at least five passes through an X-PRESS. The homogenate was clarified by centrifugation ($20\,000\times g$) for 30 min. The pale yellow supernatant was then stirred overnight with 0.4 vol. of *n*-butanol and recentrifuged to separate the layers and remove precipitated proteins. The aqueous layer was freed of *n*-butanol by flash evaporation at 65°C for 2 h during which time the volume was continually made up with distilled water. After sitting at 4°C for several hours the coagulated proteins were removed by Millipore filtration (0.2 μm) and the solution dialyzed overnight against 0.05 M phosphate buffer pH 6.4. Some samples were desalted by a second overnight dialysis against distilled water and subsequently lyophilized. Further purification was achieved via preparative native PAGE using a 6% running gel.

Spore ferritin was isolated in a similar fashion from approx. 2 g wet weight of spores. Unfortunately, only a small amount of ferritin could be isolated from these preparations due to the refractory nature of the *A. spinosa* spores which resisted efforts to disrupt them. In addition, the spore ferritin ran so slowly on native PAGE that preparative electrophoresis was unsuitable for its purification. Hence, only partially purified protein could be isolated.

The purity of the holo proteins and estimates of their molecular masses were determined by analytical PAGE using an 8% native gel and the discontinuous buffer system of Laemmli [21]. Holoprotein molecular masses were also estimated by HPLC gel filtration at 0.3 ml/min on a TSK gel G4000SW column equilibrated with 0.1 M phosphate buffer, pH 6.4, containing 0.1 M sodium sulfate and 0.05% azide. Subunit molecular weights were determined, after heating the holoprotein with a sample buffer containing 7.5% DTT and 5% SDS at 100°C for 10 min, using a 4–20% Tris-HCl gradient gel from Bio Rad. Both the high and low molecular mass standards were from Pharmacia. Proteins were visualized by staining with either Coomassie Brilliant blue R-250 (Fluka, Buchs Switzerland) or silver [22]. Iron-containing proteins could also be visualized by the method of Kuo and Fridovich [23] either alone or in combination with Coomassie staining.

N-terminal sequence analysis was performed on protein electroblotted directly from the gradient gels onto PVDF membranes (0.45 μm, Millipore). UV-Vis spectra were recorded on a Pharmacia LKB Ultraspec III spectrophotometer. Protein content was estimated by the Bradford method while iron was determined by atomic absorption on a PE 400 instrument equipped with a graphite furnace and autosampler.

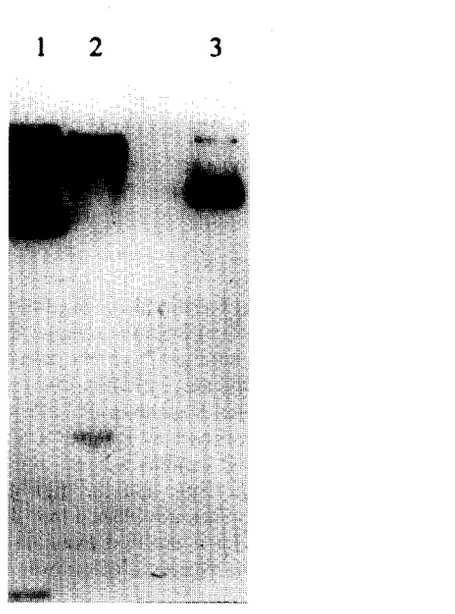

Fig. 1. Native PAGE profiles of partially purified ferritins from mycelia and spores of *A. spinosa* stained for both iron by the Fridovich method and protein with Coomassie blue. The dark ferritin bands in the upper third of the gel stained positively for both iron and protein while the remaining weak bands in the lower third of lanes 1 and 2 are non-iron containing protein impurities.

3. Results and discussion

In contrast to the results reported for *Phycomyces*, butanol extraction, heating to 65°C and dialysis using a 100 kDa cut-off membrane did not lead to highly purified ferritins from either mycelia or spores of *A. spinosa*. Native PAGE gels showed a number of protein bands, however, after partial purification only a single iron containing species was evident for each after Fridovich staining (Fig. 1). The apparent molecular masses as determined by native PAGE for the spore and mycelial ferritins were quite different with the mycelial protein migrating slightly faster than horse spleen ferritin and the spore protein running much slower. However, it is evident that the difference in apparent molecular mass between the two proteins is a result of a difference in surface charge rather than actual mass, since on gel filtration all three proteins ran coincidentally.

The mycelial ferritin could be further purified by preparative PAGE yielding an essentially homogeneous protein. The UV-Vis spectrum of the purified protein displayed bands at 417 and 530 nm in its oxidized form and at 424, 526, and 557 nm in its reduced state, features which are characteristic of the heme found in all bacterioferritins (Fig. 2). The iron loading

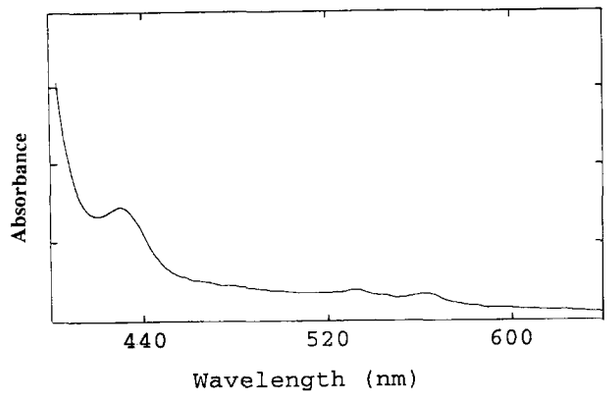

Fig. 2. Optical spectrum of reduced (sodium dithionite) and purified mycelial ferritin from *A. spinosa*.

of the holoprotein was relatively low at approx. 750 Fe atoms/molecule, another characteristic of the bacterioferritins. The holoprotein could be dissociated into its subunits by heating in the presence of a reducing agent and SDS for 10 min at 100°C. SDS gel electrophoresis on gradient gels showed two clear bands of similar intensity with molecular masses of 20000 and 19000 Da. Electroblotting these subunits onto PVDF membranes with subsequent N-terminal sequence analysis gave the sequences shown in Fig. 3.

When the N-terminal sequences reported here for the two subunits of mycelial ferritin from *A. spinosa* were checked against the sequences in the National Center for Biotechnology Information (NCBI-NLM) component data banks, all of the high scoring matches were bacterioferritins. The degree of homology of the lower band ranged from 44% identity with mycobacterial Bfr and Bfr from *A. winograskyi* to 80% identity between it and the α-subunit of Bfr from *Pseudomonas aeruginosa* [24]. Interestingly, the latter two Bfrs exhibit sequences of 20 amino acids stretching from Gly-17 to Gly-36 which are, except for one difference (Leu-26/Phe), identical. The degree of homology of the upper band of *A. spinosa* varied from 52% identity with the Bfr from *M. leprae* and *Synechocystis* to 67% between it and the β-subunit of the *Pseudomonas aeruginosa* Bfr. These data in combination with the observation of a heme spectrum in the purified protein leave no doubt that the ferritin isolated from *A. spinosa* mycelia is a bacterioferritin. Since it is has been proposed that the heme groups in Bfrs have bismethionine ligation [25,26], it is perhaps noteworthy that amongst all the Bfr sequences determined up to now, only one Met is absolutely conserved, i.e. the N-terminal Met-1 [27]. The residues of the putative ferroxidase site in human H-ferritin are Glu-27, Glu-62, His-65, Glu-107, and Gln-141 [28]. In the bacterioferritins the corresponding amino acids are Glu-18, Glu-50, His-53, Glu-93, and Leu-119(Met). To the extent that the short sequences reported here allow, the residues of the putative ferroxidase site remain absolutely conserved in all the bacterial Bfrs and in the lower fungal band. However, in the upper band of *A. spinosa* mycelial ferritin Glu-18 is replaced by a His, a substitution similar to that seen in mammalian L-chains (i.e. His/Lys/Tyr). An additional residue of H-chain ferritin stabilizing the ferroxidase center via hydrogen bonding is Tyr-34 which also remains absolutely conserved in all Bfrs analyzed so far.

There have been reports in the literature that there exists subunit heterogeneity in at least some of the bacterioferritins based on SDS-PAGE profiles [29]. For example, upon SDS-PAGE, *A. vinelandii* Bfr has been reported to give two bands of M_r 19500 and 13000. Since no evidence for sequence heterogeneity was found, the lower band was attributed to degradation [30]. Pea seed ferritin also gives two bands, the smaller of which is again thought to arise from the larger by a specific cleavage promoted by hydroxyl radicals. However, subunit sequence heterogeneity has recently been reported for the Bfr from *Pseudomonas aeruginosa* [24]. In the fungus *A. spinosa* the N-terminal amino acid sequences of the upper and lower mycelial band shown in Fig. 3 are also clearly distinct, indicating that the lower band is not a degradation product of the upper and confirming the extension of subunit heterogeneity to the Bfr class of protein. It is remarkable that the *Absidia* subunits share their greatest degree of homology with the corresponding α- and β-bands from the *Pseudomonas* Bfr. Natural mammalian ferritins are also composed of various proportions of two subunit types designated H and L which are genetically distinct but share 55% amino acid sequence homology. Similarly, the upper and lower bands of mycelial Bfr from *A. spinosa* exhibit a relatively low identity of 55%. Since Glu-18 is missing from the upper mycelial band, it is tempting to propose, that similar to the mammalian L-chain, the ferroxidase site is missing from this subunit.

The initial suggestion based on Mössbauer spectroscopy that both spores and mycelia of *A. spinosa* each contain a ferritin but that these differ from each other has been confirmed by this work. Polyacrylamide gel electrophoresis with Fridovich staining demonstrates clearly that the ferritins isolated from mycelia and spores differ significantly. Unfortunately, we have been unable to characterize rigorously the *Absidia* spore ferritin due to the small amount of material available, however, it shows no heme-type spectrum suggesting that it is a mammalian-type ferritin. This is again in concert with the earlier in vivo Mössbauer results. The presence of the two distinct types of ferritin in two different growth phases of *Absidia* is intriguing in that it suggests a different function for each. Although clearly speculative at this point, it seems reasonable to suppose that in spores the ferritin is likely to play its traditional role, i.e. that of long-term iron storage. This would be consistent with what is known about the role of other mammalian type ferritins. In the growing mycelial phase, on the other hand, iron is rapidly being used in metabolic processes and the presence of only a bacterioferritin

N-terminal Sequences of bacterioferritins

	% identity to *A. spinosa* protein band		
	lower	upper	
MRGNPEVIDY LNMLIGGELA ARDQYLIHSR MYEDWGLTK- Y	100	55	*Absidia spinosa*, mycelia lower
MKGNREVINQ LNQVLYHHLC AINQYFLHSR M-ND	55	100	*Absidia spinosa* mycelia upper
MKGDTKVINY LNKLLGNELV AINQYFLHAR MFKNWGLKRL N	49	61	*E. coli*
MKGDKIVIQH LNKILGNELI AINQYFLHAR MYEDWGLEKL G	51	58	*Azotobacter vinlandii*
MQGDPDVLRL LNEQLTSELT AINQYFLHSK MQENWGFTEL A	44	52	*Mycobacterium leprae* [27]
MQGDPDVLRL LNEQLTSELT AINQYFLHSK MQDNWGFTEL A	44	55	*Mycobacterium paratuberculosis*
MKGKPAVLAQ LHKLLRGELA ARDQYFIHSR MYQDWGLEKL Y	66	52	*Synechocystis* PCC 6803
MKGDPKVIDY LNKALRHELT AINQYWLHYR LLDNWGIKDL A	44	55	*Nitrobacter winogradskyi*
MKGDKKVIQH LNKILGNELI AINQYFLHSR MWNDWGLKRL Y	53	67	*Pseudomonas aeruginosa*-α [24]
MQGHPEVIDY LNTLLTGELA ARDQYFIHSR MYEDWGFSKL Y	80	52	*Pseudomonas aeruginosa*-β [24]
M G V LN L EL A NQY LH R M WG L			consensus

Fig. 3. N-terminal amino acid sequences of the 'heavy' and 'light' chains of denatured mycelial ferritin from *A. spinosa*. Sequences taken from [1] except where indicated. Residues marked with a [?] indicate where an assignment is uncertain.

suggests that this molecule might play a short-term iron-storage role. Finally, based on our finding of a bacterial-type ferritin in a eukaryote, the term bacterioferritin seems to us no longer appropriate for this group. Whether the term heme-containing ferritin (HCF) would be self-consistent or whether the subunit heterogeneity of the *Absidia* and *Pseudomonas* ferritins requires the definition of a new group remains an open question.

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