Food Chemistry 210 (2016) 491–499

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Limited proteolysis of myoglobin opens channel in ferrochelatase-globin complex for iron to zinc transmetallation

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ARTICLE INFO

Article history:
Received 21 September 2015
Received in revised form 20 April 2016
Accepted 22 April 2016
Available online 23 April 2016

Keywords:
Parma ham
Ferrochelatase
Myoglobin
Meat pigment
Transmetallation

ABSTRACT

Recombinant ferrochelatase (BsFECH) from *Bacillus subtilis* expressed in *Escherichia coli* BL21(DE3) was found by UV-visible spectroscopy to bind the model substrate tetraphenylporphyrin-sulfonate, TPPS, with $K_a = 3.8\ 10^5$ mol/L in aqueous phosphate buffer pH 5.7 at 30 °C, and to interact with metmyoglobin with $K_a = 1.07 \pm 0.13\ 10^5$ mol/L at 30 °C. The iron/zinc exchange in myoglobin occurring during maturation of Parma hams seems to depend on such substrate binding to BsFECH and was facilitated by limited pepsin proteolysis of myoglobin to open a reaction channel for metal exchange still with BsFECH associated to globin. BsFECH increased rate of zinc insertion in TPPS significantly and showed saturation kinetics with an apparent binding constant of Zn(II) to the [enzyme-TPPS] complex of $1.3\ 10^4$ mol/L and a first-order rate constant of $6.6\ 10^{-1}\ s^{-1}$ for dissociation of the tertiary complex, a similar pattern was found for zinc/iron transmetallation in myoglobin.

1. Introduction

Insertion of ferrous iron into protoporphyrin IX to produce protoheme is catalyzed by ferrochelatase (FECH; EC 4.99.1.1, protoheme ferrolyase) and is the terminal step in heme biosynthesis (Ajioka, Phillips, & Kushner, 2006; Ferreira et al., 1995). FECH genes have been isolated and sequenced from various organisms ranging from bacteria to higher eukaryotes and its catalytic residues are conserved. The prokaryotic and eukaryotic enzymes vary with regard to their cellular distribution and the presence or absence of an iron-sulfur cluster at the carboxyl terminal of the protein (Ajioka et al., 2006; Ferreira et al., 1995). The role of this cluster present in the mammalian enzyme is still unclear. Ferrous iron has been established as the preferential substrate for the enzyme *in vivo*. Although it was proved that FECH irreversibly catalyzes the insertion of metal ions into porphyrin ring, it was also demonstrated, *in vitro*, that FECH can catalyze iron removal from heme to create protoporphyrin and subsequently convert protoporphyrin IX to Zn-protoporphyrin IX (Chau, Ishigaki, Kataoka, & Taketani, 2010; Chau, Ishigaki, Kataoka, & Taketani, 2011; Taketani et al., 2007).

In dry cured meat products and in cooked hams the characteristic red color is usually due to nitrosylmyoglobin and nitrosylhemochromagen, respectively (Pegg & Shahidi, 1997). Because nitrosamines, generated in meat product upon addition of nitrite, are considered to be associated with cancer risk, nitrite free meat products are often preferred and considered to be a healthier alternative (Bryan, Alexander, Coughlin, Milkowski, & Boffetta, 2012; Mirvish, 1995). Parma hams are produced without the addition of nitrites but still develop a characteristic red color (Parolari, Benedini, & Toscani, 2009; Wakamatsu, Nishimura, & Hattori, 2004). The nature of the Parma ham pigment has been unexplained until 2004, when Zn-protoporphyrin IX was found to contribute to red color (Wakamatsu et al., 2004). Three possible mechanisms have been suggested for formation of the Parma ham pigment including a) a non-enzymatic reaction in which Zn-protoporphyrin is formed under anaerobic conditions (Becker, Westermann, Hansson, & Skibsted, 2012); b) enzymatic reactions where FECH is directly involved (Becker et al., 2012; Chau et al., 2011); c) bacterial enzymatic reactions (Morita, Niu, Sakata, & Nagata, 1996). We have recently suggested a novel mechanism by which Zn-protoporphyrin formation is promoted when myoglobin is partly degraded by endogenous peptidases during muscle salting and ham maturation (Grossi, do Nascimento, Cardoso, & Skibsted, 2014). The role of FECH at various stages of the degradation of myoglobin was not investigated and previously

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http://dx.doi.org/10.1016/j.foodchem.2016.04.109
0308-8146/

non-enzymatic formation of Zn-protoporphyrin IX was suggested to occur in parallel with the enzymatic exchange reaction (Becker et al., 2012). It seems timely to explore how myoglobin degradation by proteolysis modulates transmetallation catalyzed by myoglobin as substrate for FECH and may further affect Zn-protoporphyrin IX formation. We now report the results of such investigations combining experiments with tetraphenylporphyrin sulfonate as a model substrate for kinetic studies and with partly proteolyzed myoglobin as substrate using recombinant *B. subtilis* FECH as the active enzyme. FECH from prokaryotic organisms is easily obtained for such model studies and may also form the basis for future use in the meat processing industry.

2. Material and methods

2.1. Protein expression and purification

Recombinant *Bacillus subtilis* ferrochelatase (BsFECH) carrying His-tag was obtained by inserting corresponding DNA into pET28a vector. The recombinant BsFECH was expressed by *Escherichia coli* BL21(DE3) strain cultivated in the LB medium containing 50 μg/L of Kanamycin. The cultures were induced using 0.4 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, Steinheim, Germany) and incubated at 30 °C for 4 h. These cells were pelleted by centrifugation (7000 rpm at 4 °C for 30 min) and protein expression was checked by SDS-PAGE 12%. Pelletized bacteria were suspended in buffer added of 5 U of DNAse kit (Promega, Madison, WI) and 30 μg/L of lysozyme (Sigma-Aldrich, Steinheim, Germany) and incubated for 1 h in an ice-bath. Cells were then disrupted by ultrasonic cavitation and the soluble fractions separated by centrifugation (14,000 rpm at 4 °C for 30 min) and further analyzed by SDS-PAGE 12% gel stained with coomassie blue (Sigma-Aldrich, Steinheim, Germany). The recombinant BsFECH in the soluble fraction was purified by Ni^{2+} affinity chromatography using a HiTRAP chelating column in a Äkta Prime instrument (GE Healthcare Lifesciences, Uppsala, Sweden). The His-tagged protein was cleaved using 1U/mg thrombin (Sigma-Aldrich, Steinheim, Germany) for 12 h at 4 °C. Recombinant BsFECH was further purified by size-exclusion chromatography using a HiLoad Superdex 200pg 26/60 column coupled to a Äkta Prime instrument (GE Healthcare Lifesciences, Uppsala, Sweden) equilibrated with the 25 mM Tris-HCl buffer pH 7.5 (Sigma-Aldrich, Steinheim, Germany), 100 mM NaCl (Sigma-Aldrich, Steinheim, Germany) and 1 mM β-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany). Protein concentration was determined by the absorbance of the protein solution at 280 nm according to established procedures in the literature (Edelhoch, 1967).

Recombinant BsFECH was studied by analytical size exclusion chromatography in order to estimate the Stokes radius as well as oligomeric status. For that, we used a Superdex 200 10/300 GL column (GE Healthcare Lifesciences, Uppsala, Sweden) coupled to a ÄKTA Prime device, equilibrated with the 25 mM Tris-HCl buffer (pH 7.5), 100 mM NaCl and 1 mM β-mercaptoethanol. The standard protein mixture was constituted by apoferritin (480 kDa/R_s 67 Å), γ-globulin (160 kDa/R_s 48 Å), BSA (67 kDa/R_s 36 Å), ovalbumin (45 kDa/R_s 29 Å), carbonic anhydrase (29 kDa/R_s 24 Å) and cytochrome C (12 kDa/R_s 14 Å) (Sigma-Aldrich, Steinheim, Germany). The retention times were transformed to the partition coefficient K_{av} applying the following equation:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \quad (1)$$

where V_e is the elution volume of the protein; V_0 is the void volume of the column and the V_t is the total volume of the column. The $-\log K_{av}$ was plotted against the R_s of standards in order to estimate the R_s of BsFECH by linear regression. The frictional ratio (f/f_0) was estimated by the ratio of the experimental R_s to the radius of a sphere of the same mass.

2.2. Synchrotron small angle X-ray scattering

Small-angle X-ray scattering (SAXS) experiments were performed at the Brazilian Synchrotron Light Laboratory (LNLS, Campinas-SP, Brazil) using a monochromatic X-ray beam ($\lambda = 1.488$ Å) of the D02A-SAXS1 beam line. The sample-to-detector distance was ~1000 mm, which corresponds to the scattering vector range of $0.015 < q < 0.35$ Å^{-1}, where q is the magnitude of the q-vector defined by $q = (4\pi/\lambda)\sin\theta$ (2θ is the scattering angle). The protein samples were placed in a 1-mm path-length cell formed by two mica windows, and the scattering curves were recorded at 0.6 mg/mL in buffer solutions, as indicated below. The sample and buffers were subjected to X-ray frames of 100 s, and the scattering curves were corrected for the detector response and scaled by the incident beam intensity and the sample attenuation. The corrected scattering sample was subtracted from the scattering buffer curve. The Guinier law was used to estimate both protein radius of gyration (R_g) and irradiation intensity I(0). The GNOM program (Semenyuk & Svergun, 1991) was used to generate the pair distance distribution curves (p(r) curves), which supply information about protein size and shape.

2.3. UV-vis absorption spectroscopy and spectral titrations

Spectrophotometric titrations were carried out in an Shimadzu UV3600 spectrophotometer equipped with a TCC-100 Thermoelectrically Temperature Controlled Cell Holder (Shimadzu Co., Kyoto, Japan) and using 0.2 × 0.2 quartz cell (Hellma Gmbh, Müllheim, Germany) or using a 96-well plate reader spectrophotometer Multiskan GO (Thermo Scientific, Vartaa, Finland). The spectrophotometric studies of the metal exchange reactions were done using the same spectrophotometer with thermostated cell holder. The sodium tetraphenylporphyrin sulfonate, TPPS, and the zinc acetate used for the kinetic studies were from Sigma-Aldrich (Steinheim, Germany).

2.4. Circular dichroism (CD) spectroscopy

CD spectra were recorded in J-815 spectropolarimeter (Jasco Corp., Tokyo, Japan) coupled to the Peltier system PFD 425S for temperature control and using a quartz curvet of 1 mm × 1 mm (Hellma-Gmbh, Müllheim, Germany) for protein concentration of 0.17 mg/mL and of 0.5 mg/mL. CD spectra were normalized to the mean residue ellipticity ([θ]), and the protein secondary structure content was estimated using the CDNN deconvolution program (Bohm, Muhr, & Jaenicke, 1992). Half-denaturation concentrations (C_m) of urea as denaturant was determined by parameter fitting accordingly to the denaturation equilibrium:

$$\text{native} - \text{BsFECH} + \text{n urea} \rightleftharpoons \text{denaturated} - \text{BsFECH} \cdot (\text{urea})_n \quad (2)$$

with the following equilibrium expression:

$$K_{N\to D} = \frac{[denaturated - BsFECH \cdot (urea)n]}{[urea]^n * [native - BsFECH]} \quad (3)$$

$$\log \frac{[native - BsFECH]}{[denaturate - BsFECH \cdot (urea)n]} = \log K_{N\to D} + n * \log[urea] \quad (4)$$

Thermal-induced unfolding was monitored by CD spectroscopy and were performed with 1 mg/mL protein in a 1 mm quartz cuvette using a heating rate of 1 °C/min and data acquisition at