Heme Synthase (Ferrochelatase) Catalyzes the Removal of Iron from Heme and Demetalation of Metalloporphyrins†

Shigeru Taketani,*,‡,§ Mutsumi Ishigaki,‡ Atsushi Mizutani,‡ Masashi Uebayashi,|| Masahiro Numata,|| Yoshiko Ohgari,‡ and Sakihito Kitajima‡

Department of Biotechnology and Insect Biomedical Center, Kyoto Institute of Technology, Kyoto 606-8585, and Ito Ham Inc., Ibaraki 302-0104, Japan

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ABSTRACT: The red pigments in meat products, including cooked cured ham, arise from the reaction of myoglobin with nitric oxide generated from exogenous nitrite. Since carcinogenic nitrosoamines may be generated by the treatment of meats with nitrite, the production of nitrite-free meat products is an attractive alternative. Raw dry-cured (Parma) hams are produced by the treatment of meats with salts other than nitrite. Analysis of pigments in raw dry-cured hams reveals that the main pigment is zinc protoporphyrin, suggesting that the conversion of heme to zinc protoporphyrin occurs via an iron-removal reaction from myoglobin heme during the processing of raw hams. Purification of the iron-removal enzyme showed that it was identical to ferrochelatase. Recombinant ferrochelatase in combination with NADH-cytochrome b_5 reductase catalyzed NADH-dependent iron-removal reaction from hemin and hemoproteins. Metal ions such as zinc and cobalt were also removed from the corresponding metalloporphyrins. The addition of zinc ions led to the formation of zinc protoporphyrin. In cultured cells, the conversion of zinc mesoporphyrin to mesoheme was observed to be dependent on ferrochelatase and could be markedly induced during erythroid differentiation. This is the first demonstration of a new enzyme reaction, the reverse reaction of ferrochelatase, which may contribute to a new route of the recycling of protoporphyrin and heme in cells.

Ferrochelatase (protoheme ferrolyase, heme synthase, EC 4.99.1.1), the terminal enzyme of the heme-biosynthetic pathway, catalyzes the insertion of ferrous ion into protoporphyrin IX to form protoheme, and the animal enzyme is located at the inner membrane of the mitochondria (*1*). Both cDNA and genes for ferrochelatase have been isolated from various organisms (*2*−*4*). Ferrous ions are a substrate of the enzyme in vivo, but divalent metal ions such as zinc, cobalt, and nickel ions are also utilized as substrates for the purified enzyme in vitro (*5*, *6*). Thus, the enzyme is able to synthesize metalloporphyrins, but the reverse reaction of heme synthase in biological systems has not been reported.

Color formation in cured meat products also involves, basically, the reaction of the endogenous pigment in muscle, myoglobin, with nitric oxide from exogenously added nitrite (*7*, *8*). Nitrosomyoglobin is a typical pigment of cured meat and is converted to red nitrosohemochrome by the cooking process. On the other hand, European dry-cured hams, Parma ham, and Jamon Serrano are produced by the use of only sea salt as an artificial additive. The bright red color in the hams is very stable (*8*, *9*). The extraction of the pigment with organic solvent revealed that zinc protoporphyrin is a major component of the hams (*10*). However, the reaction mechanism of the conversion of myoglobin heme (iron protoporphyrin) to zinc protoporphyrin in the ham is not clear.

We set out to purify the enzyme involved in the removal of iron from heme or hemoproteins. In this paper, we demonstrate the NADH-dependent removal of iron from heme in mouse mitochondria and show that the reaction is catalyzed by ferrochelatase in combination with b_5 reductase.[1] In the presence of zinc ions, the direct conversion of heme to zinc protoporphyrin was also observed. These findings about the demetalation of metalloporphyrin may provide a new pathway for the reutilization of the heme moiety of hemoproteins in cells.

MATERIALS AND METHODS

Materials. Mesoporphyrin IX, zinc protoporphyrin, cobalt protoporphyrin, tin protoporphyrin, and zinc mesopophyrin were products of ICN. Hemin−imidazole complex was prepared by mixing hemin (10 mM) dissolved in Me_2SO with 20 mM imidazole. Phenyl-Sepharose and Blue-Sepharose were products of GE Healthcare Biosciences, and Red-Agarose was a product of Amicon Co. Hematoporphyrin−Sepharose CL-4B was prepared by the reaction of AH-

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* To whom correspondence should be addressed. Phone: 81-75-724-7789. Fax: 81-75-724-7760. E-mail: taketani@kit.ac.jp.

‡ Department of Biotechnology, Kyoto Institute of Technology.

§ Insect Biomedical Center, Kyoto Institute of Technology.

|| Ito Ham Inc.

[1] Abbreviations: b_5 reductase, NADH-cytochrome b_5 reductase; Me_2SO, dimethyl sulfoxide; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue; kDa, kilodalton(s); MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MEL, mouse erythroleukemia; DMEM, Dulbecco's modified Eagle's medium.

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Sepharose (GE Healthcare Biosciences) with hematoporphyrin, using *N*-cyclohexyl-*N*′-2-(4′-methylmorpholinium)ethylcarbodiimide-*p*-toluenesulfonate. Cured and raw dry-cured hams were obtained from Ito Ham Inc. Anti-ferrochelatase was prepared as previously described (*11*, *12*). Anti-b_5 reductase was a kind gift of Dr. T. Ogihara, Kyushu University. Restriction endonucleases and DNA-modifying enzymes were from Takara Co. and Toyobo Co. All other chemicals were of analytical grade.

Analysis of Heme Pigments. Metalloporphyrins and porphyrins were extracted from minced cured hams (1 g wet weight) with ethyl acetate/acetic acid (3:1, v/v; 5 mL) (*5*, *13*). Heme was determined by the reduced−oxidized difference spectrum of pyridine hemochromogen (*13*). Total porphyrins were determined by fluorospectrophotometry (*5*, *13*). Zinc protoporphyrin and protoporphyrin were extracted from hams with acetone/ethanol (2:1, v/v) and estimated by fluorospectrophotometry (*5*, *13*). Protoporphyrin and metalloporphyrins were analyzed by HPLC with a Cosmosil 5C18-AR column (4.6 × 160 mm) (Nacalai Tesque, Kyoto), as described previously (*14*).

Purification of the Iron-Removal Enzyme. Mouse liver mitochondria were suspended in 10 mM Tris−HCl, pH 7.8, containing 10% glycerol, 5 mM 2-mercaptoethanol, 2 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride (designated solution A) plus 100 mM KCl. Mitochondria were solubilized with 1% sodium cholate. After centrifugation at 20000*g* for 30 min at 4 °C, the supernatant was fractionated by 35−55% saturation of ammonium sulfate. The 55% saturated pellets were dissolved in solution A containing 0.5% Tween 20 (designated solution B) and 0.6 M ammonium sulfate. The solution was applied to a Phenyl-Sepharose CL-4B column (32 × 50 mm) equilibrated with solution B plus 0.6 M ammonium sulfate. The column was washed with the starting solution (100 mL), and the enzyme was eluted with solution B plus 0.3 M ammonium sulfate. The fractions exhibiting iron-removal activity were collected and directly applied to Red-Agarose (15 × 20 mm) equilibrated with solution B. The column was subsequently washed with 20 mL of solution B and 20 mL of solution B containing 1 M NaCl. The enzyme was eluted with solution A containing 1% sodium cholate and 1 M NaCl. The pooled fractions containing the enzyme activity were diluted 4-fold with solution A, and this solution was applied to hematoporphyrin−Sepharose (12 × 15 mm) equilibrated with solution B. After the column was washed with 20 mL of solution B plus 1 M NaCl, the enzyme was eluted with solution A containing 1% sodium cholate and 1 M NaCl and the fractions exhibiting iron-removal enzyme activity were collected and concentrated. The proteins were separated by SDS−PAGE and then visualized by CBB staining. The stained bands were subjected to in-gel trypsin digestion, and the resulting peptides were analyzed by MALDI-TOF MS (*15*). Ferrochelatase was also purified to homogeneity using Blue-Sepharose, as described previously (*5*).

Enzyme Assay. The reaction mixture for iron-removal activity contained 25 mM potassium phosphate buffer, pH 5.7, 50 μM hemin−imidazole, and 50 μM NADH, in a final volume of 1.0 mL in a Thunberg vacuum tube. The air in the tube was replaced with nitrogen gas, and the dissolved gas was removed in vacuo (*14*). The reaction was carried out at 45 °C for 1 h. For the measurement of zinc protoporphyrin, zinc ions (50 μM) were added to the reaction mixture. The reaction mixture (0.5 mL) was mixed well with 50% acetone in ethanol (2 mL). After the resulting mixture was centrifuged at 1000*g* for 10 min at room temperature, fluorescence was measured in the supernatant by scanning 550−700 nm fluorescent emission with excitation at 400 nm. The conventional iron-chelating and zinc-chelating ferrochelatase activities were measured as previously described (*14*).

Recombinant Enzymes. The cDNA for mouse b_5 reductase with N-terminal truncation (*16*, *17*) was amplified by PCR with primers 5′-AAGGATCCTCAGCGCTCCACACCGGC-3′ and 5′-AAAAGCTTCAGAAGGTGAAGCATC-3′. The resulting DNA was subcloned into the *Bam*HI−*Hin*dIII site of the multicloning site 1 (MCS1) of the expression vector pETDuet-1 (Novagen Co.) and transformed into *Escherichia coli* (BL21) to express His-tagged b_5 reductase. The human wild-type ferrochelatase carrying the His tag and the truncated form of the ferrochelatase (C395Δ) which lacked in the enzyme activity were as previously described (*11*).

Incubation of Cultured Cells with Zinc Mesoporphyrin. Mouse fibroblast Balb/3T3 cells highly expressing ferrochelatase (*12*), mock-DNA-transfected Balb/3T3 cells, and MEL cells were cultured in DMEM containing 10% fetal calf serum and antibiotics. Human erythroleukemia M-TAT cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, 10 ng/mL GM-CSF, and antibiotics and induced to undergo erythroid differentiation with 10 U/mL erythropoietin (EPO) (*18*). These cells were incubated for 4−16 h with zinc mesoporphyrin (10 μM) in the media and collected. The hemoglobin concentration was examined by staining with diaminofluorene (DAF) (*19*).

Immunoblotting. Aliquots from purification steps of the iron-removal enzyme were subjected to SDS−PAGE and electroblotted onto poly(vinylidene difluoride) membrane. Immunoblotting was done with anti-ferrochelatase and anti-b_5 reductase antibodies as the primary antibodies (*11*, *12*).

RESULTS

Content of Heme and Zinc Protoporphyrin in Cured and Raw Dry-Cured (Parma) Hams. We first compared the content of metalloporphyrins of cooked cured and raw dry-cured hams. A prominent pink fluorescence was examined in the acetone/ethanol extracts from the raw ham (Figure 1A), showing the maximum fluorescence at 590 nm with excitation at 400 nm. This agreed with the fluorescence of authentic zinc protoporphyrin. The blue fluorescence of the left tube can be derived from artificial additives. Figure 1B summarizes the content of heme pigments in both types of hams. Heme was a predominant pigment of cured ham, comprising more than 99% of the total heme pigments. In raw dry-cured hams, zinc protoporphyrin comprised up to 67% of the total pigment, while 23% of the pigment corresponded to free protoporphyrin and less than 10% of the pigment was heme. These results were consistent with previous findings (*9*) by others and suggested that zinc protoporphyrin is formed from heme compounds including myoglobin during the processing of raw dry-cured hams.

Characterization and Purification of the Iron-Removal Enzyme from Mouse Liver Mitochondria. When mouse liver

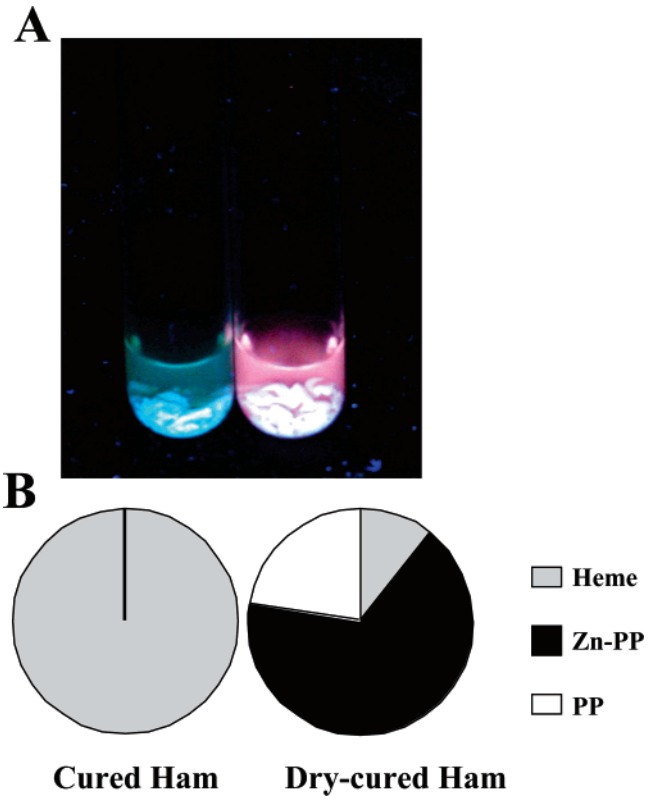

FIGURE 1: Composition of heme pigments of cured and raw dry-cured hams. (A) Fluorescent images of the acetone/ethanol extracts of hams: left tube, cured ham; right tube, raw dry-cured ham. (B) Composition of heme pigments. Heme pigments were extracted from hams. The amounts of heme were measured by the reduced−oxidized difference spectrum of pyridine hemochromogen, and those of zinc protoporphyrin and protoporphyrin were determined by fluorescence spectrophotometry. Heme pigments including heme, protoporphyrin, and zinc protoporphyrin in both hams amounted to 60−70 nmol/g wet weight: (cured hams) heme (>99%), zinc protoporphyrin (<1%); (raw dry-cured hams) zinc protoporphyrin (67%), protoporphyrin (23%), heme (10%).

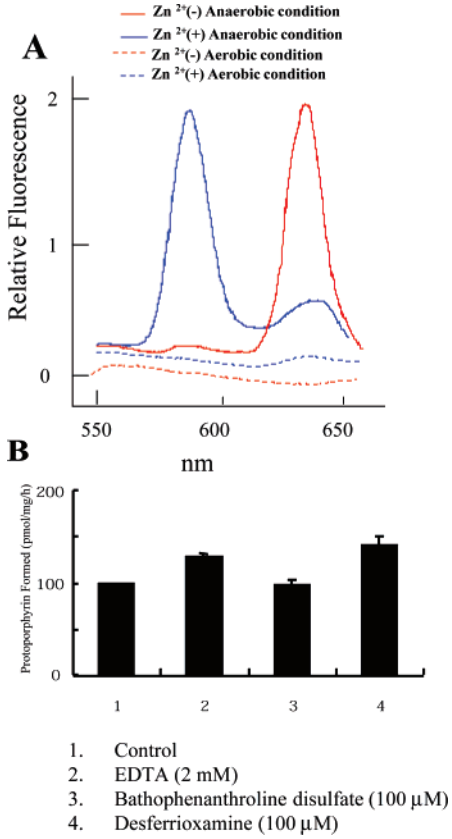

FIGURE 2: Fluorescence profile of NADH-dependent formation of zinc protoporphyrin and protoporphyrin with mouse liver homogenates. (A) Homogenates (20 mg of protein) of mouse liver were incubated with myoglobin (50 μM) at 45 °C for 1 h under the indicated conditions. The reaction mixture was mixed well with acetone/ethanol. After centrifugation, the fluorescence of the extracts was measured by excitation at 400 nm and scanning emission at 550−670 nm. (B) Effect of chelators of metal ions on the formation of protoporphyrin from myoglobin. Mitochondria (1 mg of protein) were incubated anaerobically in the presence of myoglobin (50 μM) and 50 μM NADH without other reagents (1) or with EDTA (2 mM) (2), bathophenanthroline disulfate (100 μM) (3), and desferrioxamine (100 μM) (4), as above. Data are expressed as the mean ± SD of triplicate experiments.

homogenates were incubated anaerobically with NADH (50 μM) and zinc ions (50 μM), the de novo formation of zinc protoporphyrin from heme was observed (Figure 2A). An increased formation of protoporphyrin, accompanied by a decrease of zinc protoporphyrin, was observed when EDTA at a final concentration of 2 mM was added. Testing of fractionated mouse liver homogenates showed that most activity was localized in the mitochondrial fraction. The iron-removal activity was slightly increased in the absence of zinc ions and in the presence of both general metal ion chelators, including EDTA, and iron chelators such as desferrioxamine and bathophenanthroline (Figure 2B). Protoporphyrin could also be formed from demetalation of heme complexes such as hemin−BSA as well as hemin−imidazole (K_m = 14 μM). The reaction occurred optimally at a pH of 5.5−6.0 (Figure 3A) and proceeded at temperatures higher than 25 °C, and for a 1 h incubation the highest activity was observed at 45 °C (Figure 3B). The heme moiety of hemoproteins such as myoglobin, hemoglobin, and catalase was also converted to protoporphyrin, but to a lesser extent (Figure 3C). The addition of rotenone, an inhibitor of mitochondrial NADH dehyrogenase, did not inhibit the removal reaction, indicating that the reduction of heme compounds occurs by rotenone-insensitive b_5 reductase located at the outer membrane of mitochondria (*16*). The formation of protoporphyrin did not occur without NADH; however, it could be restored by exogenous reductants such as 2 mM ascorbate or 2 mM dithiothreitol. The reaction was totally canceled under aerobic conditions (Figure 2A).

Next we tried to purify the enzyme. Purification of the iron-removal activity was undertaken, and the results of a five-step purification of the iron-removal enzyme from mouse liver mitochondria are summarized in Table 1. After the fourth Red-Agarose step of purification, b_5 reductase was required for the full enzyme activity. Immunoblotting with anti-b_5 reductase confirmed that this protein was almost absent after Red-Agarose column chromatography (Figure 4B). After the final step with hematoporphyrin−Sepharose, the iron-removal enzyme was purified about 410-fold with a 3% yield. The purified proteins were analyzed by SDS−PAGE. CBB staining showed five major protein bands (Figure 4A). Each protein band in the gel was identified by trypsin digestion and analysis of the resulting peptides by MALDI-TOF MS. The higher molecular weight proteins were identified as carbamoyl-phosphate synthetase 1, catalase, and glutaryl-CoA dehydrogenase. The 42 kDa protein

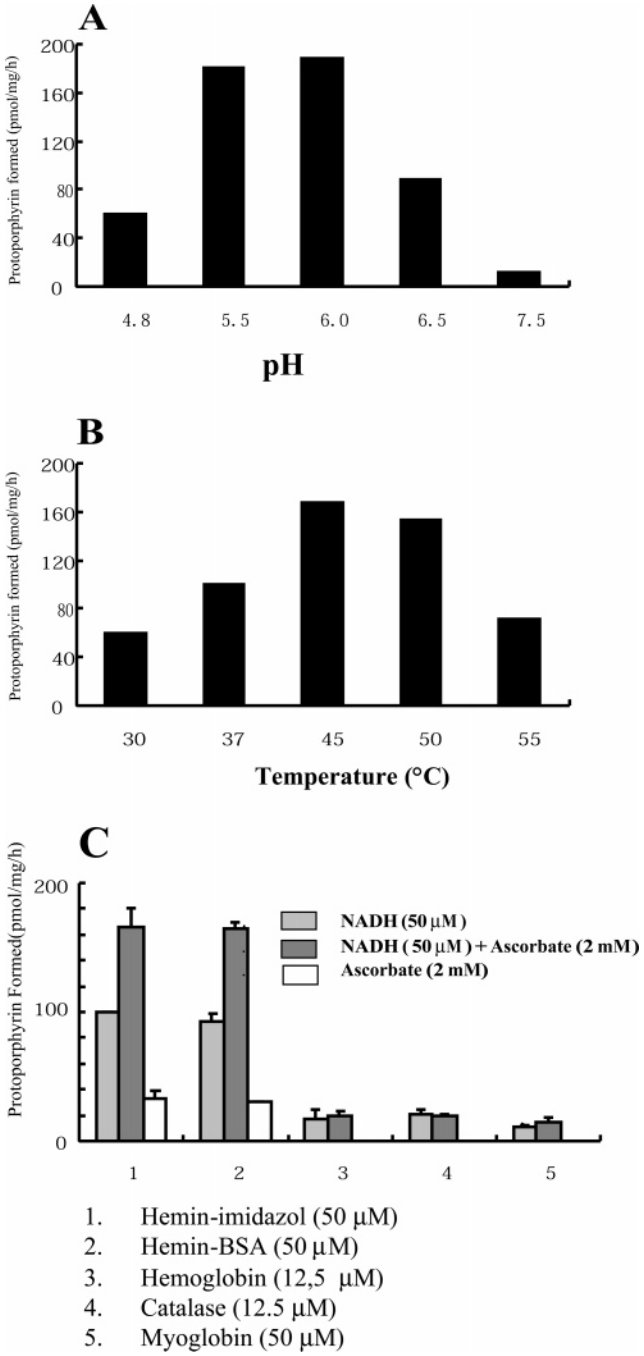

FIGURE 3: Characterization of the iron-removal reaction from heme or hemoproteins. (A) Effect of pH. Mitochondria (1 mg of protein) were incubated in the reaction mixture containing hemin−imidazole (50 μM), 50 μM NADH, and 25 mM potassium phosphate buffer of the indicated pH at 45 °C for 60 min. Data are expressed as the mean of duplicate experiments. (B) Effect of temperature. The reaction conditions were similar to those described above, except for the use of the indicated temperature. (C) Substrate specificity. The reaction mixture containing the indicated heme or hemoproteins was incubated in the presence of 50 μM NADH or 2 mM ascorbate. The heme moiety of hemin−imidazole (1), hemin−BSA (2), hemoglobin (3), catalase (4), or myoglobin (5) was used at 50 μM as the substrate. Data are expressed as the mean ± SD of triplicate experiments.

corresponded to ferrochelatase. The lower main band was retinal dehydrogenase. Of critical importance was the observation that iron-chelating (ferrochelatase) activity increased in parallel with an increase in the reverse iron-removal activity (Table 1), which was also confirmed by immunoblotting with anti-ferrochelatase, which demonstrated increased ferrochelatase during purification (Figure 4B). We and others (*5*, *20*−*22*) previously purified ferrochelatase to homogeneity using Blue-Sepharose. Homogeneous ferrochelatase thus obtained also exhibited the iron-removal activity (data not shown).

Properties of the Iron-Removal Reaction with Recombinant Ferrochelatase. Recombinant ferrochelatase (wild type) purified with Ni^{2+} beads from homogenates of *E. coli* expressing His-tagged ferrochelatase (*11*) also exhibited iron-removal activity when added together with purified recombinant b_5 reductase (Figure 4C). During the incubation, the absorbance of the Soret band of heme at 430 nm decreased and that of protoporphyrin below 400 nm increased (Figure 4D). When the products of the iron-removal reaction were analyzed, ferrous ions and protoporphyrin increased in parallel (Figure 5A), indicating that heme Fe(III) was first reduced by b_5 reductase, followed by the removal of ferrous ions from heme by ferrochelatase. The iron-removal reaction from heme and myoglobin could be demonstrated in the absence of b_5 reductase by adding 2 mM ascorbate (Figure 5B). When purified ferrochelatase was incubated with hemin in the presence of zinc ions, heme was converted to zinc protoprphyrin, although the enzyme was capable of removing ferrous ions from heme in the absence of zinc ions (Figure 5C). Similarly, hemoproteins such as myoglobin and hemoglobin produced protoporphyrin when zinc ions were absent and zinc protoporphyrin in the presence of zinc ions. Finally, the substrate specificity of the metal ion removal reaction was examined with various metalloprotoporphyrins as substrates. Metal ions of cobalt, zinc, and tin propoporphyrins were removed to a similar extent, but cadmium protoporphyrin proved to be a poor substrate (Figure 5D). These demetalations were completely inhibited by a 10-fold excess of hemin. These results were in accord with the observations that the same metalloprotoporphyrins can be synthesized by ferrochelatase (*5*). Deletion of the C-terminus of the enzyme (C395Δ) where the iron−sulfur is located resulted in the loss of both ferrochelatase activity (*11*) and iron-removal activity (Figure 4C).

Conversion of Zinc Mesoporphyrin to Mesoheme in Cultured Cells. We have shown that ferrochelatase catalyzes the removal of zinc ions from zinc protoporphyrin. If the iron-removal reaction of hemoproteins takes place in living cells, we can expect the conversion of zinc mesoporphyrin to mesoheme. Balb/3T3 cells highly expressing mouse ferrochelatase (*12*) were incubated with zinc mesoporphyrin for 4 h. Heme compounds were extracted, and the mesoheme formed was separated from endogenous protoheme by HPLC. As shown in Figure 6A, the formation of mesoheme was shown to be dependent on the expression of ferrochelatase, since control Balb/3T3 cells have much lower expression of ferrochelatase than the ferrochelatase transfectants. When the analogous experiments were carried out with MEL cells, zinc mesoporphyrin was clearly converted to mesoheme (Figure 6B). A marked increase in the conversion was observed in erythroid differentiation-induced MEL cells, in which ferrochelatase was induced, indicating the formation of mesoporphyrin and the insertion of ferrous ions into mesoporphyrin to form mesoheme. Since heme is a competitive inhibitor of the demetalation of zinc mesoporphyrin, the formation of mesoheme was apparently inhibited by the

Table 1: Purification of the Iron-Removal Enzyme of Heme from Mouse Liver Mitochondria

fraction	total protein amt, mg	iron-removal activity[a]		iron-insertion activity[b]	
		total activity, nmol/h	specific activity, (nmol/mg)/h	total activity, nmol/h	specific activity, (nmol/mg)/h
sodium cholate extracts	4230	63	0.02	677	0.25
ammonium sulfate (35−55%)	1740	56	0.03	661	0.38
Phenyl-Sepharose	378	16	0.04	192	0.51
Red-Agarose	0.94	1.0	1.1	23.9	25.4
hematoporphyrin−Sepharose	0.03	0.26	8.5	3.2	106.7

[a] Assay was carried out with hemin−imidazole and NADH by adding b_5 reductase (1 μg). [b] The incubation with mesoporphyrin and ferric citrate was performed anaerobically.

treatment of the cells with hemin. Furthermore, erythopoietin-responsive M-TAT cells induced to undergo erythroid differentiation converted more zinc mesoporphyrin to mesoheme than control cells, accompanied by an increase in hemoglobin-synthesizing cells (Figure 6C).

DISCUSSION

In the present study we first demonstrated the reverse reaction of heme synthase in vitro and in vivo. Hams throughout the world are mainly made from pig legs, and the colors of hams are derived from myoglobin in the muscle cells. We showed that there were different contents of metalloporphyrins between cured and raw dry-cured hams, in accord with previous observations by others (*9*, *10*) that zinc protoporphyrin is a major color component of raw hams. The hams are produced by incubation of raw meats at room temperature for a long time after they are treated only with salts. These results suggest that the conversion of myoglobin heme to zinc protoporphyrin occurs in raw tissues during the incubation. It is interesting that our findings suggest that while the use of ferrous ions to make protoheme in living cells is strictly controlled, after the cells die, zinc ions can be readily used for the formation of zinc protoporphyrin. We also showed that the formation of protoporphyrin is required for the subsequent insertion of zinc ions into porphyrin. We then purified the iron-removal enzyme and identified it as ferrochelatase. Although the iron-removal activity in mitochondria was stable at pH 6.0−6.5, the enzyme activity after solubilization of the enzyme was very unstable under acidic conditions. Therefore, we used buffer at pH 7.8 containing 20% glycerol for the purification of the iron-removal enzyme, which was similar to the conditions used previously for the purification of ferrochelatase. The use of hematoporphyrin−Sepharose chromatography was a powerful tool for the purification, although hemin−Sepharose was without effect (data not shown). Analysis by MALDI-TOF MS identified all of the major five proteins present at the final step of the purification and revealed that one of them was ferrochelatase. We (*5*, *20*) previously found that chromatography using blue dye resulted in the homogeneous purification of ferrochelatase. The enzyme homogeneously purified with Blue-Sepharose from mouse liver mitochondria and the recombinant ferrochelatase also exhibited the iron-removal activity. Although the precise mechanism of the iron-removal reaction of heme by ferrochelatase is unknown, the acidic pH (5.5−6.0) weakens the chelation bond between ferrous ions and the porphyrin ring and facilitates the enzyme-mediated removal of iron.

NADH-dependent reduction of the heme moiety is catalyzed by b_5 reductase (also named methemoglobin reductase) (*16*, *17*). In combination with b_5 reductase, ferrochelatase catalyzed the NADH-dependent removal of ferrous ions from hemin and myoglobin. b_5 reductase is located at the outer membrane of mitochondria or in the cytoplasm and is abundant in animal muscle cells (*23*, *24*), suggesting that this enzyme is essential for the iron-removal reaction which we postulate occurs during the processing of raw dry-cured hams. The conventional ferrochelatase reaction, insertion of divalent metal ions into porphyrins, showed an optimum at pH 7.8−8.0, whereas the removal of iron from heme required anaerobic conditions and had an optimum pH of 5.7. Considering that the pH in raw meats is 5.5−6.0 (*25*), the removal of iron from heme myoglobin would readily proceed in the raw tissues. Furthermore, the concentration of zinc ions in muscle cells is higher than that of iron (*26*), and the insertion of zinc ions into protoporphyrin could easily occur during the processing of raw dry-cured hams. It is relevant to consider that the supply of ferrous ions to synthesize heme in living cells is strictly controlled, whereas after cell death zinc ions are readily used for the ferrochelatase reaction (*3*, *5*). Ferrochelatase from yeast and *E. coli* also catalyzed the iron-removal reaction (Ishigaki, M., and Taketani, S., unpublished observations). Since the treatment of meats with nitrite in the production of cooked hams potentially allows the formation of carcinogenic nitrosoamines, it is important to develop an alternative procedure for processing meat products. Using a high concentration of active ferrochelatase, we can duplicate the bright red color of cooked cured hams without the formation of nitrosomyoglobin.

Under normal conditions, preferential formation of zinc protoporphyrin does not occur to a significant extent in vivo except in lead poisoning (*27*) and iron-deficient mutants (*28*). The inhibition of the reduction of ferric ions by lead causes the unavailability of ferrous ions for the ferrochelatase reaction, and the more plentiful zinc ions are thus directly utilized for the ferrochelatase reaction, resulting in the accumulation of zinc protoporphyrin (*2*, *5*). These are similar to the mechanisms involved in the formation of zinc protoporphyrin by the efficient use of zinc ions as the ferrochelatase substrate (*5*) after cells die. Therefore, once the enzyme-catalyzed removal of iron from heme occurs, the insertion of zinc ions readily proceeds. Conversely, zinc mesoporphyrin was converted to mesoheme in Balb/3T3 and erythroid cells, suggesting that removal of divalent metal ions from metalloporphyrins was catalyzed by ferrochelatase in vivo.

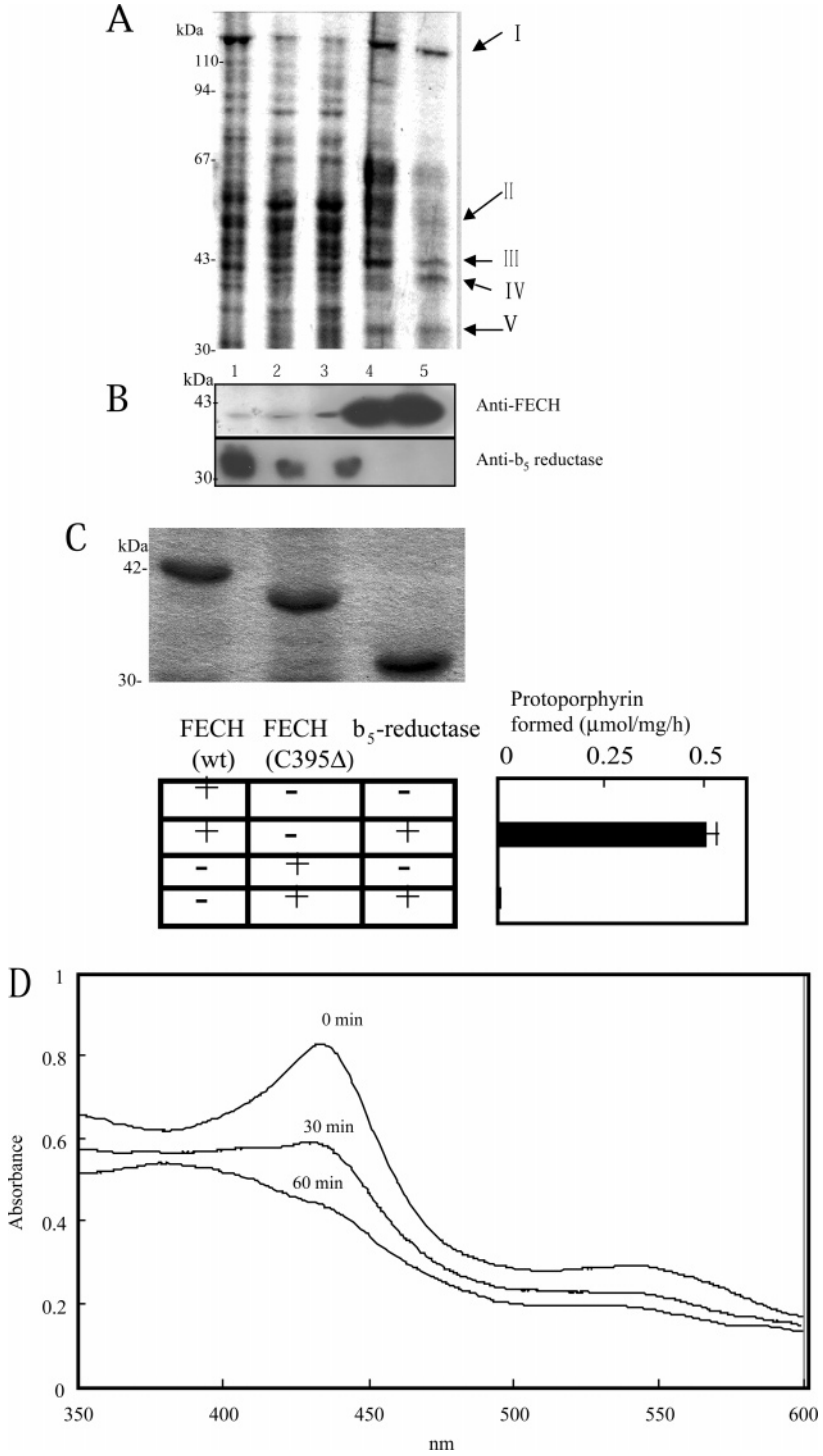

FIGURE 4: Identification of the iron-removal enzyme as ferrochelatase. (A) SDS−PAGE analysis of the purified proteins. Purification steps shown are sodium cholate extract (1), 35−55% saturated ammonium sulfate (2), Phenyl-Sepharose (3), Red-Agarose (4), and hematoporphyrin−Sepharose (5). MALDI-TOF MS analysis showed that the indicated proteins were carbamoyl-phosphate synthetase 1 (I), catalase (II), glutaryl-CoA dehydrogenase (III), ferrochelatase (IV), and retinal dehydrogenase (V). (B) Immunoblot analysis. The samples at each step of purification were separated by SDS−PAGE and transferred onto a membrane. Immunoblotting was performed with anti-ferrochelatase and anti-b_5 reductase. (C) Iron-removal activity of the recombinant enzymes. His-tagged ferrochelatase (wild type and mutant C395Δ) and His-tagged b_5 reductase were separately purified (upper panel). The enzyme assay was carried out with the purified enzymes (1 μg of protein) in the presence of 50 μM NADH (lower panel). (D) Spectrophotometric change during the iron-removal reaction from heme. The absorption spectra were taken during the iron-removal reaction of hemin−imidazole with the recombinant enzymes as above.

Since heme is the only physiologically important metalloporphyrin, the iron removal reaction may occur in living cells. The protoporphyrin ring of the heme moiety in hemoproteins is recycled and reutilized for new synthesis of hemoproteins after the reinsertion of ferrous ions. This recycling system of protoporphyrin heme is markedly induced, accompanied by the induction of de novo biosynthesis of heme, during erythroid differentiation, suggesting

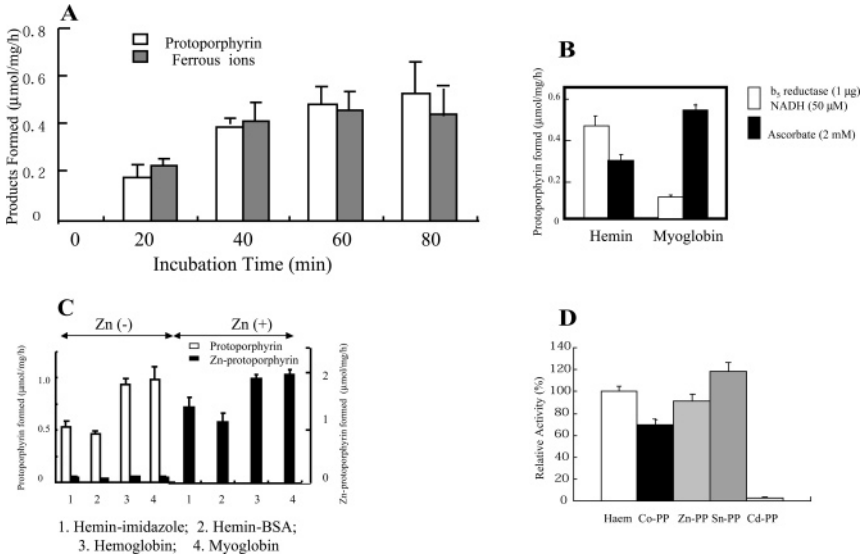

FIGURE 5: Properties of the iron-removal activity of the purified ferrochelatase. (A) Stoichiometry. The purified enzymes were incubated with hemin−imidazole (50 μM) and 50 μM NADH. Ferrous ions and protoporphyrin produced were determined by established methods. Data are expressed as the mean ± SD of four experiments. (B) Removal of the iron of myoglobin heme. Myoglobin (50 μM) or hemin−imidazole (50 μM) was incubated with the purified ferrochelatase under the indicated conditions. (C) Formation of zinc protoporphyrin by ferrochelatase. Hemin−imidazole (1), hemin−BSA (2), hemoglobin (3), and myoglobin (4) at 50 μM were incubated with purified ferrochelatase in the presence or absence of 50 μM zinc ions. Ascorbate (2 mM) was also added to reduce heme. (D) Metalloporphyrin specificity. Metal protoporphyrins (50 μM) as indicated were incubated with ferrochelatase. The formation of protoporphyrin was determined by fluorospectrophotometry.

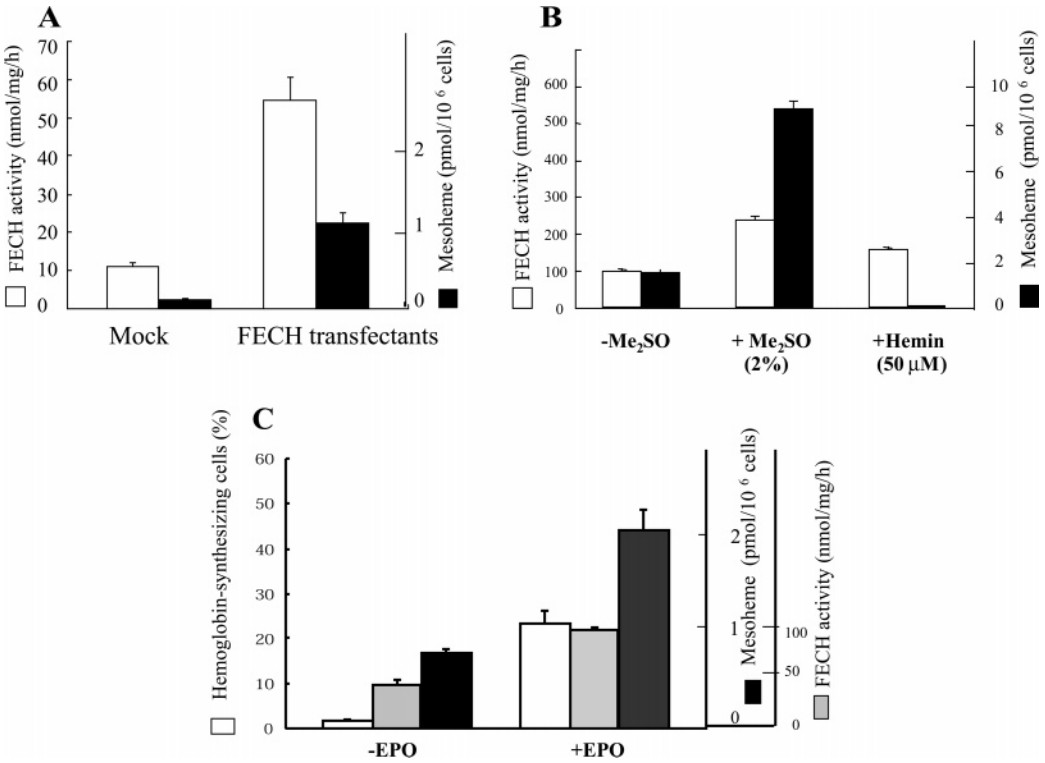

FIGURE 6: Conversion of zinc mesoporphyrin to mesoheme in Balb/3T3 cells and erythroid cells. (A) Balb/3T3 cells stably expressing ferrochelatase or transfected with mock DNA were incubated with 10 μM zinc mesoporphyrin for 4 h. (B) MEL cells not treated or treated with 2% Me_2SO and 50 μM hemin for 30 h were incubated with 10 μM zinc mesoporphyrin for 16 h. (C) Human erythroleukemia M-TAT cells induced to differentiate with 10 U/mL erythropoietin for 56 h were treated with 10 μM zinc mesoporphyrin for 16 h. Heme compounds were extracted from the cells with ethyl acetate/acetic acid and analyzed by HPLC. The ferrochelatase activity and hemoglobin-synthesizing cells were measured. Data are expressed as the mean ± SD of triplicate experiments.

that this recycling may be necessary for the supply of heme to hemoglobin from the original proteins of different compartments. In addition, heme is toxic until combined with proteins (*29*). The recycling of the heme moiety could contribute to the maintenance for lowering the intracellular level of the uncommitted heme after bleeding, in addition to the induction of heme degradation by heme oxygenase and the sequestering of the released iron by ferritin (*30*).

This mechanism could be important in the detoxification of heme in hemorrhagic stroke or severe muscle breakdown, as in rhabdomyolysis.

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