Ferrochelatase consisting of wild-type and mutated subunits from patients with a dominant-inherited disease, erythropoietic protoporphyria, is an active but unstable dimer

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Erythropoietic protoporphyria (EPP) is an autosomal inherited disease of heme biosynthesis caused by a partial deficiency of the enzyme ferrochelatase. Patients with EPP show only 20–30% normal activity because of mutations in one of the alleles of the ferrochelatase gene. To clarify the molecular mechanisms of this low level of activity, we co-expressed human ferrochelatase carrying His- and HA-tags in a tandem fashion in *Escherichia coli*. Purification of the His-tag-containing enzyme revealed that the His-enzyme forms an oligomer in association with the HA-enzyme, and analysis by gel-filtration confirmed that the enzyme is a dimer (~80 kDa). Then we expressed homo- and heterodimers composed of the wild-type and engineered mutants of the enzyme (C395Δ, H157A, H263A, H388A) or mutants from EPP patients (I186T, M267I). The levels of homodimeric enzymes produced were low, and the activities of the purified homodimeric mutants were abolished. On the other hand, the heterodimers with wild-type and mutated subunits exhibited potential, but weak, activities without a marked change of $K_m$ values for substrates. These results showed that heterodimers containing normal and mutated subunits retain the enzymic activity, which is inconsistent with the hypothesis that ferrochelatase is only active when the dimer contains two normal subunits. Pretreatment at 42°C led to a rapid inactivation of the heterodimeric mutants, indicating instability. Thus, we provide evidence that the instability of the heterodimer containing normal and mutated ferrochelatase as well as the low production levels due to the structural defect of the mutant protein, not the abolishment of the enzymic activity of the heterodimer, causes the weak activity in EPP patients.

INTRODUCTION

Erythropoietic protoporphyria (EPP) is an inherited disease caused by a deficiency of ferrochelatase, the terminal enzyme of the heme biosynthetic pathway. The enzyme catalyzes the insertion of ferrous ion into protoporphyrin to form protoheme and is located at the inner membrane of the mitochondria (1). In patients with EPP, the accumulation of protoporphyrin due to the deficiency of ferrochelatase takes place predominantly in the erythropoietic tissues (2). Clinically, the disorder manifests at childhood, mainly as a painful photosensitivity caused by an excess amount of free protoporphyrin in the skin. The mode of inheritance of EPP is mainly autosomal dominant with variable penetrance. In EPP, different degrees of enzyme deficiency can be seen between patients and asymptomatic gene carriers. Both the cDNA and the gene for human ferrochelatase have been isolated (3,4). The gene contains 11 exons spanning 45 kb and is assigned to chromosome 18q21.3 (5). The mutation abrogating ferrochelatase activity is heterogeneous. Missense and nonsense mutations, as well as exon skipping, of the ferrochelatase gene have been reported in EPP patients (6,7).

The human enzyme is nuclear encoded as a precursor form (48 kDa) and translocated into the mitochondrion where it is proteolytically processed to its mature size of 41–42 kDa (3,4). The mammalian enzyme is active as an ~80 kDa homodimer as suggested by radiation inactivation (8) and confirmed...
by X-ray crystallography (9). The mammalian enzyme contains a 2Fe–2S cluster in the C-terminal region. The Fe–S cluster is essential for the enzymic activity, although the cluster is distinct from the binding region of the ferrous ion substrate (8,9).

Site-directed mutagenesis studies have demonstrated that a conserved and essential histidine residue is involved in the binding of the metal substrate (10) and the conserved region plays a role in the binding of porphyrin (11). Furthermore, several amino acid residues essential for catalysis have been demonstrated (12). A puzzling feature of dominant-inherited EPP is that enzymic activities are reported at 15–30% of normal, although 50% activity of the control might be expected for a dominantly inherited disorder (13,14). One possible explanation is that the enzyme is active when the dimer contains two normal subunits. An alternative model is that a mutant monomer interacts with a wild-type monomer in a dominant-negative manner to decrease the activity level by >50%.

In this study, we achieved the over-expression of human ferrochelatase in Escherichia coli with wild-type and mutated subunits. We provide direct evidence that the human ferrochelatase is a dimer and the dimeric enzyme containing mutant and wild-type subunits is active. The findings that a normal subunit interacted with a mutated one from a patient with EPP will facilitate the molecular understanding of enzymic defects in patients with EPP.

RESULTS

Expression of recombinant ferrochelatase with the pET-Duet system

Several strategies for the expression of recombinant human ferrochelatase in bacteria have been developed (9–11). To examine the interaction of the enzyme subunits, we prepared a bicistronic construct expressing ferrochelatase containing a His-tag and HA-tag, as outlined in Figure 1. We first expressed separately ferrochelatase containing His-tag, HA-tag and His-/-HA-tags. Figure 2A shows the Coomassie brilliant blue (CBB) staining of homogenates and immunoblot analysis of recombinant ferrochelatase with anti-HA antibody. HA-tag ferrochelatase was precisely expressed. When ferrochelatase was purified with Ni²⁺-beads, and the amount of enzyme was evaluated with CBB staining, only the ferrochelatase containing the His-tag was purified (Fig. 2B). Interestingly, anti-HA also reacted with the protein eluted from nickel-beads on the co-expression of ferrochelatase containing the His- and HA-tag (lane 5). After the incubation of bacterial extracts of the cells expressing His-tagged ferrochelatase with those expressing the HA-tagged ferrochelatase, the enzyme was purified with Ni²⁺-beads and the amount of enzyme was evaluated with CBB staining, only the ferrochelatase containing the His-tag was purified (Fig. 2B). Interestingly, anti-HA also reacted with the protein eluted from nickel-beads on the co-expression of ferrochelatase containing the His- and HA-tag (lane 5). After the incubation of bacterial extracts of the cells expressing His-tagged ferrochelatase with those expressing the HA-tagged ferrochelatase, the enzyme was purified with Ni²⁺-beads and then the proteins were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by immunoblotting with anti-HA antibody. No band was detected in the co-expression of ferrochelatase containing the His- and HA-tag (lane 5). The enzyme was purified from nickel-beads with Ni²⁺-agarose. The purified enzymes were stained with CBB and anti-HA antibody.

Functional dimer containing wild-type and mutated ferrochelatase

We previously reported that a conserved histidine residue of the human ferrochelatase is involved in the maintenance of the structure and the binding of the metal substrate (10), by replacing histidine-157, -263 and -388 with alanine (H157A, H263A and H388A, respectively). Furthermore, deletion of the C-terminal of the enzyme (C395D) where the Fe–S cluster is located resulted in a loss of the activity (6,15). To clarify whether the wild-type and mutated subunits can form a ‘heterodimer’, we then expressed His-C395D/HA-wild type (heterophilic form), His-C395D/HA-C395D (homophilic form), His-H157A/HA-wt, His-H157A/HA-H157A, His-H263A/HA-wt, His-H263A/HA-H263A, His-H388A/HA-wt and His-H388A/HA-H388A. The enzymic
activity in homogenates of cells expressing mutated homophilic enzymes (C395Δ, H157A, H263A and H388A) was dramatically decreased (Fig. 4A). The level of these mutants except H263A/H263A was also decreased (Fig. 4A, lower panel). When the enzyme was purified with Ni²⁺-beads, the specific activity of the homodimeric wild-type enzyme increased by 25-fold, and mutated homodimeric enzymes (C395Δ, H157A, H263A and H388A) showed very low levels of activity (Fig. 4B). Immunoblot analysis revealed that all mutated enzymes reacted with anti-HA (Fig. 4B, lower panel). Figure 4C and D shows the activity levels and immunoblots of heterodimers of normal and mutated enzymes. The activity with homogenates of cells expressing the C395Δ/wt enzyme was 60% that of wild-type-expressing cells. The level of activity in cells with H157A/wt, H263A/wt and H388A/wt was 23–30% of the control. When the enzymes were purified, no H157A/wt was recovered, indicating that the structure of the H157A mutant changed dramatically and its function was lost. Interestingly, purified C395Δ/wt, H263A/wt and H388A/wt enzymes exhibited a heterodimeric form and 37.5–43% of the activity of the wild-type enzyme.

Next, we constructed plasmids where the order of cDNAs of wild-type and mutated enzyme was reversed, namely, heterophilic forms of His-wild-type and HA-H263A (wt/H263A) and His-wild-type and HA-H388A (wt/H388A) were expressed. Purification with Ni²⁺-beads resulted in the isolation of His-tag-containing wild-type enzyme and the purified enzymes containing HA-H263A and HA-H388A (Fig. 4E). The enzymic activities of the purified wt/H263A and wt/H388A were 40 and 62% that of the control, respectively. Table 1 shows Kₘ values of wild-type/mutant enzymes for zinc ions and mesoporphyrin. The affinity of the H263A/H263A enzyme for zinc ions was decreased (10), but no marked change in the Kₘ value was observed between wild-type and heterodimeric forms. These results indicated that the heterodimer made from wild-type and mutated subunits exhibits weak but significant activity.

Heterodimer of wild-type and mutated ferrochelatase subunits from patients with EPP

Several missense mutations of ferrochelatase cDNA have been reported in patients with EPP (7). We then selected two missense mutants, I186T (16,17) and M267I (18). When His-tag I186T/HA-tag I186T and His-tag M267I/HA-tag M267I enzymes were expressed, no activity was detected in the homogenates and the level of mutated ferrochelatase was lower than that of wild-type enzyme (Fig. 4F). Homogenates with His-I186T/HA-wt and His-M267I/HA-wt showed 20–30% of the level of activity of the wild-type enzyme. After purification with Ni²⁺-beads, immunoblot analysis showed that M267I and I186T mutated subunits were associated with the wild-type subunit as the band corresponding to the wild-type enzyme was detected with anti-HA antibody (Fig. 4G). Thus, purified heterodimeric enzymes retained 20% (M267I) and 27.5% (I186T) of the activity of the wild-type enzyme. Kₘ values for zinc ions and mesoporphyrin did not vary markedly between the M267I/wt and I186T/wt enzymes (Table 1). Finally, we examined the stability of heterodimeric enzymes in comparison with the homodimeric wt/wt enzyme. When the enzymes were treated at 42°C, the activity of M267I/wt and I186T/wt rapidly decreased in a time-dependent manner (Fig. 5). All other C395Δ/wt, H263A/wt and H388A/wt heterodimeric mutants were unstable, when compared with the wild-type enzyme.

DISCUSSION

Of all the different mutations of the human ferrochelatase gene, ~80% are null-allele mutations, including nonsense and frame-shift mutations, which lead to the formation of a shortened mRNA (6,7). The rest (~20%) are missense mutations (7,17). Mainly missense mutations cause the formation of the heterodimeric ferrochelatase in the dominant-inherited disease EPP. The present study provides the first direct evidence of the formation of a homodimer of human ferrochelatase using a bacterial co-expression system. We also achieved the formation of a homodimer with mutated ferrochelatase (H157A/H157A, H263A/H263A, H388/H388A and C395Δ/C395Δ), and heterodimers containing normal and mutated ferrochelatase (wt/H157A, wt/H263A, wt/H388A and wt/C395Δ). Homodimers of the mutated enzyme were produced in small amounts and showed very low levels of activity, whereas the recovery of the corresponding heterodimers differed depending on the amount of functional mutated ferrochelatase. Interestingly, all the heterodimers purified exhibited ferrochelatase activity without marked changes in the Kₘ values for the substrates. The enzymic activity of the heterodimers was <50% than that of the wt/wt homodimer, indicating that only the normal subunit functions in the catalytic reaction.

If the present duet-expression system theoretically produces an equal amount of both subunits, the amounts of wild-type/wild-type, mutant/wild-type and mutant/mutant dimers by co-expression of wild-type and mutant enzymes can be 25, 50 and 25% in the same cell, respectively. When the mutant subunit with His-tag is purified by Ni²⁺-beads, the amounts of the mutant/wt heterodimer and the mutant/mutant homodimer are 67 and 33%, respectively. Thus, the relative specific enzymic activity of this mixture would be expected to be significantly <50% of the wt/wt homodimer (i.e. 34%) when the mutant/mutant homodimer does not exhibit any enzymic activity and one of the wt/mutant heterodimers exhibits 50% activity. On the other hand, when the wild-type subunit carrying
His-tag co-expressed with the mutant subunit is purified, the elution mixture contains 33% of wt/wt homodimer and 67% of wt/mutant heterodimer, and the resulting activity corresponds to 67% of the wt/wt homodimer. The above estimated values are compatible with the experimental data, as compared with the enzymic activities between the purified H388A/wt (Fig. 4D) and the wt/H388A dimers (Fig. 4E).

The dimeric structure of human ferrochelatase has potentially profound implications with respect to the human genetic disease EPP. A majority of clinical EPP patients show only 20–30% residual ferrochelatase activity rather than the 50% that one would expect from a disease that is dominantly inherited (7,13,14). One possible explanation for this low level of activity is that ferrochelatase is active only when the dimer contains two normal subunits. On the other hand, the present data clearly demonstrated that heterodimers consisting of normal and mutated subunits exhibited significant activity. The activity of these heterophilic forms was weak and varied, depending on the positions of the mutations.

It is reported that ferrochelatase truncated at the C-terminal is inactive (19), and the mutation of the phenylalanine at 417 (F417S) diminished the characteristics of the Fe–S cluster.
spectra of the human enzyme with concomitant loss of the activity (20). Our previous study (21) showed that the lack of a Fe–S cluster at the C-terminal of ferrochelatase led to a loss of activity as measured using bacterial cell lysates. These results have suggested that the cluster was essential for the ferrochelatase reaction. However, the present study showed that the purified truncated enzyme (C395Δ/C395Δ) did exhibit Zn-chelating activity although the level of activity was low. The cluster is not necessary for the enzyme reaction, but may promote it. Furthermore, when the wt/C395Δ was expressed and purified, the structure of the heterodimer was retained, a significant activity (40% of control) without any changes in the $K_m$ values for the substrates was observed. This is inconsistent with the hypothesis by Wu et al. (9), which suggested that the Fe–S cluster plays a role in the dimerization of the mammalian ferrochelatase. The structure of human ferrochelatase resolved by crystallization and X-ray analysis showed that the active sites of the two monomers are positioned adjacent to one another (9). The authors suggest that the entrance to the active site pocket is formed by a hydrophobic mouth composed of an ‘upper lip’ (a

Table 1. $K_m$ values of the recombinant ferrochelatase for zinc ions and mesoporphyrin

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Mesoporphyrin (µM)</th>
<th>Zn$^{2+}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt/wt</td>
<td>8.5</td>
<td>11.4</td>
</tr>
<tr>
<td>C395Δ/wt</td>
<td>9.3</td>
<td>18.0</td>
</tr>
<tr>
<td>H263A/H263A</td>
<td>12.0</td>
<td>40.0</td>
</tr>
<tr>
<td>H263A/wt</td>
<td>12.0</td>
<td>9.5</td>
</tr>
<tr>
<td>H388A/H388A</td>
<td>7.7</td>
<td>10.8</td>
</tr>
<tr>
<td>H388A/wt</td>
<td>9.2</td>
<td>10.5</td>
</tr>
<tr>
<td>I186T/wt</td>
<td>11.1</td>
<td>11.0</td>
</tr>
<tr>
<td>M267I/wt</td>
<td>10.5</td>
<td>24.0</td>
</tr>
</tbody>
</table>

Figure 4. Continued.
hydrophobic segment formed by residues from the N-terminal part of the polypeptide) and a ‘lower lip’ (composed of the conserved hydrophobic residues from the active site pocket). The lips are probably responsible for acquiring the hydrophobic porphyrin substrate from the membrane and may also facilitate the exit of the product heme. Thus, a multiple structure or hydrophobic interaction in the ferrochelatase molecule may be required for the dimerization, as suggested by data obtained with yeast ferrochelatase (22). In any case, the wild-type subunit of the heterodimer mainly contributed to the enzymic reaction even when the truncated ferrochelatase as the other subunit was dimerized.

The expression of heterodimeric forms in bacteria using the duet-expression system is likely to elucidate the expression mode of enzymes in dominant-inherited diseases. Ferrochelatase I186T and M267I are derived from EPP patients and exhibited a loss of activity (16–18). Here we confirmed defects of M267I and I186T enzymes, using bacterial expression system. As EPP patients generally exhibit an autosomal dominant trait (6,7), the normal enzyme was expressed as one allele. Wild-type/I186T and wt/M267I enzymes exhibited activity, equivalent to ~20-30% of the control level. The values obtained from crude and purified enzyme were <50% and similar to those reported for the ferrochelatase activity of lymphocytes from EPP patients. The reason for such weak activity by the heterodimer is unknown, but one possible explanation is instability of the heterodimer, since preincubation for a short period led to the inactivation of the enzyme (Fig. 5).

Data obtained from the modeling of the human ferrochelatase dimer helped us to elucidate the role of the mutated amino acids in the enzymic activity. The impaired dimerization in the C395Δ mutant can provide an explanation for the diminishment of its activity. Theoretically, if a dominant-negative interaction existed that abolished all enzymic activity by the heterodimer, there would still remain 25% of normal activity from the wild-type homodimers (13,14). If the level of ferrochelatase produced and the catalytic activity were also compatible, it is hypothesized that most EPP patients exhibit 25% of the activity of the control (14). However, even in EPP patients with the null mutation which leads to ferrochelatase protein not being produced, the activity was <50%. Furthermore, as several patients with EPP exhibit <25% of normal activity, it is possible that a second mechanism including instability of the mutated enzyme exists that would decrease the activity to <25%. To account for the lowered ferrochelatase activity in EPP patients, we provide a hypothetical scheme for the heterodimer of mutant and normal enzymes (Fig. 6), namely, heterodimeric ferrochelatase made from missense and normal alleles. A heterodimer is also made with the truncated and normal polypeptides. These asymmetric ferrochelatases retain the activity, but because of instability the level of activity is lowered.

Studies using the yeast two-hybrid system (22) suggested that the dimer is the functional form for the yeast ferrochelatase. The present results, with regard to the expression and purification of the heterodimer of wild-type and mutated ferrochelatase or homodimer of mutated enzyme, indicated that this is true also for the human enzyme. The present study also showed that one of the active site pockets of such a dimer might be active, whereas the other is disabled by the mutation. These results are consistent with previous findings (22) that the only plausible explanation for the allelic complementation is that two defective monomers are able to form a functional dimer. Our analysis of model structures has shown that such non-symmetrical mutant dimers are more likely to form than the homodimers consisting of mutant subunits, and then the part of the dimeric molecule with the undisturbed active center is able to carry out the enzymic reaction. However, we cannot rule out the existence of an active monomer of the enzyme.

As demonstrated by a recent study (23), the presence of a C at IVS3-48 in the human ferrochelatase gene causes low ferrochelatase expression because of a partially aberrant splicing of pre-mRNA and therefore is responsible for the clinical manifestations of EPP. The variation of IVS-48C/T in the ferrochelatase gene also explains the difference in the residual enzymic activities in asymptomatic and symptomatic mutant carriers. Among Japanese patients with EPP, all mutations were found in IVS-48C of the normal allele (data not shown). Lymphocytes ferrochelatase activity in Japanese healthy individuals with C/C and C/T genotypes decreased to 42 and 68%, respectively, compared with the ferrochelatase activity in individuals with a T/T genotype. Heterozygotes with the low-expressed allele (IVS3-48C) in combination with a null allele would produce ferrochelatase in a small amount when compared with the normal group. Similarly, a low-expressing allele combined with a missense allele could explain the weak ferrochelatase activity observed in patients with EPP. Conversely, the ferrochelatase activity in healthy controls varied, suggesting that the level of activity in EPP patients differs, depending on the different activities from the IVS-48 genotypes of the ferrochelatase gene among controls. In addition to the effect of the low-expressing allele, the instability of the heterodimer containing normal and
mutated ferrochelatase caused the decreased activity in EPP patients. Nevertheless, multiple steps including the translocation of the precursor of ferrochelatase to mitochondria and the precise localization of ferrochelatase in the inner membrane are required for the maturation of the enzyme in humans.

MATERIALS AND METHODS

Materials
Restriction endonucleases and DNA modifying enzymes were from Takara Co. (Tokyo, Japan) and Toyobo Co. (Tokyo, Japan). Mesoporphyrin IX was from Porphyrin Products (Logan, UT, USA). Antibodies for ferrochelatase were as previously described (10). Monoclonal antibody for HA-tag was from Roche Applied Sciences Co., (Mannheim, Germany). All other chemicals were of analytical grade.

Construction of ferrochelatase expression plasmids
The cDNA for human ferrochelatase with a N-terminal truncation (10) was subcloned into the EcoRI–HindIII site of multicloning site 1 (MCS1) of the expression vector pET-Duet-1 (EMD Biosciences, Darmstadt, Germany). Then to make human ferrochelatase containing a HA-tag at the N-terminal, the truncated ferrochelatase was ligated into the XbaI–HindIII site of the vector pCG-HA (24). The insert was amplified by adding NdeI and EcoRV cloning sites, after which it was ligated into the NdeI–EcoRV cloning sites of the MCS2 of pET-Duet-1. Thus, the co-expression vector carrying wild-type His-ferrochelatase/wild-type HA-ferrochelatase (pET-His-wt/HA-wt) was obtained. cDNAs for mutated human ferrochelatase, H157A, H263A, H388A and C395D (6,10,15) were inserted into pET-Duet. pET-H157A/H157A, −H157A/wt, −C395Δ/C395Δ, −C395Δ/wt, −H263A/H263A, −wt/H263A, −H263A/wt, −H388A/H388A, −wt/H388A and −H388A/wt were thus obtained. cDNAs for mutated human ferrochelatase I186T and M267I were prepared as follows: in the first round of PCR, we used a pAR-HF plasmid (10) as a template. Primer pairs used were primer A (5′-AAGAATTCGGTGCAAACCTCAAGT-3′) as a 5′ primer, the mutagenic primer (5′-GCCACCAAACACTCAGTCT-3′) and primer B (5′-AAAGCTTACAAGCTGCTGCTGCTG-3′) as a 3′ primer, and the mutagenic primer (5′-AGCTGAGTTTGGTGGTC-3′) for the substitution I186T. On the other hand, in the preparation of the substitution M267I, we used 5′-AGGACTGAAAGCATTCTCTGT-3′ and 5′-ACAGATGACTTTGCAGTCT-3′ as mutagenic primers. In the second round, the (A) and (B) primer pairs were used to amplify the full-length human ferrochelatase with the mutation, and the DNA fragments were purified, sequenced and inserted into pET-Duet, as described previously, to give pET-Duet I186T/I186T, I186T/wt, M267I/M267I and M267I/wt. All plasmids were introduced into E. coli strain BL21. Proteins were over expressed as described previously (10).

Purification and gel-filtration of recombinant ferrochelatase
Wild-type, mutated and chimeric mutated ferrochelatase expressed with a six-His tag at the N-terminal were purified using Ni²⁺-NTA Agarose (Quagen Inc. Valencia, CA, USA), according to the manufacturer’s recommendations. The elution of the enzyme from Ni²⁺-beads was performed with 10 mM Tris–HCl, p. 8.0, containing 250 mM imidazole and 10% glycerol. The eluted proteins were gel-filtered by HPLC with a YMC-PAK DIOL A-7155-5-20 column (8.0 ø 500 mm, Yamamura Chemicals Lab. Ltd., Kyoto, Japan). The elution solution used was 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl.

Immunoblots
The lysates of strain BL21 and purified ferrochelatase were subjected to SDS–PAGE and electrophoresed onto polyvinylidene difluoride membrane. Immunoblotting was done with anti-ferrochelatase and anti-HA as the primary antibodies (10,24).
Enzyme assays
Ferrochelatase activity was measured using mesoporphyrin and zinc acetate as substrates as previously described (10). One unit of ferrochelatase activity was designated as 1 nmol of Zn-mesoporphyrin formed per minute at 37°C. The protein concentration was estimated by the method of Bradford (25).

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REFERENCES