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Expression of a Carboxy-Terminal Deletion Mutant of Recombinant Tadpole H-Chain Ferritin in *Escherichia coli*

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Abstract: In order to study the role of the protein shell in both iron uptake and iron core formation of ferritin, we constructed a deletion mutant of the ferritin gene and expressed the mutant gene in *Escherichia coli*. This mutant was obtained by introducing an amber mutation at position Pro-157 and a deletion of the 19 amino acid residues at the carboxy-terminus of the recombinant tadpole H-chain ferritin. The deleted amino acids correspond to E-helix forming the hydrophobic channel in the protein. *E. coli* harboring the plasmid pTHP157, which contains the deleted gene, was grown at 23℃ in the presence of 0.1 mM IPTG, and the induced protein appeared to be partly soluble. Nondenaturing polyacrylamide gel electrophoresis showed that the expressed mutant H-chains coassemble into holoprotein, suggesting that E-helix is not necessary for assembly of the subunits as reported for human H-chain ferritin. Its ability in iron core formation was proven in an Fe staining gel, the result disagreeing with the observation that the hydrophobic channel is necessary for iron core formation in human H-chain ferritin.

Key words: carboxy-terminal deletion mutant, tadpole H-chain ferritin.

Iron is an essential trace element for most organisms, but it presents aerobes with the dual problem of bioavailability and cytotoxicity. Ferritin is an almost spherical form of iron storage or binding protein of which major function is to store and detoxify intracellular iron (Kim *et al.*, 1986; Harrison *et al.*, 1989; Theil, 1990). It consists of a protein shell of 24 subunits and arranged in 432 symmetry with the capacity to accumulate a core of up to 4500 atoms of iron. Two types of hydrophilic and hydrophobic channels lay on molecular 3-fold and 4-fold symmetry axes. The 3-fold and 4-fold channels are possible routes for passing in and out iron, water and other small molecules into the core.

Ferritins are composed of various proportions of two subunit types, H (heavy)- and L (light)-chains, depending on the tissues and sources. Although H- and L-subunit assemblies are known in most ferritins studied, three distinct types of subunits (H, L, and M) have only been reported in tadpole red cell ferritins (Dickey *et al.*, 1987). When the sequences of the above subunits are compared, there are blocks of conserved amino acids as well as variable sequences (Harrison *et al.*, 1989). The entire 4-fold channel region (E-helix) from residues 158 to 174 is conserved in L-subunits of horse, human and rat, but not tadpole. In addition, the H-chains show variability at the inner end of the E-helices (Harrison *et al.*, 1989). Tadpole H- and M-chains end in Glu-Ser-Ser whereas tadpole L chain terminates with Leu-Gly at residue 172 (Dickey *et al.*, 1987). Our knowledge of functional differences between the two subunits in human ferritins and the three in tadpole ferritins is very limited. Therefore, it was necessary to produce homopolymers of H-chain or L-chain ferritins (H-ferritin or L-ferritin, respectively) to compare their structures and functions. These homopolymers have been expressed in *Escherichia coli* using the genes from various sources including *E. coli* (Andrews *et al.*, 1993), tadpole (Waldo *et al.*, 1993; Kim and Kim, 1994), horse (Takeda *et al.*, 1993) as well as human being (Costannzo *et al.*, 1984; Levi *et al.*, 1987; Levi *et al.*, 1992). In human H-ferritin, rather extensive studies on the relationship of structure and structure/function were carried out using various recombinant mutants. These results indicate that the E-helix forming the hydrophobic channel is not essential for proper assembly of the subunits although it is still possibly considered that in the native molecule the interaction along the 4-fold has an important kinetic role (Luzzago and Cesareni, 1989; Levi *et al.*, 1993). It has also been

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suggested that the carboxy terminus (C-terminus) including the E-helix is necessary for iron core formation (Levi *et al.*, 1988). Considering the sequence differences especially in the E-helix of tadpole ferritin, comparative studies are quite important.

An almost full-length (817 bp) cDNA (pJD5F12) for tadpole red cell ferritin H-chain was cloned and sequenced (Disbury *et al.*, 1986). Tadpole H- and L-ferritins have been expressed by using T7 polymerase promoter in *E. coli* (Waldo *et al.*, 1993). Expression of tadpole H-ferritin was also achieved by *tac* promoter in *E. coli* and the cells produce large amounts of active recombinant H-ferritin (Kim and Kim, 1994). Partial characterization of recombinant H-ferritin including an iron uptake experiment was performed (Chang *et al.*, 1995). The recombinant tadpole H-ferritin was analogous to the natural ferritin, which was folded into a spherical form and contained an iron core.

In this study, a deletion plasmid pTHP157 was constructed and the ferritin mutant gene was expressed in *E. coli*. The mutant introduces an amber mutation at position Pro-157 and deletes E-helix involving 19 amino acid residues from the C-terminus in the recombinant tadpole H-ferritin. To examine the effect of C-terminal residues on expression of recombinant H-ferritin, the above mutant was analyzed by performing nondenaturing polyacrylamide gel electrophoresis. In both protein and iron stains, the results showed that expressed mutant H-ferritin assembled into holoprotein *in vitro* and has ability to form an iron core as in recombinant H-ferritin. Our results also suggest that this mutant seems heat stable.

Materials and Methods

Materials

In order to construct a deletion plasmid pTHP157, the plasmid pVUTFH10 containing the coding region of the cDNA for tadpole ferritin H-chain was used (Kim and Kim, 1994). The plasmid pUC18 was used for subcloning and sequencing genes. Isopropyl-β-D-thiogalactoside (IPTG) and all restriction enzymes used in the experiment were obtained from Promega Corp. (Madison, USA). Sodium dodecyl sulfate (SDS), tris buffer, sodium chloride, urea and TEMED (N,N,N',N'-tetramethylethylenediamine) were purchased from Sigma Chemical Co. (St. Louis, USA). Bacto-trypton, bacto-agar, and yeast extract were from Difco Laboratories (Detroit, USA). Acrylamide, N,N'-methylene bisacrylamide, ammonium persulfate, agarose and molecular size markers were from Bio-Rad Laboratories (Richmond, USA).

Synthesis and purification of oligonucleotides

A deletion mutant of tadpole H-ferritin gene was constructed by using two oligonucleotides as follows: MY1, 5'-CCGGAATTCATGGTATCCCAGGTACGCCA-GAACTTCCAC-3'; MY2, 5'-CCCAAGCTTCTACAGACC-AAGGCGCTTCAGGTTGGT-3'. The oligonucleotides were prepared with a DNA synthesizer (Pharmacia LKB, Gene Assembler Special, Uppsala, Sweden) according to the manufacturer's procedure. Treatment of the oligonucleotide-support cassette with 35% ammonia solution brings about cleavage of oligonucleotide from the support and deprotect it in one step. After deprotection sample purification was achieved by passing the crude oligonucleotide through a NAP column. It was confirmed by 20% acrylamide gel (data not shown).

Polymerase chain reaction (PCR)

Target DNA sequence was selectively amplified by PCR reaction utilizing the simultaneous primer extension of complementary strands of DNA. The reaction was performed after adjusting the reaction mixture to 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.2 mM dNTP, 0.1 μg of the primers MY1, MY2 and 2 ng of template DNA. Reaction condition was for 35 cycles on a Heat Block Thermocycler (Thermolyne, Duburue, USA) as follows: 30 s at 95℃, 30 s at 66℃ and 1 min at 72℃. Taq DNA polymerase (2.5 unit) was used.

Analysis of DNA sequence

DNA sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase version 2.0 kit from United States Biochemical Co. (Cleveland, USA). All the steps were carried out according to the manufactures' protocols. Reaction mixtures were heated at 75℃ for 20 min, quickly chilled and immediately loaded on a 6% polyacrylamide sequencing gel containing 8 M urea in TBE buffer.

Expression of mutant ferritin

E. coli JM109 harboring the plasmid pTHP157 was grown to an absorbance of 0.5 at A_{600}. Expression from *tac* promoter was induced, when necessary, by the addition of IPTG into LB culture and the cells were incubated for an additional times. The cells were harvested by centrifugation at 7,000×g for 10 min. The cellular proteins were analyzed by polyacrylamide gel electrophoresis. Induction experiments were performed at different temperatures including 23℃ and 37℃.

Electrophoresis

Discontinuous-SDS polyacrylamide gel electrophore-

sis (SDS-PAGE) was carried out as described by Laemmli (1970). Samples were resuspended in SDS-sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.01% bromophenol blue). Each supernatant was obtained after being heated at 95℃ for 15 min, followed by centrifugation at 15,000×g for 10 min. The supernatants were applied to 12% SDS-PAGE gels. In order to examine subunit assembly of expressed mutant H-chains, the supernatants of cell lysates were also preincubated with 1 mM ferrous ammonium sulfate and heated at 70℃ for 7 min. Nondenaturing polyacrylamide gel electrophoresis was performed basically following Davis (19 64). Samples mixed with sucrose were applied to 7.5% polyacrylamide gels. Gels were stained for protein using 0.2% Coomassie brilliant blue and for iron using $K_4Fe(CN)_6$ (2%) and HCl (2%) mixed (1 : 1, v/v) immediately before use.

Results and Discussion

We developed a scheme that would allow us to produce a deletion mutant. A deletion mutant gene that lacks the 19 amino acid residues from the C-terminus was cloned. The deleted residues are known to form a hydrophobic channel in the protein. As a result, the mutant clone was named as pTHP157 which was derived from the plasmid pVUTFH10 for the recombinant H-ferritin. The plasmid pVUTFH10 which we constructed makes it possible to produce large amounts (30%) of tadpole ferritin H-chains (TFH). The expression vector pVUCH-1 contains the strong *trp-lac* (*tac*) promoter (de Boer *et al.*, 1983). Since the *tac* promoter can be repressed by the *lac* repressor and induced by the addition of IPTG to the medium in a *lac* host, *E. coli* strain JM109 was used as a host for pVUTFH10 and the deletion plasmid pTHP157.

To optimize the efficiency of translation, synthetic oligonucleotides were subjected to changes in preferential codon usage in *E. coli* (Sprengart *et al.*, 1990). For a synthetic oligonucleotide encoding the amino terminal (N-terminal) region a single change in the codon GTC was made to GTA (Fig. 1A). In case of the C-terminal region, a single change in the codon GGG was made to GGT (Fig. 1B). The codon CCA encoding the amino acid proline-157 (horse spleen L subunit numbers) was altered to the termination codon TAG. Although the synthetic DNAs were changed for highly used codons in *E. coli*, these encode the identical amino acids of the corresponding sequence of ferritin. The synthesized oligonucleotides had an *Eco*RI overhang at one end and a *Hin*dIII linker at the other, which could allow them to be inserted into the

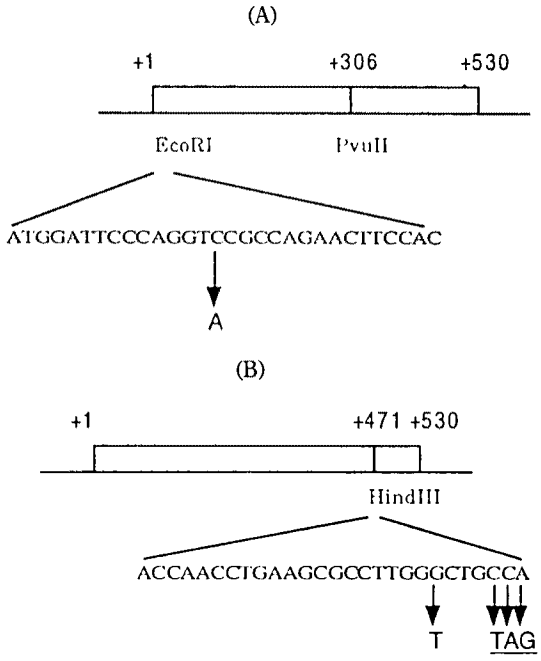

Fig. 1. Synthetic oligonucleotide encoding the N-terminal (A) and the C-terminal (B) regions of tadpole H-ferritin gene, respectively. Panel (A) shows a change in nucleotides of the natural ferritin gene using the synthetic gene. The change was made with consideration of *E. coli* codon usage. Panel (B) shows changes in nucleotides of the natural ferritin gene using the synthetic gene. The changes were made for codon usage and termination codon.

*Eco*RI/*Hin*dIII site of pVUTFH10 to generate the plasmid pTHP157 (Fig. 2).

A PCR reaction was carried out to amplify the structural gene of mutant ferritin H-chain and the amplified product was 471 bp. In the plasmid pTHP157, the spacing between the S/D sequence of the vector and the AUG of synthetic DNA is 10 nucleotides. The correct initiation and termination regions were verified by nucleotide sequencing according to the chain termination method (data not shown).

E. coli JM109 harboring pTHP157 was examined for the production of mutant tadpole ferritin H-chain from the plasmid upon induction with IPTG. The plasmid contains *tac* promoter that directs transcription of the deleted gene and transcription terminator (amber codon UAG). Cells harboring pTHP157 accumulated protein having molecular weight of ca. 20 kDa in the cell (Fig. 3). The plasmid pTHP157 overproduced ferritin even in the absence of IPTG although the production was much less than in the presence of IPTG. Because of number inferiority of the *lac* repressor generated by a single copy of the *lac* I^Q, it is likely that the repressor could not repress all the *tac* promoter in multicopy plasmids. At the induction condition of 1 mM IPTG and 37℃, the solubility of the expressed protein

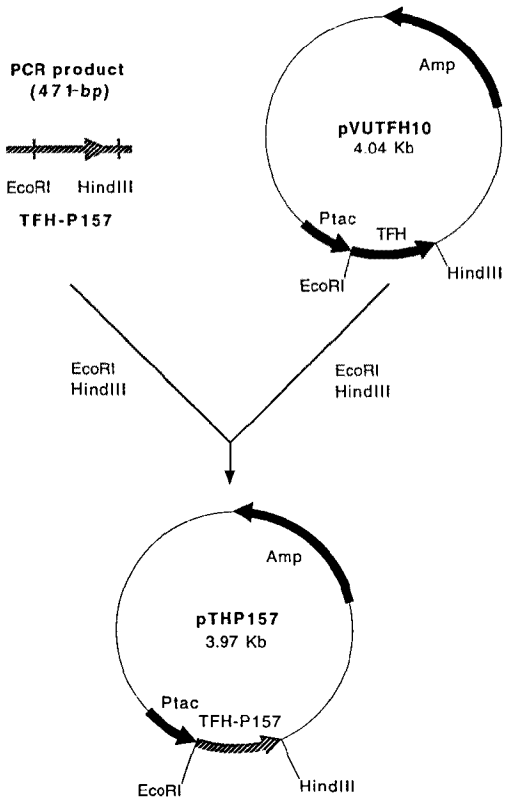

Fig. 2. Construction of the mutant ferritin expression vector pTHP157. The plasmid was constructed by ligation of the *Eco*RI/*Hin*dIII treated fragment of PCR product (471 bp) into the *Eco*RI/*Hin*dIII site of pVUTFH10.

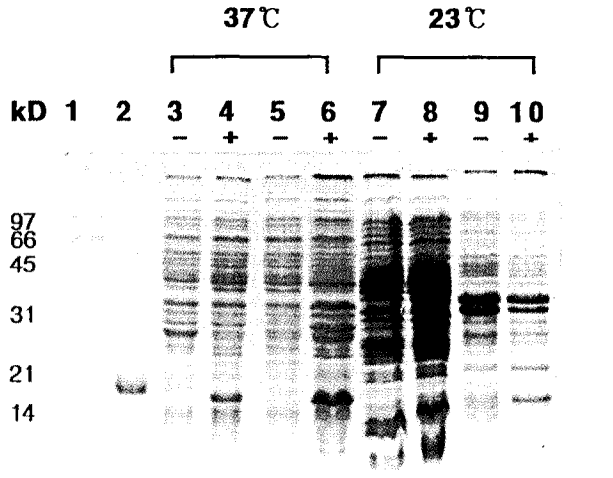

Fig. 3. Comparison of expression depending on induction conditions in a SDS-PAGE gel. Lane 1, size markers; lane 2, horse spleen ferritin; lane 3, total cellular protein (−); lane 4, total cellular protein (+); lane 5, soluble protein after sonication (−); lane 6, precipitate after sonication (+); lane 7, soluble protein after sonication (−); lane 8, soluble protein after sonication (+); lane 9, precipitate after sonication (−); lane 10, precipitate after sonication (+); (−), without IPTG; (+), with IPTG.

Pro-157_{am} was examined after sonification of the cells. As shown in Fig. 3, the precipitate (lane 6) showed a major band of ferritin subunit, suggesting that most of the expressed protein remained in the insoluble fraction. In case of expression of recombinant ferritin H-chain using the plasmid pVUTFH10 in *E. coli*, the extracts from the induced cells showed a major soluble protein band of 23 kDa (Chang *et al.*, 1995). With the mutant plasmid pTHP157 the changes in preferential codon usages resulted in increase of total expressed protein and thus may have caused formation of inclusion body. The *E. coli* JM109 induced by IPTG was examined under phase contrast microscope and inclusion bodies were observed at the end of the cells. To obtain more soluble protein, we carried out induction in different conditions as suggested by Von Darl *et al.* (1994). At a growth temperature of 0.1 mM IPTG and 23℃, the soluble fraction (lane 8) showed a subunit band with increased intensity. However, there is still indication of the subunit fraction in the precipitate (lane 10), suggesting that some of the expressed protein remained insoluble. Further optimal conditions to obtain the expressed protein in a soluble form are being sought. For comparison, another amber mutant gene which deletes two more amino acids (Gly-Leu) from the C-terminus of the Pro-157_{am} mutant was constructed. However, the mutant protein was not expressed in *E. coli* under the identical conditions as for pTHP157 (data not shown). It is reported that some mutant variants appear to distribute to native soluble structures and inclusion bodies so that both forms are present in the same cells (DeLoskey *et al.*, 1994). A great part of the protein contained within these inclusion bodies are known to be in a denatured form, in part due to the reducing environment of the *E. coli* cytoplasm (Tuggle and Fuchs, 1985). Inclusion bodies can be overcome by its solublization in alkaline solution and immobilization of proteins on a strong and resistant anion exchanger (Suttnar *et al.*, 1994). A method involving the use of osmotic stress was proposed to produce recombinant protein in a soluble form which normally accumulates as inclusion bodies in *E. coli* (Blackwell and Horgan, 1991).

The ferritin-like assembly in nondenaturing gels showed a band with a mobility similar to that of wild type ferritin (Fig. 4). It is suggested that E-helix is not necessary for assembly of the subunits as reported for human H-ferritin (Luzzago and Cesareni, 1989). The sample loaded on the gels had been applied to heat denaturation at 70℃ for 7 min, implying that this mutant seems heat stable at this temperature. In the recombinant human H-ferritin, the similar mutant Pro-161_{am}

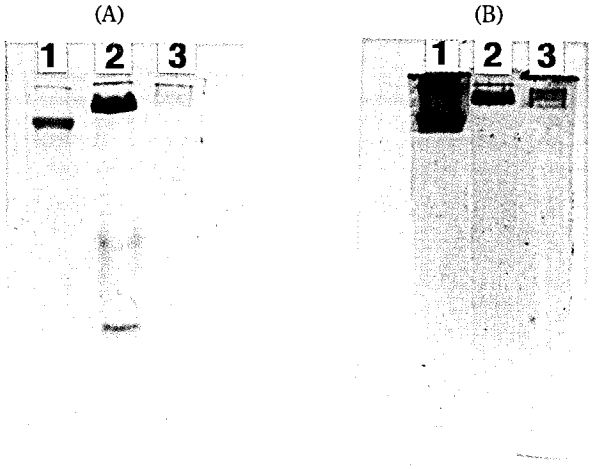

Fig. 4. Electrophoresis in 7.5% polyacrylamide gels. For tadpole H-ferritin (lane 2) and mutant H-ferritin (lane 3), cells harvested were resuspended in 20 mM Tris-HCl, pH 7.4, and sonicated. The supernatant was incubated with ferrous ammonium sulfate for 2 h and centrifuged at 8,000×*g* for 10 min, followed by heat denaturation at 70℃ for 7 min. After heat denatured proteins were removed, clear fractions were loaded in duplicate on the gel, which cut into two parts for protein and iron stainings, respectively. Horse spleen ferritin was loaded as a control. (A): Stained for protein with Coomassie brilliant blue. (B): Stained for iron with ferrocyanide. Lane 1, horse spleen ferritin (6 μg in (A) and 13 μg in (B)); lane 2, heat denatured tadpole H-ferritin (~12 μg); lane 3, heat denatured mutant H-ferritin (~5 μg).

protein was reported to precipitate and denature after being heated at 75℃ (Levi *et al.*, 1988). However, it is known that Pro-161_{am} of human H-chain ferritin is still relatively resistant to heat denaturation (Luzzago and Cesareni, 1989). The observation seems consistant with our result. In iron staining gels a striking difference was observed between mutant tadpole H-ferritin and mutant human H-ferritin. Mutant tadpole H-ferritin showed a positive Fe band with the same mobility that the mutant ferritin has in the protein stain (Fig. 4). In case of mutant human protein, it formed no Fe band and thus no stable iron core although its ability to catalyze iron oxidation was maintained (Levi *et al.*, 1988). To understand such differences, further molecular characterization on the mutants should be carried out.

In summary, *E. coli* JM109 harboring the deletion plasmid pTHP157 was grown at an IPTG concentration of 0.1 mM and 23℃, and the induced protein existed as some amount of soluble protein. Assembly of the expressed mutant subunits was identified by performing nondenaturing polyacrylamide gel electrophoresis. It is thus suggested that E-helix forming the hydrophobic channel is not essential for assembly of the subunits as reported for human H-ferritin. This mutant seems rather heat stable. Its ability in iron core formation was proven in an Fe staining gel, the result disagreeing with the observation that the hydrophobic channel is necessary for iron core formation in human H-ferritin. To confirm whether the hydrophobic interactions along the 4-fold axes are essential for iron uptake and core formation, further studies are required using a series of tadpole ferritin mutants. These can be achieved by inserting amber mutations at different positions of the loop region between D- and E-helices.

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