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Influence of Site-Directed Mutagenesis on Protein Assembly and Solubility of Tadpole H-chain Ferritin

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In order to understand the influence of ferroxidase center on the protein assembly and solubility of tadpole ferritin, three mutant plasmids, pTH58K, pTH61G, and pTHKG were constructed with the aid of site-directed mutagenesis and mutant proteins were produced in *Escherichia coli*. Mutant ferritin H-subunits produced by the cells carrying plasmids pTH58K and pTHKG were active soluble proteins, whereas the mutant obtained from the plasmid pTH61G was soluble only under osmotic stress in the presence of sorbitol and betaine. Especially, the cells carrying pTH61G together with the plasmid pGroESL harboring the molecular chaperone genes produced soluble ferritin. The mutant ferritin H-subunits were all assembled into ferritin-like holoproteins. These mutant ferritins were capable of forming stable iron cores, which means the mutants are able to accumulate iron with such modified ferroxidase sites. Further functional analysis was also made on the individual amino acid residues of ferroxidase center.

Key words: mutagenesis, ferritin assembly, solubility

INTRODUCTION

Ferritin is a multimeric protein that makes a spherical shell surrounding an iron core of inorganic complexes [1, 2]. The mechanisms of iron uptake *in vivo* are largely unknown, but *in vitro* studies on iron core formation suggested that the protein shell has a catalytic role in a complex series of hydrolytic polymerization reaction of Fe(III) [2, 3]. The reactions involve the binding and oxidation of Fe(II), the nucleation of ferric oxide mineral, and the mineral growth in the core. Generally ferritins consist of two main subunits, heavy (heart; H) and light (liver; L). From experiments using recombinant ferritins, homogeneous H-chain ferritins (H-ferritin) show iron uptake and ferroxidase kinetics faster than homogeneous L-chain ferritins (L-ferritin) do [4, 5]. It was proposed that the metal ligand site identified by X-ray analysis in H-chains is responsible for the ferroxidase activity and was identified as Glu23, Glu58, His61 (L-chain numbering), and the fourth ligand is possibly a water molecule [6, 7]. Those three residues are conserved in all known H-chain sequences, although the residues are substituted with Tyr, Lys, and Gly, respectively, in L-chains. However, these residues are not substituted in tadpole ferritin L-chains as other ferritin L-chains, and in fact the residues Glu58 and His61 are conserved [1, 2]. The reason for the faster iron uptake kinetics by tadpole H-ferritin than by L-ferritin was not clearly elucidated [5].

Site-directed modifications of Glu58 and His61 resulted in complete loss of ferroxidase activity in human H-ferritin, although core formation by modified H-ferritin occurred slowly at pH 7.0 [6, 8]. From this result, it was suggested that Fe(II) can be directly oxidized on the mineral surface of iron core once nucleation of mineral has occurred, apparently making the ferroxidase sites of the shell redundant for mineral growth [1, 2, 4]. However, mutagenesis at each site has not been performed yet.

Active recombinant tadpole H-ferritin has been produced in *Escherichia coli* in large amounts [9]. In this study site-directed H-ferritin mutants were prepared and the ferroxidase sites were individually analyzed in tadpole ferritin. Therefore, specific modifications were performed as follows: Glu58 → Lys; His61 → Gly; Glu58, Arg59, and His61 → Lys, Ser, and Gly. Our investigation is to understand whether the ferroxidase center of ferritin is at all required for not only iron uptake but also protein assembly and solubility.

MATERIALS AND METHODS

Materials and Plasmids

The mutagenesis kit and enzymes were purchased from Promega(USA) or New England Biolabs(USA). The plasmid pVUTFH10 containing tadpole ferritin H-chain gene was used [9]. Plasmid pGroESL, which harbors the GroES and GroEL genes of molecular chaperone, was gifted by Dr H.-C. Shin (Hanhyo Institute of Technology).

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Synthesis of Oligonucleotides and Site-directed *in vitro* Mutagenesis

In order to perform site-directed mutagenesis of *tadpole H-ferritin gene, segregated oligonucleotides* were designed as follows:
E58K, 5'- CAAAGTCACGAAAAGCGCGAGCATG CTGAGAA - 3';
E61G, 5'- GAAGAGAGGGAGGGTGCTGAGAAAT TGATG - 3';
KG, 5'- CAAAGTCACGAAAAGAGCGAGGGTGC TGAGAAATTGATG -3' (The underlined sequences indicate the substituted codons). The oligonucleotides were prepared with a DNA synthesizer (Pharmacia LKB, Gene Assembler Special) *using 0.2 μmol* columns according to the manufacturer's procedure.

Site-directed *in vitro* mutagenesis was performed by employing a phagemid, pSELECT-1 [10]. Sequences of the mutant ferritin genes were confirmed by dideoxynucleotide sequencing [11] using Sequenase version 2.0 kit from U.S. Biochemicals. The mutant plasmids resulted from mutagenesis are named as pTH58K, pTH61G, and pTHKG, respectively. *E. coli* JM109 cells transformed with *pTH58K, pTH61G, or pTHKG are denoted as 58K,* 61G, and KG, respectively.

Expression of the Ferritin Mutant Genes

E. coli strains 58K, 61G, and KG were induced by addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactoside) and incubated for further 4 hr. In addition, for 61G, cells were grown under osmotic stress by adding the appropriate concentration of sorbitol and 2.5 mM betaine to produce the protein in a soluble form. Especially, molecular chaperone was applied to 61G to examine the solubility of induced protein. Therefore, the plasmid pGroESL harboring the GroES and GroEL genes was cotransformed into above cells carrying the plasmid pTH61G. The addition of IPTG to cells induced tadpole ferritin-61G as well as molecular chaperone.

The culture was harvested and cellular proteins were analyzed by both 12%(w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE; [12]) and 7.5% polyacrylamide gel electrophoresis in nondenaturing gels [13]. In order to stimulate ferritin assembly, ferrous ammonium sulphate was added to the mutant cell lysates and the samples left for 2 hr before the native PAGE. The gels were stained for protein using Coomassie brilliant blue and for iron using potassium ferrocyanide as reported previously [14].

RESULTS AND DISCUSSION

Construction of Mutant Plasmids by Site-directed Mutagenesis

Influence of site-directed mutagenesis on the protein assembly and solubility was investigated in tadpole H-ferritin. Construction of the mutant plasmids, which modified the catalytic sites for Fe(II) oxidation (ferroxidase site), is shown in Fig. 1. We constructed three mutant plasmids pTH58K, pTH61G, and pTHKG for recombinant ferritin H-chain by site-directed mutagenesis. To obtain high efficiency of translation, synthetic oligonucleotides were applied to changes in preferred codon usage in *E. coli* [15]. Changes in codons encoding the amino acids of the N-terminal region were made to Glu58 (GAG) → Lys(AAG) for pTH58K, His61(CAT) → Gly(GGT) for pTH61G, and Glu58(GAG), Arg59 (AGG) and His61(CAT) → Lys(AAG), Ser(AGC) and Gly(GGT) for pTHKG, respectively. Genes mutated at the ferroxidase center were cloned and identified. The fragments of the mutant genes were all 540 bps.

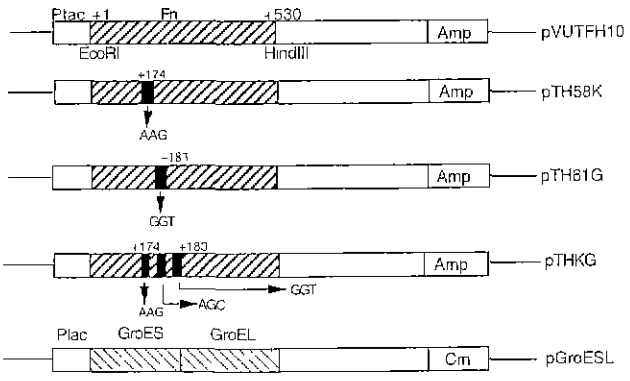

Fig. 1. Construction of the plasmids pVUTFH10, pTH58K, pTH61G, pTHKG and pGroESL

Expression of Mutant Genes of Ferritin in *E. coli*

In order to express mutant genes of tadpole H-ferritin the expression vector pVUTFH10 was used, which contains a *tac* promoter. The strong *trp-lac (tac) promoter of the expression vector was described* by de Boer *et al.* [16]. *E. coli* JM109 cells carrying the plasmids were induced with 0.1 mM IPTG for expression of the mutant genes. The production of mutant ferritins was examined (Fig. 2). Cells harboring the plasmids accumulated a protein with molecular weight of ca. 23 kDa in the cells. The mobility of the protein bands was similar to that of recombinant H-ferritin [14]. However, relative productivity of ferritin mutants was lower than

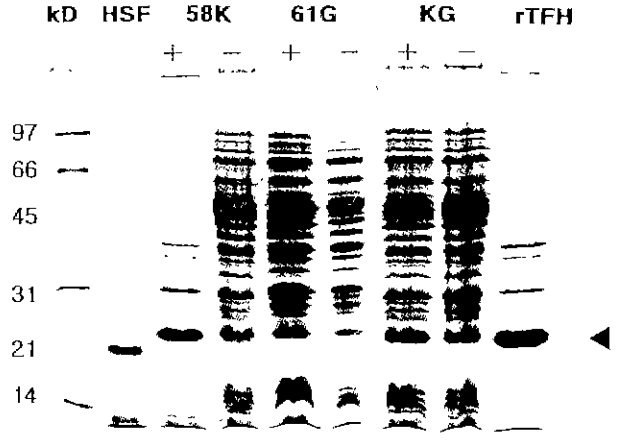

Fig. 2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of tadpole mutant H-chain ferritins (H-ferritins). *E. coli* JM109 strains 58K, 61G, and KG were grown and induced *with 0.1 mM IPTG. Horse spleen ferritin (HSF) and* recombinant H-ferritin (rTFH) were used for comparison The arrow indicates mutant ferritins. M, molecular size marker; (+), induction; (-), no induction.

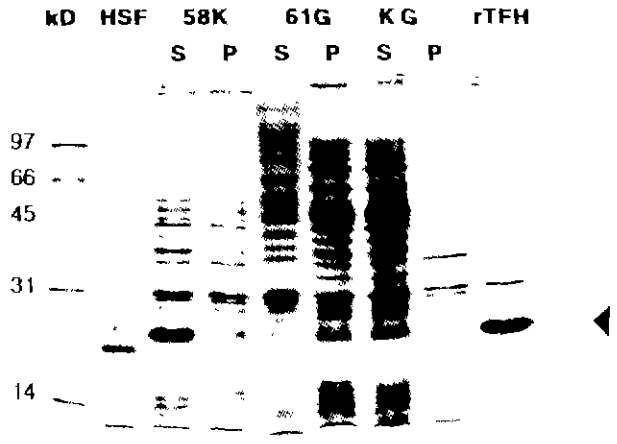

Fig. 3. Solubility analysis of expressed mutant proteins Supernatant was obtained from cell lysate by centrifugation at 10,000×g for 10 min and precipitate was collected and redissolved in running buffer. S, supernatant; P, precipitate. Other descriptions are specified in Fig. 2.

that of recombinant H-ferritin and variable depending on the plasmids. In the supernatant fraction, mutant ferritins from plasmids pTH58K and pTHKG were produced as soluble form (Fig. 3). In contrast, the mutant ferritin produced from plasmid pTH61G was present as an insoluble form under the same induction condition. We could not obtain soluble-protein from plasmid pTH61G at various concentrations of IPTG (0.1-2 mM) or various temperatures (23-37℃).

Solubilization of Mutant Ferritin-61G

Recombinant DNA technology has allowed the expression of foreign proteins in various host cells. One of the major problem involved is that a higher yield of soluble protein expressed in host cells could raise inclusion bodies in cells [17]. Various techniques to recover soluble active proteins have been reported [18, 19] and some have succeeded. We examined two methods to obtain soluble protein from plasmid pTH61G. One was to use osmotic-pressure regulating agents which caused preferential hydration of protein and thus stabilized protein structure [14]. Therefore, *E. coli* cells harboring plasmid pTH61G were grown under osmotic stress in the presence of sorbitol and betaine. As the result, the mutant ferritin was solubilized (Fig. 4a). The amount of the expressed protein increased as the concentration of sorbitol increased from 300 to 1000 mM. The other method was an induction of protein in the presence of molecular chaperones. Molecular chaperones bind to and maintain the unfolded polypeptides and timely release them following ATP hydrolysis to direct refolding [20]. As shown in Fig. 4b, most of the mutant ferritin was present as soluble protein.

Protein Assembly of Mutant Ferritin Subunits

On PAGE under nondenaturing conditions, the mutant ferritins produced from plasmids pTH58K, pTH61G, and pTHKG showed the band with a similar migration to purified recombinant tadpole H-ferritin (rTFH; Fig. 5). A little difference in migration rate are observed between rTFH and the

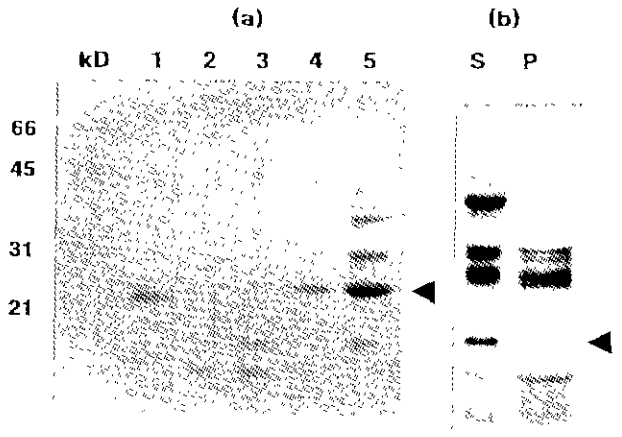

Fig. 4. SDS-PAGE analysis of cell lysate from mutant ferritin 61G induced with either (a) 2.5 mM betaine and various concentrations of sorbitol or (b) molecular chaperone. Lane 1, horse spleen ferritin, 2,300 mM; 3,600 mM; 4, 1000 mM; 5, recombinant H-ferritin; S, supernatant; P, precipitate. Arrows indicate mutant ferritins.

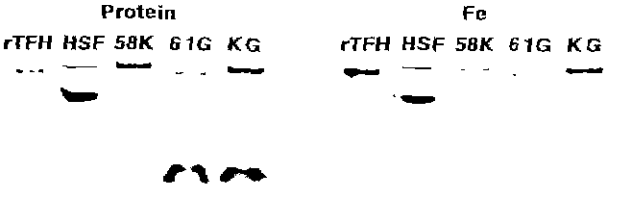

Fig. 5. Electrophoresis on 7.5% polyacrylamide gels with protein staining and Fe staining. rTFH, recombinant H-ferritin; HSF, horse spleen ferritin.

mutant ferritins (58K and KG). Obviously, molecular weights of the mutant ferritins are not very different. In addition, surface charges of the mutant ferritins seem not to be changed as the residue Glu58 is located within the subunit. It might be caused by differences in the Van der Waals volumn of the amino acid which leads some changes in molecular dimension on assembly of 24 subunits into holoprotein. Relation between the mobility and the solubility is obscure in the mutant ferritins. The mutant ferritins were stained for protein and iron. Comparing the relative migration to rTFH, the mutant subunits were assumed to assemble in a similar manner to native ferritin (i.e. ferritin-like holoproteins). Mutant ferritins had taken up some iron, although the intensity of the Prussian blue stain was somewhat inconsistent with that of the protein stain. The result of the iron staining gel implies that the mutants lacking the ferroxidase sites are still capable of forming stable iron core. It thus appears that ferroxidase sites (Fe(II) oxidation site) are separated from iron hydrolysis sites. The result is consistent with the previous report

obtained using a recombinant human ferritin mutant, in which the modification of the ferroxidase center, namely both Glu58 and His61, resulted in complete loss of ferroxidase activity although iron core was formed [6]. Modification of these ferroxidase sites has not been carried out in other ferritin sources. Nevertheless, this is the first report to analyze individual amino acid residues of Glu58 and His61 in ferritin. Further studies on iron uptake and ferroxidase activity in relation to those residues should be performed.

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REFERENCES

[1] Theil, E. C. (1987) Ferritin; structure, gene, regulation and cellular function in animals, plants and microganisms. *Ann. Rev. Biochem.* 56, 289-315.

[2] Harrison, P. M., S. C. Andrews, P. J. Artymiuk, G. C. Ford, D. M. Lawson, J. M. A. Smith, A. Treffry, and J. L. White (1990) Ferritin, In: *Iron transfer and storage* (Ponka, P., H. M. Schulman and R. C. Woodworth, eds.) p. 81-101 CRC press.

[3] Harrison, P. M. and P. Arosio (1996) The ferritins: molecular properties, ion storage function and cellular regulation. *Biochim. Biophys. Acta* 1275, 161-203.

[4] Sun, S., P. Arosio, S. Levi, and N. D. Chasteen, (1993) Ferroxidase kinetics of human liver apoferritin, recombinant H-chain apoferritic and site-directed mutants. *Biochemistry* 32, 9362-9369.

[5] Waldo, G. S., J. Ling, J. Sanders-Loehr, and E. C. Theil (1993) Formation of an Fe(III)-tyrosinate complex during biomineralization of H-subunit ferritin. *Science* 259, 796-798.

[6] Lawson, D. M., A. Treffry, P. J. Artymiuk, P. M. Harrison, S., J. Yewdall, A. Luzzago, G. Cesareni, S. Levi and P. Arosio (1989) Identification of the ferroxidase centre in ferritin. *FEBS Lett.* 254, 207-210.

[7] Levi, S., P. Santambrogio, A. Cozzi, E. Rovida, B. Corsi, E. Tamborini, S. Spada, A. Albertini, and P. Arosio (1994) The role of the L-chain in ferritin iron incorporation. *J. Mol. Biol.* 238, 649-654.

[8] Levi, S., A. Luzzago, G. Cesareni, A. Cozzi, F. Franceschinelli, A. Albertini and P. Arosio (1988) Mechanism of ferritin iron uptake: Activity of the H-chain and deletion mapping of the ferroxidase site. *J. Biol. Chem.* 263, 18086-18092.

[9] Kim, Y.-T. and K.-S. Kim (1994) Synthesis of active tadpole H-chain ferritin in *Escherichia coli*. *Mol. Cells* 4, 125-129.

[10] Kramer, B., W. Kramer and H. J. Fritz (1984) Different base-base mismatches are corrected with different efficiencies by the methyl-directed DNA mismatch-repair system of *E. coli*. *Cell* 38, 879.

[11] Sanger, F. (1981) Determination of nucleotide sequences in DNA. *Science* 214, 5463-5467.

[12] Laemmli, U. K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

[13] Hames, B. D. and D. Rickwood (1983) In: *Gel electrophoresis of proteins*. IRL press. Oxford.

[14] Chang, S.-R., Y.-T. Kim, and K.-S. Kim (1995) Purification and characterization of recombinant tadpole H-chain ferritin in *Escherichia coli*. *J. Biochem. Mol. Biol.* 3, 238-242.

[15] Spreugart, M. L., H. P. Fatscher and E. Fuchs (1990) The initiation of translation in *E. coli*: apparent base pairing between the 16 srRNA and downstream sequences of the mRNA. *Nucleic Acids Res.* 18, 1719-1723.

[16] De Boer, H. A., L. J. Comstock and M. Vesser (1983) The *tac* promoter: A functional hybrid derived from the *trp* and *lac* promoters. *Proc. Natl. Acad. Sci. USA* 80, 21-25.

[17] Keshavarz-Moore, E., R. Olbrich, M. Hoare and P. Dunnill (1991) Application of biochemical engineering principles to develop a recovery process for protein inclusion bodies. p. 307-314 In: Prokop, A. and R. K. Bajpai (ed.) *Recombinant DNA technology I. Annals of NY academy of Sciences*. NY.

[18] Blackwell, J. R. and R. Horgan (1991) A novel strategy for production of a highly expressed recombinant protein in an active form. *FEBS Lett.* 295, 10-12.

[19] Darby, N. J. and T. E. Creghton (1990) Folding protein. *Nature* 334, 715-716.

[20] Georgopoulos, C. (1992) The emergence of the chaperone machines. *TIBS* 17, 295-299.