Recombinant ferritin: modulation of subunit stoichiometry in bacterial expression systems

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We describe a strategy for the creation of recombinant ferritin heteropolymers which mimic the natural heterogeneity of this protein. This method entailed the co-expression of cDNA for both ferritin H and ferritin L subunits in a single bacterium using either a bicistronic vector, in which both cDNAs were expressed from the vector, or a dual vector expression strategy, in which each subunit was expressed from a separate compatible plasmid in a single bacterial host. Electron microscopy and sucrose density gradient centrifugation demonstrated that ferritin assembled spontaneously in such bacteria to form catalytically active proteins of the expected size and shape. Isoelectric focusing revealed that protein isolated from any of these bacteria exhibited a restricted heterogeneity in subunit composition. Such multi-subunit recombinant ferritins spontaneously assembled in bacteria may be useful in further studies of ferritin assembly and function. Our results further suggest that varying expression levels is a simple way to alter levels of individual components within a multi-subunit recombinant protein, and that this approach may be of general utility in assessing the contribution of individual components to the function of multi-subunit proteins or protein complexes.

Key words: ferritin/iron/protein assembly/recombinant protein/subunit stoichiometry

Introduction

Ferritin is a 24 subunit protein. It plays a key role in intracellular iron storage and has a highly conserved structure [see Theil (1987) and Harrison and Arosio (1996) for reviews]. Two major subunit types are found in the assembled molecules, H and L. In vivo, these associate in various ratios, forming heteropolymers which vary in composition in a consistent and tissue-specific manner: heart, for example, contains ferritins of predominantly the H-subunit type, whereas in liver and spleen, L-rich ferritins predominate (Powell et al., 1975). In vivo, these associate in various ratios, forming heteropolymers which vary in composition in a consistent and tissue-specific manner: heart, for example, contains ferritins of predominantly the H-subunit type, whereas in liver and spleen, L-rich ferritins predominate (Powell et al., 1975). In order to study the biochemical properties of these heteropolymers, we wished to produce a series of recombinant proteins which recapitulated this heterogeneity. Desired characteristics of the proteins were appropriate size (i.e. assembly into 24mers) and a range of subunit composition (e.g. H:L ratios of 22:2, 14:10, 4:20, etc.), as found in vivo. However, we also wished ferritins produced in any one expression system to possess a relatively restricted subunit composition (i.e., proteins of a 12:12 average subunit composition should not reflect the equal production of two different proteins, 0:24 and 24:0), such that their biochemical properties could be studied.

Here we demonstrate that by combining two different bacterial expression strategies, a family of ferritin proteins which largely satisfy these criteria can be produced. Using both a bicistronic expression system (two subunits encoded in a single plasmid) or a dual vector strategy (expression of individual subunits from two compatible plasmids in the same bacterium), we have produced three human and four mouse heteropolymers, and also three interspecies proteins, all of differing subunit composition. In addition to providing a useful tool in the analysis of ferritin subunit function, these results suggest that varying expression levels is a simple way to alter levels of individual components within a multi-subunit recombinant protein. Such an approach may be of general utility in assessing the contribution of individual components to the function of multi-subunit proteins.

Materials and methods

Construction of plasmids

Diagrams of the plasmid constructs are shown in Figure 1. Monocistronic expression plasmids for all cDNAs were first constructed by cloning into the pET21C vector (Novagen). Mouse ferritin L [MFL] cDNA was obtained from pMLF27 (a gift from C. Beaumont, Paris), mouse ferritin H [MFH] cDNA from clone MFH (Torti et al., 1988), human ferritin L [HFL] from pLF108 (a gift from J. Drysdale, Tufts University) and human ferritin H [HFH] from reverse transcription of human fibroblast RNA (MRC-5 cells, a gift from H. Blau, Stanford University) followed by PCR amplification. All cDNAs were modified by PCR to contain an NdeI restriction site at the initiator methionine and a BamHI site at the stop codon prior to cloning into Ndel/BamHI digested pET21C vector. These vectors direct the synthesis of intact, non-fusion proteins in host strains carrying T7 RNA polymerase (BL21DE3, Novagen). Bicistronic expression plasmids were constructed for murine ferritin by digesting either the mouse ferritin H or mouse ferritin L pET21C construct with XbaI, followed by T4 DNA polymerase fill-in and SacI digestion. The resulting fragment was ligated to a recipient pET21C construct containing ferritin H or ferritin L that had been digested with BamHI, followed by T4 DNA polymerase fill-in and SacI digestion. This yielded two constructs with the functional order T7 promoter/ribosomal binding site/coding region/translational stop/ribosomal binding site/coding region/translational stop/T7 terminator, one with an H–L coding region orientation and the other with an L–H coding region orientation. A human bicistronic expression plasmid was constructed similarly, except that SacI was used in place of ScaI. pACYC177-based expression plasmids of MFL, MFH, HFL and HFH were constructed by isolating the entire expression cassette of the four pET21C constructs using BspEII digestion, T4 DNA polymerase fill-in, BglII digestion and ligation into a BamHI, SacI-digested pACYC177 vector (New England Biolabs). This destroyed the ampicillin resistance gene of pACYC177 while leaving the kanamycin resistance
Fig. 1. Genetic design of expression constructs. (A) pET21 monocistronic expression plasmids. These have the pET21C vector backbone with coding regions for MFH (murine ferritin H), MFL (murine ferritin L), HFH (human ferritin H) and HFL (human ferritin L) inserted as NdeI/BamHI fragments. (B) pACYC expression plasmids. These have the pACYC177 vector backbone and the entire expression cassettes from the pET21 vectors shown in (A) cloned into the BamHI/ScaI restriction site as a BglII/BsPEI (blunt) fragment. These plasmids were used in a dual-vector strategy in conjunction with the pET21 plasmids to express two proteins in the same bacterial cell. (C) pET21 bicistronic ferritin expression plasmids. These were constructed by ligating a fragment containing the ferritin expression cassette from the pET21 vector into a second pET21 vector as described in Materials and methods. These plasmids were used to co-express two proteins from the same plasmid in one bacterium.

gene intact. The cloning junctions of all constructs and the entirety of the HFH, HFL, MFH and MFL coding regions were sequenced by the dideoxy chain termination method.

Expression from dual vector constructs
The strategy for achieving dual vector expression was to transform Escherichia coli host cells sequentially with two ferritin subunits, each cloned in a different vector. Vectors were chosen to have different origins of replication, so that they could co-exist in the same bacterial cell, as well as a different copy number, so that encoded ferritin subunits might be unequally expressed. In addition, a recombination-deficient host (BLR/DE3, Novagen) was used to minimize recombination between plasmids. Finally, each plasmid was constructed to encode resistance to a different antibiotic, such that only cells expressing both plasmids would be resistant to both kanamycin and ampicillin. For example, to create an HFH/HFL dual vector construct, BLR/DE3 cells were transformed with pET21C–HFH, followed by selection on plates containing 100 µg/ml ampicillin. Ampicillin-resistant transformants were then picked and transformed with pACYC177–HFL, followed by selection on plates containing 50 µg/ml kanamycin.

Isolation of recombinant proteins and assay of iron content
Ferritin protein was purified from bacterial cultures grown in the presence of 1.0 mM IPTG (isopropyl-β-D-thiogalactopyranoside, Sigma) for 2.5 h according to published procedures (Santambrogio et al., 1993; Rucker et al. 1996). Briefly, cells were pelleted and sonicated and the supernatant was heated at 70°C for 15 min. Ferritin was then isolated from the clarified supernatant by centrifugation on a 10–40% sucrose density gradient or by ammonium sulfate precipitation followed by column chromatography on Sephacryl S300 (for ferritin L-containing proteins) or Sepharose 6B (for ferritin H-containing proteins). The iron content of isolated proteins was low (<0.01 µg iron/µg protein) (Fish, 1988).

Determination of subunit composition
Intraspecies heteropolymers could be resolved by SDS–PAGE into H and L subunits, and the composition of these proteins was determined by staining with silver or Coomassie blue, followed by scanning densitometry. However, interspecies proteins containing human H and mouse L subunits or human L and mouse H subunits were difficult to resolve electro- phoretically. The subunit composition of these proteins was therefore determined by Western blotting. Polyclonal antibodies against HFH, MFL and MFL were prepared by immunizing rabbits with the appropriate ferritin. IgG fractions of immune serum were isolated by protein A column chromatography (Calbiochem) and then adsorbed overnight against heterologous ferritins conjugated to Affiprep-10 (Bio-Rad). A commercial anti-liver ferritin antibody (Dako) was used as an anti-HFL antibody. The specificity of these antibodies was assessed by dot blotting (Figure 2). Note that these antibodies exhibited some cross-reactivity. For example, anti-MFL antibody recognized HFL, anti-HFH recognized HFL and anti-HFL recognized HFH and MFL. Nevertheless, these antibodies were adequate to distinguish the subunits present in interspecies hybrids. Thus, HFH could be distinguished from MFL because anti-HFH antibody did not cross-react with MFL and anti-MFL did not cross-react with HFH. Similarly, MFH could be distinguished from HFL because anti-MFH antibody did not cross-react with HFL and anti-HFL antibody did not cross-
initiation and termination codons) was introduced in a cassette (including a ribosome binding site and cDNA with the stop codon of the first expression cassette). These bicistronic expression plasmids directed the synthesis of both subunits of ferritin, as shown in Table I. Expression levels of ferritin protein were similar in bacteria harboring these plasmids and those isolated from the same bacterium composing the dual-expression plasmid (Figure 3 and Table I).

**Results**

Co-expression of recombinant ferritin H and ferritin L subunits in vivo

In order to achieve a greater range in ferritin subunit composition, a second strategy for the expression of two ferritin subunits in the same bacterium was explored. For these experiments, a dual expression vector system was designed. cDNA for each of the human and murine ferritin subunits was cloned into an expression vector, pACYC, which contains a ribosome binding site and a downstream region encoding murine ferritin L (MFH–MFH), the downstream region encoded murine ferritin L (MFL), and the downstream region encoded murine ferritin H and the downstream region encoded human ferritin H or human ferritin L, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG and chemiluminescent substrate (ECL, Amersham Corp.).

Subunit composition (given as % ferritin H subunit, with the balance representing ferritin L) of ferritins generated using various expression constructs. Ferritin composition of purified mouse and human proteins (A and B) was estimated following SDS–PAGE, staining with Coomassie blue and scanning densitometry. Composition of mouse/human hybrid proteins (C) was estimated by Western blot analysis as described in Materials and methods.

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**Electron microscopy**

Following incubation with 0.1 mM ferrous ammonium sulfate in 0.1 M HEPES buffer, pH 6.5, a purified recombinant human ferritin heteropolymer (43% H chain, Table I) was deposited on a carbon-stabilized Formvar-coated copper grid, stained with 2% phosphotungstic acid, and viewed in a Philips TEM400 electron microscope.

**Isoelectric focusing**

IEF was performed in a Rotofor preparative isoelectric focusing cell (Bio-Rad). Approximately 200 µg of a density-purified ferritin preparation were diluted into 2% ampholytes (the ampholytes used were a 1:4 mixture of Biolyte 3/10 and Biolyte 5/7, Bio-Rad) and focused for 3 h at 4°C. Fractions of 0.9 ml were collected and the pH, protein concentration and subunit composition of each fraction determined.

**Table I.** Expression of multiple ferritin subunits in ferritin expression constructs

<table>
<thead>
<tr>
<th>cDNA orientation</th>
<th>Composition (%H)</th>
<th>Expression ratio (first:second)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFL–MFH</td>
<td>24</td>
<td>3.0:1</td>
</tr>
<tr>
<td>MFH–MFL</td>
<td>33</td>
<td>0.5:1</td>
</tr>
<tr>
<td>HFL–HFH</td>
<td>43</td>
<td>1.3:1</td>
</tr>
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</table>

**B) Intraspecies dual-vector constructs**

<table>
<thead>
<tr>
<th>pET</th>
<th>pACYC</th>
<th>Composition (%H)</th>
<th>Expression ratio (pET:pACYC)</th>
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</thead>
<tbody>
<tr>
<td>HFH</td>
<td>HFL</td>
<td>69</td>
<td>2.2:1.0</td>
</tr>
<tr>
<td>HFL</td>
<td>HFH</td>
<td>20</td>
<td>4.0:1.0</td>
</tr>
<tr>
<td>MFH</td>
<td>MFL</td>
<td>40</td>
<td>0.7:1.0</td>
</tr>
<tr>
<td>MFL</td>
<td>MFH</td>
<td>9</td>
<td>9.0:1.0</td>
</tr>
</tbody>
</table>

**C) Interspecies dual-vector constructs**

<table>
<thead>
<tr>
<th>pET</th>
<th>pACYC</th>
<th>Composition (%H)</th>
<th>Expression ratio (pET:pACYC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFH</td>
<td>MFL</td>
<td>90</td>
<td>9.0:1.0</td>
</tr>
<tr>
<td>MFH</td>
<td>HFL</td>
<td>48</td>
<td>0.9:1.0</td>
</tr>
<tr>
<td>MFH</td>
<td>HFH</td>
<td>14</td>
<td>6.1:1.0</td>
</tr>
</tbody>
</table>
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Fig. 3. Effect of orientation on expression of ferritin subunits in bicistronic constructs. MFH and MFL were cloned into the pET21C vector as described in Materials and methods. Lysates were prepared from IPTG-induced cultures and analyzed by SDS–PAGE and staining with Coomassie blue. L–H and H–L indicate order (5’→3’) of MFL and MFH cDNA in the expression vector. A lysate from a control culture lacking plasmid is shown on the right.

This vector, pACYC, is also present in lower copy number than the pET21 plasmid (Chang and Cohen, 1978). Bacteria were then transformed sequentially with ferritin cDNA cloned in the pET21 expression vector and a different ferritin cDNA cloned in the pACYC expression vector. It was expected that these plasmids should be able to co-exist in the same bacterial cell, but that expression from the pET21 plasmids might greatly exceed that from the pACYC plasmids, allowing the synthesis of ferritin heteropolymers of predominantly one subunit type. In most cases, these predictions were borne out, as shown in Table IB. In this case, expression of two subunits was much more unequal, and proteins with a 4–9-fold difference in relative subunit abundance were produced (Figure 4). The murine ferritin H subunit again proved an exception, resulting in a protein with only 40% ferritin H subunit content even when expressed in the pET21 vector. Nevertheless, expressing ferritin subunits from separate vectors was a more effective method for generating dramatic alterations in the final subunit composition of the protein than manipulation of the bicistronic expression system: as seen in Table I, reversing the cistron order of MFH and MFL in the bicistronic expression system increased the H content by 38% (from 24 to 33%), whereas expressing the same subunits in the pET21 versus pACYC vector increased the relative ferritin H content by 344% (from 9 to 40%). The dual vector system was also effective in the production of interspecies hybrid proteins, suggesting that at least between mouse and humans, subunits are sufficiently conserved to allow the formation of mixed species proteins (Table IC).

As shown in Table I, by combining these methods, both human and murine ferritins with a broad range of subunit composition were expressed in bacteria.

pET21. The SDS–PAGE analysis used in the assessment of ferritin subunit expression described above did not address whether these subunits in fact co-assembled. In order to determine whether ferritin heteropolymers expressed in bacteria assembled correctly, ferritins were purified and analyzed by sucrose density gradient centrifugation. Figure 5 demonstrates that horse spleen apoferritin migrates on these gradients at a position intermediate between aldolase (158 kDa) and thyroglobulin (669 kDa), consistent with its molecular mass of ~480 kDa. Recombinant ferritin produced in either a bicistronic or dual vector expression system migrated similarly, demonstrating that the ~20 kDa subunits assemble appropriately in vivo (Figure 5). All proteins, including interspecies hybrid proteins, demonstrated a similar ability to assemble (data not shown). Since iron-rich ferritin pellets in these gradients (not shown), this result corroborates direct measurements of ferritin iron content, which indicate that ferritin isolated under these conditions is iron-poor (<0.01 µg iron/µg protein). Subunit assembly was also evident from the ability of recombinant ferritins to co-migrate with commercially procured ferritin in gel filtration columns and native gel electrophoresis (data not shown). The fact that the subunit composition and yield of purified proteins did not differ substantially from that predicted by SDS–PAGE of bacterial lysates (Table II and Figure 6) also suggests that the majority of ferritin subunits assemble appropriately.

Further evidence for subunit assembly as well as functionality was provided by electron microscopy. Ferritin catalyzes iron oxidation and deposition, and in vitro kinetic analyses of the recombinant ferritins described here have revealed that they are fully competent in iron uptake (Rucker et al., 1996). In order to verify that the iron in recombinant proteins accumulated in a central iron core, and also to determine whether ferritin subunits assembled appropriately in bacteria,
Recombinant ferritin heteropolymers

Fig. 5. Density gradient analysis of recombinant ferritin heteropolymers purified from bicistronic and dual-vector expression systems. The following proteins were layered onto 10–40% sucrose gradients and centrifuged at 100,000 g for 18 h at 4°C: 40% MFH/60% MFL ferritin (purified protein from a bicistronic vector), 43% HFH/57% HFL (total protein isolated from a dual-vector construct following heat treatment at 70°C (protein migrating at the top of the gradient represents bacterial proteins present in this partially purified preparation). Fractions were collected and assayed for protein content. Under these conditions aldolase (158 kDa) migrated at fraction 10 (left arrow) and thyroglobulin (669 kDa) at fraction 28 (right arrow). The migration of horse spleen apoferritin is indicated by an F. Fraction 1 represents the top of the gradient.

Table II. Subunit composition assessed in bacterial lysates and purified proteins

<table>
<thead>
<tr>
<th>Expression system</th>
<th>Subunits</th>
<th>Lysate</th>
<th>Purified protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dual vector</td>
<td>HFH/HFL</td>
<td>70</td>
<td>69</td>
</tr>
<tr>
<td>Dual vector</td>
<td>HFL/HFH</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Dual vector</td>
<td>MFH/MFL</td>
<td>37</td>
<td>40</td>
</tr>
<tr>
<td>Dual vector</td>
<td>MFL/MFH</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Bicistronic</td>
<td>HFL-HFH</td>
<td>33</td>
<td>43</td>
</tr>
</tbody>
</table>

Ferritin composition of proteins was estimated by electrophoresis of total bacterial lysates or purified proteins by SDS–PAGE followed by staining with Coomassie blue and scanning densitometry. Composition is given as % ferritin H subunit, with the balance representing ferritin L.

Fig. 6. Composition of ferritin in bacterial lysates is similar to that of purified proteins. *E. coli* harboring a bicistronic expression plasmid encoding HFH–HFL were lysed in SDS sample buffer and analyzed by SDS–PAGE (A). Ferritin was purified from the same culture and electrophoresed on the same gel (B).

Fig. 7. Negative staining of a recombinant ferritin heteropolymer. Ferritin was isolated from bacteria harboring a ferritin bicistronic expression plasmid (43% human ferritin H; Table I) and visualized by electron microscopy. Bar indicates 40 nm.

a negatively stained preparation of a human ferritin protein derived from a bicistronic expression system was viewed by electron microscopy following iron-loading *in vitro*. As seen in Figure 7, this protein exhibited characteristic features of native ferritin, including a particle diameter of ~13 nm and a central iron core (Massover, 1993).

Ferritin heteropolymers assembled in vivo have a restricted subunit composition

These results demonstrated that bacterially expressed ferritin subunits can assemble into multi-subunit heteropolymers. However, these experiments did not define the range of composition of ferritin heteropolymers formed under these conditions, i.e. does a particular ferritin heteropolymer represent a single
species of protein of defined subunit composition, or does the average subunit composition reflect a range of widely divergent subunit compositions? To address this question, recombinant ferritins were analyzed by isoelectric focusing. This procedure separates ferritin proteins according to their charge, with H-subunit-rich proteins being most acidic and L-subunit-rich ferritins more basic (Arosio et al., 1976). As shown in Figure 8, recombinant human ferritins assembled in vitro exhibited a similar property: the lowest average pI (~5.1) was seen in a protein composed of 100% ferritin H; as the L content increased, so did pI. Further, in all cases analyzed, ~90% of the ferritin focused within a pH range of 0.5 units. In addition, analysis of subunit composition across the pH gradient revealed that the species of ferritin formed did not deviate substantially in composition. Thus, in a ferritin preparation of 69% average H-chain content derived from a dual vector expression system, 94% of the protein had an H-subunit content between 70 and 64.7% and focused between pH 5.1 and 5.6 (Figure 6). Similar results were seen with a human protein of 43% average H-chain content produced from a bicistronic expression system (data not shown). This compares well with ferritin isolated from human tissue, which exhibits a similar range in pI (Bullock et al., 1980; Yoda and Abe, 1980). Hence recombinant ferritin heteropolymers synthesized in bacteria are relatively homogeneous, but display a microheterogeneity in subunit composition reminiscent of tissue ferritin.

**Discussion**

These studies demonstrate that using either a bicistronic or a dual vector expression strategy, it is possible to produce multisubunit ferritins in vivo. By manipulating these systems, an array of heteropolymers that mimic the wide range of ferritin subunit composition found in different mammalian tissues were isolated as natively assembled proteins (Figures 5 and 7). Production of these proteins by spontaneous assembly in bacteria obviates the need for denaturants and the in vitro reconstitution of heteropolymers (Otsuka et al., 1980; Santambrogio et al., 1993). These recombinant ferritins synthesized in bacteria permitted the exploration of functional differences between mouse and human ferritin subunits (Rucker et al., 1996).

Although the expression systems described here have been used previously, to our knowledge this is the first attempt to use such vector systems to alter systematically subunit stoichiometry in a multi-subunit protein. Through extensive characterization of the protein by electron microscopy, sucrose density gradient centrifugation and isoelectric focusing, we observed that ferritins produced by either expression system assemble into a relatively homogenous population of proteins whose final composition is largely dictated by intracellular subunit abundance. In the dual-vector system, this in turn is primarily dependent on plasmid copy number, although intrinsic translatability also appears to play a role. Comparison
of the two expression systems with each other revealed that the bicistronic expression system produced proteins of relatively equivalent subunit composition, although there was some bias towards increased expression of the first relative to the second cistron (Table I). The two-vector system allowed the isolation of proteins with a much greater differential in final subunit content.

The proteins described here have revealed a strict dependence of ferritin function on subunit composition, and will be of utility in further studies of the role of subunit composition in ferritin function and assembly. More generally, the strategy described here may be applicable to studies of the role of subunit stoichiometry in the assembly or function of other multimeric proteins or protein complexes.

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